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Given the steady rise in obesity worldwide, it is important to identify dietary compounds that prevent adiposity. One dietary strategy is supplementation with conjugated linoleic acid (CLA), which has been demonstrated to reduce body fat mass. However, side effects associated with CLA supplementation include inflammation, insulin resistance, and dyslipidemia. Elucidation of the antiobesity mechanism of CLA is critical for evaluating its efficacy and safety as a dietary supplement for treating obesity. Therefore, this research examined the upstream mechanism by which CLA induced inflammation, insulin resistance, and delipidation of human adipocytes.

Our research group has previously demonstrated that trans-10, cis-12 (10,12) CLA causes delipidation of human adipocytes via activating nuclear factor kappa B (NFκB) and mitogen-activated protein kinase / extracellular signal-regulated kinase kinase (MEK/ERK) signaling, leading to inflammation and the suppression of peroxisome proliferator activated receptor (PPARγ) and decreased glucose and fatty acid uptake. Based on these findings, the following questions were addressed using primary cultures of newly differentiated human adipocytes as a cell model 1) How does CLA impact PPARγ activity?, 2) What upstream mechanisms activate ERK, NFκB and induce inflammation?, and 3) Does resveratrol, a phenolic phytochemical with antioxidant properties, attenuate CLA-induced inflammation, insulin resistance, and delipidation?

Answers to these questions were as follows. 1) 10,12 CLA antagonized ligand-dependent PPARγ activity, possibly via PPARγ phosphorylation by ERK. 10,12 CLA suppression of PPARγ and insulin-stimulated glucose uptake, along with delipidation were partially rescued by co-supplementation with the PPARγ agonist BRL, further supporting CLA antagonizing PPARγ. 2) Cultures treated with TMB-8, an inhibitor of calcium release from the endoplasmic reticulum or KN-62, an inhibitor of calcium/calmodulin-dependent kinase II (CAMKII) attenuated 10,12 CLA-mediated reactive oxygen species (ROS) production, mitogen-activated protein kinase (MAPK) activation, inflammatory gene induction, and insulin resistance. These data suggested that 10,12 CLA-mediated inflammation and insulin resistance are dependent on calcium release from the endoplasmic reticulum or CAMKII. 3) Treatment with resveratrol prevented 10,12 CLA-mediated inflammation and insulin resistance by attenuating intracellular calcium, ROS, and inflammation, by increasing PPARγ activity. Collectively, these data suggest that one of the antiobesity mechanisms of 10,12 CLA is inducing cellular stress and inflammation which antagonize PPARγ, leading to insulin resistance and delipidation of human adipocytes.

THE ANTIOBESITY MECHANISM OF CONJUGATED LINOLEIC ACID

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To my mother, who loves me unconditionally.

APPROVAL PAGE

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CHAPTER I

INTRODUCTION

<u>Overview</u>

Obesity rates have risen in the U.S. over the past 20 years. In 2007, the CDC estimated that of the 51 states in the U.S., 30 had rates of obesity greater than 25% based on a body mass index (BMI) greater than 30 (CDC 2007).   Obesity is the leading risk factor for developing chronic diseases such as type 2 diabetes, stroke, cancer, and cardiovascular disease. These metabolic disorders are major health concerns in the U.S., especially among minority populations. This has resulted in the U.S. spending ~100 billion dollars on medical expenses related to obesity. Although reducing caloric intake relative to energy expenditure and increasing physical activity are established means for preventing or treating obesity, the incidence of obesity among children, adolescents, and adults continues to increase dramatically.   Therefore, research has been aimed at identifying alternative strategies that prevent or attenuate adiposity, and identify underlying mechanisms impacting the growth, differentiation, or metabolism of adipocytes, the main cell types in white adipose tissue (WAT).

One potential strategy for reducing adiposity is dietary supplementation with conjugated linoleic acid (CLA), an unsaturated fatty acid naturally found in ruminant meats and dairy products (Fig. 1.1). Numerous animal studies and several human studies

have demonstrated that supplementation with a mixture of trans-10, cis-12 (10,12) and cis-9, trans-11 (9,11) isomers of CLA, or 10,12 CLA alone, reduces body weight and fat deposition. Precisely how CLA reduces adiposity remains unclear, and thus will be the focus of this dissertation.

Our research group has demonstrated that 10,12 CLA, but not 9,11 CLA, decreases fatty acid and glucose uptake and triglyceride (TG) synthesis in primary cultures of human adipocytes by activating mitogen-activated protein kinase / extracellular signal-regulated kinase kinase (MEK/ERK) and nuclear factor kappa B (NFκB) signaling that induces interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF) α production. These cytokines, in turn, attenuate peroxisome proliferator activated receptor (PPAR) γ target gene expression in adipocytes, reducing glucose and fatty acid uptake. The mechanism by which 10,12 CLA produces upstream signals that activate NFκB and ERK1/2, and cause delipidation and insulin resistance is unknown. Elucidating this mechanism will provide valuable information on the efficacy, specificity, and potential side effects of CLA isomers as a dietary strategy for preventing or treating obesity. Lack of such knowledge is an important problem, because until it becomes available, the effective and safe use of CLA supplements to control obesity can not ensue.

Central Hypothesis and Specific Objectives

The central hypothesis for the proposed research is that 10,12 CLA elevates the levels of intracellular calcium $[Ca^{2+}]_i$ and reactive oxygen species (ROS), which lead to the activation of calcium/calmodulin-dependent protein kinase II (CAMKII), MEK/ERK,

and NFκB-induced inflammation, resulting in the suppression of PPARγ activity and target gene expression, insulin resistance, and delipidation in primary cultures of human adipocytes.

To test this hypothesis, the following three specific aims were investigated using primary cultures of newly differentiated human adipocytes.

1. Determine the mechanism by which 10,12 CLA suppresses the expression of PPARγ and its target genes (Chapter III).

2. Identify upstream mechanism(s) by which 10,12 CLA induces inflammation, and insulin resistance (Chapter IV).

3. Determine the extent to which resveratrol prevents 10,12 CLA induced cellular stress, inflammation, insulin resistance and delipidation (Chapter V).

trans-10, cis-12 octadecadienoic acid

cis-9, trans-11 octadecadienoic acid

cis-9, cis-12 octadecadienoic acid

Figure 1.1. Conjugated linoleic acid isomers (CLA).
From Am. J. Clin. Nutr. 2004:79 p1132S-1136S

CHAPTER II

REVIEW OF THE LITERATURE

Background and Significance

Conjugated linoleic acid (CLA) refers to a group of conjugated octadecadienoic acid isomers found in ruminant meats and dairy products. Conjugated linoleic acid is a derivative of linoleic acid, which contains 18 carbons and two double bonds in the cis configuration at the 9th and 12th carbons, i.e., cis-9, cis-12 octadecadienoic acid. In the gastrointestinal tract of ruminant animals, microbes 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 (9,11) or trans-10, cis-12 (10,12) octadecadienoic acid.

Commercially, CLA is produced from linoleic acid derived from safflower or sunflower oils in the presence of alkaline substances. This type of processing yields a CLA mixture containing ~40% of the 9,11 isomer and 44% of the 10,12 isomer (reviewed in Pariza et al. 2001). Commercial preparations also contain ~4-10% trans-9, trans-11 CLA and trans-10, trans-12 CLA, as well as trace amounts of other isomers.

In natural foods, the predominant form of CLA is the 9,11 isomer, also known as rumenic acid. 9,11 CLA makes up ~ 90% of CLA found in ruminant meats and dairy products, with the remaining 10% of CLA as the 10,12 isomer. Although several other isoforms of CLA have been identified (i.e., trans-9, trans-11; cis-9, cis-11; trans-10, trans-11; and cis-10, cis-12), the 9,11 and 10,12 isomers appear to be the most biologically active (Wallace et al. 2007).

The content of CLA in milk and other dairy products ranges between ~0.34 – 1.07% total fat, and from 0.12 – 0.68% total fat in raw or processed beef products (reviewed in Dhiman et al. 2005 and Silveira et al. 2007, Mendis et al. 2008). However, the CLA content of beef is dependent on several factors including the breed, season, nutrition, and age of the animal (reviewed Dhiman et al. 2005). The average daily intake of CLA is ~152 and 212 mg for non-vegetarian women and men, respectively (Ritzenthaler et al. 2001). Physiological levels in human serum range from 10 to 70 μmol/L (Mougios et al. 2001, Petridou et al. 2003).

Antiobesity properties of CLA

CLA was initially discovered in 1987 by Pariza and colleagues and it was identified to be an anti-carcinogen (Hay et al. 1987). Subsequently, CLA was shown to exhibit anti-atherosclerotic (reviewed in Mitchell et al. 2008) and anti-obesity properties (reviewed in Whigham et al. 2007). Due to the substantial rise of obesity prevalence over the past 30 years (CDC 2008), more interest in CLA has developed for its use as a weight loss treatment. For example, supplementation with a CLA mixture (i.e., 10,12 + 9,11

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isomers in equal concentrations) or the 10,12 isomer at ~3-6 g/d decreases body fat mass (BFM) in animals and some humans (reviewed in Wang et al. 2004 and Whigham et al. 2007). Of the two major isomers of CLA, the 10,12 isomer possess most of the anti-obesity properties compared to the 9,11 isomer (Park et al. 1999, Brown et al. 2001, House et al. 2005, Brandebourg et al. 2005, Miller et al 2008).

CLA regulation of body weight

Park et al. (1997) were the first 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 (Sisk et al. 2001, Clement et al. 2002, Meadus et al. 2002, Yamasaki et al. 2003, Poirier et al. 2006). For example, dietary supplementation with 1% (wt/wt) CLA mixture for 28 d decreased body weight and peri-uteral white adipose tissue (WAT) mass in C57BL/6J mice (Poirier et al. 2005). Dietary supplementation with a 1.5% (wt/wt) mixture of CLA for 4 wk decreased body weight and WAT mass in male ob/ob mice (Wendel et al. 2008).

CLA reduces BFM in animals more consistently than in humans. Whereas some studies show that CLA decreases BFM and increases lean body mass (LBM) (reviewed in Wang et al. 2004 and House et al. 2005), others have shown no effect of CLA supplementation on body composition in humans (reviewed in Wang et al. 2004). For example, supplementation of 3-4 g/d of a CLA mixture for 24 wk decreased BFM and

increased LBM in overweight and obese people (Gaullier et al. 2007). On the other hand, supplementation of 3.76 g/d of a CLA mixture in yogurt for 14 wk in healthy adults had no effect on body composition (Nazare et al. 2007). Larsen et al. (2006) investigated the potential role of CLA for preventing body weight regain in moderately obese subjects who lost ~10 kg after an 8-wk dietary intervention on a low calorie diet. Supplementation for 1 year with a CLA mixture did not prevent body weight regain compared to controls. Laso et al. (2007) found that supplementation with 3.2 g/d of a CLA mixture decreased total BFM and trunk fat compared to placebo in overweight subjects, but not obese subjects. 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.

The main discrepancy between animal and human studies may be due to the dose of CLA used. For example, moderately overweight humans with an average weight of 72.5 kg supplemented with 3.76 g of a CLA mixture per d for 14 wk displayed no decrease in body weight, BMI, or BFM (Nazare et al. 2007). In contrast, C57BL/6 mice supplemented with 1.5% (w/w) CLA mixture for 4 wk weighed less than controls (Purushotham et al. 2007). However, when comparing the amount of CLA given on a body weight basis (g CLA consumed/ kg body weight), humans in Nazare's study received ~0.05 g/kg body weight, whereas mice in Purushotham's study received 1.07 g/kg body weight. Thus, the mice received ~20 times more CLA per kg body weight than the human subjects.

Based on CLA's potential to reduce BFM, it is important to understand the mechanism of action of CLA. Therefore, this literature review will examine potential mechanisms by which the CLA mixture or 10,12 CLA alone reduces adiposity, with particular emphasis on WAT. Potential mechanisms to be discussed include CLA regulating 1) energy metabolism, 2) adipogenesis, 3) lipid metabolism, 4) inflammation, and 5) apoptosis. Some data presented in the next sections were taken from Chapters III-V to demonstrate how my research integrates with the current literature on CLA.

Antiobesity Mechanisms of Conjugated Linoleic Acid

CLA regulation of energy metabolism

CLA decreases energy intake. Energy balance is a function of energy intake relative to energy expenditure. When energy intake exceeds energy expenditure, body weight and BFM increase, and vice versa. One proposed mechanism by which CLA reduces BFM is by decreasing energy intake or increasing energy expenditure (**Fig. 1**). Park et al. (1997) were the first to demonstrate that supplementation of mice with a CLA mixture or enriched 10,12 CLA for 4 wk reduced food intake. There are a number of studies demonstrating that feeding a CLA mixture reduces energy intake in rodents (Miner et al. 2001, Hargrave et al. 2002, Takahashi et al. 2002, House et al. 2005, So et al. 2009). However, CLA does not appear to decrease food intake in humans (Zambell 2000, Medina 2000, Lambert et al. 2007, Tholstrup et al 2008).

So et al. (2009) reported that food intake was reduced by 23.6% in mice fed a low-fat diet supplemented with 10,12 CLA. The hypothalamic gene expression ratio of

proopiomelancortin (POMC) to neuropeptide Y (NPY) was decreased by 10,12 CLA supplementation, suggesting CLA reduced food intake through suppression of hypothalamic appetite-regulating genes. In support of these findings, administration of mixed isomers of CLA to the hypothalamus decreased the gene expression of NPY and agouti-related protein, neuropeptides that robustly increase food intake (Cao et al. 2007). Alternatively, CLA could be reducing the palatability of the diet or increasing satiety.

In contrast, a number of studies have reported no changes in energy intake following administration of a CLA mixture in mice (Azain et al. 2000, West et al. 2000, Terpstra et al. 2002, Terpstra et al. 2003,). For example, supplementation of mice with a CLA mixture for 42 d decreased total body weight, without reducing food intake (Terpstra et al. 2003). These data suggest that CLA suppression of body fat is not solely dependent on reductions of food or energy intake.

CLA increases energy expenditure. Energy expenditure is a function of basal metabolic rate (BMR), adaptive thermogenesis, and physical activity. CLA has been proposed to reduce adiposity by elevating energy expenditure (**Fig. 1**) via increasing BMR, thermogenesis, or lipid oxidation in animals (West et al. 2000, Miner et al. 2001, Ohnuki et al. 2001, Terpstra et al. 2002, Terpstra et al. 2003, Nago et al. 2003). In BALB/c male mice fed mixed isomers of CLA for 6 wk, body fat was decreased by 50% and was accompanied by increased energy expended as heat as compared to controls (Terpstra et al. 2003). Enhanced thermogenesis is associated with mitochondria uncoupling. During uncoupling, trans-membrane proteins known as uncoupling proteins (UCP), which span

the inner mitochondrial membrane, uncouple the oxidation of fuels to ATP formation, leading to dissipation of energy as heat instead of ATP synthesis. UCP2 is the most abundant and active form in WAT, while UCP1 and 3 are primarily expressed in brown adipose tissue (reviewed in Ricquier et al. 2000). Supplementation with a CLA mixture or 10,12 CLA in rodents induced UCP 2 gene expression in WAT (Takahashi et al. 2002, Ealey et al. 2002, Kang et al. 2004, House et al. 2005, LaRosa et al. 2006). CLA increased the expression of carnitine palmitoyl transferase 1 (CPT1), an important enzyme in the FA oxidation in WAT (Martin et al. 2000, LaRosa et al. 2006). Consistent with these findings, 10,12 CLA increased beta oxidation in 3T3-L1 preadipocytes (Evans et al. 2002). Similarly, CLA supplementation induced UCP gene expression and elevated beta oxidation in muscle and liver (Takahashi et al. 2002, Roche et al. 2002, Choi et al. 2007, Ribot et al. 2007, Prior et al. 2007, Ferramosca et al. 2008).

Activation of UCP decreases the ATP/ADP ratio, which stimulates the activation of 5'-AMP-activated protein kinase (AMPK) (reviewed in Rossmeisl et al. 2004). Activated AMPK can phosphorylate hormone sensitive lipase (HSL), leading to activation of lipolysis in WAT (Anthony et al. 2009), and phosphorylation of acetyl CoA carboxylase (ACC), resulting in suppression of lipogenesis in isolated white adipocytes (Gaidhu et al. 2008) (**Fig. 2**). Supplementation of ob/ob mice with 1.5% (w/w) CLA mixture for 6 wk increased AMPK activity in WAT compared to controls (Wendel et al. 2007), although UCP-1 and CPT1 gene expression were not induced. While there are limited studies demonstrating that CLA activates AMPK, ACC mRNA levels were decreased in adipocytes treated with 10,12 CLA (Brown et al. 2003). Consistent with

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these findings 10,12 CLA inhibited lipogenesis (Brown et al. 2004) and increased acute lipolysis in cultures of newly differentiated human adipocytes (Chung et al. 2005a).

Results from human studies concerning CLA regulation of energy expenditure are mixed. For example, supplementation of humans with 3.9 g/d of CLA mixture for 12 wk did not alter BMR or alter BFM (Lambert et al. 2007). Similar findings have been reported in humans supplemented with a CLA mixture (Zambell et al. 2000, Steck et al. 2007). In contrast, healthy, moderately-overweight humans supplemented with 3.76 g/d CLA mixture in yogurt for 14 wk had higher BMR levels, but had no differences in UCP 2 gene expression in WAT or altered body weight (Nazare et al. 2007). Similarly, resting metabolic rate and fat-free mass were higher in humans supplemented with CLA mixture for 13 wk, but CLA had no effect on BFM (Kamphuis et al. 2003). Thus far, only one human study has demonstrated that CLA increases energy expenditure and decreases body weight (Close et al. 2007). In this study, humans supplemented for 6 mo with 4 g/d of a CLA mixture had decreased body weight and increased fat oxidation and energy expenditure while sleeping.

Other studies have demonstrated that CLA supplementation increases LBM, which is associated with higher levels of energy expenditure. For example, healthy obese humans supplemented with 6.4 g/d CLA mixture for 12 wk had increased LBM by 0.64 kg compared to controls (Steck et al. 2007). Similarly, mice fed a 0.4% (w/w) CLA mixture had increased LBM compared to controls (Bhattacharya et al. 2005). Two proposed mechanisms by which CLA increases LBM are via increasing bone or muscle

mass. 10,12 CLA supplementation for 10 wk with a 0.5% (wt/wt) CLA mixture increased bone mineral density (BMD) and muscle mass in C57BL/6 female mice. CLA supplementation has been proposed to increase BMD via increasing osteogenic gene expression and decreasing osteoclast activity (Banu et al. 2006, Rahman et al 2007). Furthermore, CLA supplementation alone or with exercise increased BMD compared to control mice (Banu et al. 2008). An alternative mechanism could be that CLA decreases adipogenesis of pluripotent mesenchymal stem cells (MSC) in bone marrow, and instead enhances 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 (Platt et al. 2008). 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, which decrease muscle and bone mass, prevented reductions in LBM, BMD, and bone mineral content (Roy et al. 2008). Collectively, these findings suggest CLA may reduce adiposity through increased energy expenditure via increased UCP and CPT1 activity in WAT or increased muscle or bone mass. However, the extent to which CLA regulates BMR or LBM, and how this contributes to the reduction in body weight or fat in humans, remains to be determined.

CLA regulation of adipogenesis

CLA inhibits adipogenesis. The conversion of preadipocytes to adipocytes involves the activation of key transcription factors such as CAAT/ enhancer binding

proteins (C/EBPs) and peroxisome proliferators-activated receptor (PPAR)γ. During the differentiation process, increased C/EBPβ and δ activity promote the activation of C/EBPα and PPARγ, the master regulators of adipocyte differentiation (**Fig. 3**). There is much evidence showing that CLA suppresses preadipocyte differentiation in animal (Brodie et al. 1999, Satory et al. 1999, Choi et al. 2000, Evans et al. 2000, Granlund et al. 2003, Kang et al. 2003, Miller et al. 2008) and human preadipocytes (Brown et al. 2003, Brown et al. 2004) treated with a CLA mixture or 10,12 CLA alone. 10,12 CLA treatment has been reported to decrease the expression of PPARγ, C/EBPα, sterol regulatory element binding protein 1c (SREBP-1c), liver X receptor (LXRα), and adipocyte fatty acid binding protein (aP2), thereby reducing adipogenesis or lipogenesis (Brown et al. 2003, Kang et al. 2003, Granlund et al. 2005, House et al. 2005, LaRosa et al. 2007).

In rodents, supplementation of 10,12 CLA decreased the expression of PPARγ and its target genes (Kang et al. 2003, LaRosa et al. 2006, Liu et al. 2007). In contrast, humans supplemented with a CLA mixture had higher mRNA levels of PPARγ in WAT, but no difference in body weight or BFM (Nazare et al. 2007).

Reduced PPARγ protein levels were reported in cultures of newly differentiated human and 3T3-L1 adipocytes, respectively, when treated with 10,12 CLA as compared to 9,11 CLA (Kennedy et al. 2008-Chapter III, Miller et al. 2008). While 10,12 CLA has been demonstrated to suppress PPARγ gene expression and protein level, data are inconclusive on CLA regulation of PPARγ activity. Several CLA isomers, including

9,11, have been shown to activate PPARγ (Yu et al. 2002, Granuland et al 2003). In contrast, in the presence of a PPARγ agonist such as rosiglitazone or darglitazone, CLA isomers have been reported to decrease the activity of the liver X receptor (LXRα)-PPRE-LUC reporter (Granuland et al. 2003) and the acyl-CoA binding protein (ACBP)-PPRE-LUC reporter (Brown et al. 2003) in 3T3-L1 adipocytes. Recently, we reported that under ligand-stimulated conditions, PPRE-LUC reporter activity was decreased in the presence of 50 μmol/L 10,12 CLA compared to 9,11 CLA (Kennedy et al. 2008). Similarly, Miller et al. 2008 demonstrated in CHO-K1 cells that 100 μmol/L and 200 μmol/L 10,12 CLA antagonized troglitazone activation of a PPRE reporter. CLA suppression of PPARγ and target genes was also attenuated by rosiglitazone *in vivo* and *in vitro* (Liu et al. 2007, Kennedy et al. 2008), further supporting CLA antagonism of PPARγ function.

PPARγ activity may also be impacted by phosphorylation. Phosphorylation of Ser-112 of PPARγ2 leads to ubiquination and proteosome degradation of PPARγ (Hu et al. 1996). PPARγ phosphorylation can occur by activation of mitogen activated kinase (MAPK) pathway, which has been demonstrated to inhibit adipogenesis (Diradourian et al. 2005). We demonstrated that 10,12 CLA increases phosphorylation of ERK and PPARγ (Kennedy et al. 2008), suggesting ERK may play a role in phosphorylating and inactivating PPARγ. Consistent with these data, Brown et al. (2004) demonstrated that ERK activation is a key player in CLA's suppression of adipogenic gene expression and insulin-stimulated glucose uptake. Therefore, it is tempting to speculate that CLA

antagonizes PPARγ activity, possibly via activation of MAPKs like ERK, thereby suppressing (pre)adipocyte differentiation (**Fig. 4**). However, the impact of CLA on the activity of other transcription factors involved in adipogenesis and lipogenesis (i.e., LXRα, C/EBPs, SREBP-1c) may also contribute to CLA's anti-obesity actions.

CLA regulation of lipid metabolism

CLA suppresses lipogenesis. Storage of FA as TG is a major function of adipocytes. Genes involved in lipogenesis, such as a lipoprotein protein 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 (**Fig. 4**) (Evans et al. 2000, Brown et al. 2003, Brown et al. 2004, LaRosa et al. 2006). PPARγ is a major regulator of LPL, and LPL gene expression decreased in adipocytes treated with 10,12 CLA (Xu et al. 2003, Kennedy et al. 2008). Consistent with the important role of LPL in delivery of FAs for TG synthesis in adipocytes, 10,12 CLA treatment decreased FA uptake in human adipocytes (Brown et. al 2004, Kennedy et al. 2009-Chapter V). Thus, the anti-lipogenic actions of CLA may be partially due to decreased PPARγ activity.

The major function of SCD is to synthesize oleate and palmitoleate, monounsaturated FAs important for neutral lipid and phospholipid synthesis from stearate and palmitate. Reduced levels of monounsaturated FAs have been reported following treatment with a CLA mixture or 10,12 CLA *in vivo* (House et al. 2005, Martin et al. 2007) and *in vitro* (Brown et al. 2003). It has also been demonstrated that body fat loss in mice fed a CLA mixture is dependent on Δ6-desaturase (Hargrave-Barnes et al.

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2008). However, in SCD-1 knockout mice, 10,12 CLA supplementation reduced body weight along with increasing the ratio of 16:0/16:1 and decreasing the ratio of 18:0/18:1 (Kang et. al. 2004), suggesting the antiobesity properties of CLA are not entirely dependent on SCD-1.

CLA causes insulin resistance. Insulin-stimulated glucose uptake in WAT is typically mediated via the insulin-dependent glucose transporter GLUT4. Defects in insulin signaling or suppression of GLUT4 translocation to the plasma membrane are major causes of insulin resistance in adipocytes, leading to reduced TG synthesis (**Fig. 5**). Insulin resistance has been reported *in vivo* (Riserus et al. 2002, Moloney et al. 2004, La Rosa et al. 2006, Thrush et al. 2007, Ingelsson et al. 2008) and *in vitro* (Brown et al. 2003, Chung et al. 2005b, Kennedy et al. 2008, Kennedy et al. 2009) following supplementation with a CLA mixture or 10,12 CLA alone. *In vivo*, supplementation with a CLA mixture or 10,12 CLA has been shown to induce hyperinsulinemia (Reviewed in Wang et al. 2004). Mechanisms by which CLA induces insulin resistance include inhibiting insulin signaling, increasing ceramide levels, or reducing the expression of genes (i.e, adiponectin, GLUT4) or activation of proteins (i.e, GLUT4, AKT, IRS-1) involved in glucose uptake.

CLA has been reported to inhibit insulin signaling via increasing the expression of suppressor of cytokine signaling (SOCS)-3 in 3T3-L1 (Poirier et al. 2006) and cultures of newly differentiated human adipocytes (Kennedy et al. 2009). SOCS-3 promotes phosphorylation of serine 307 on insulin receptor substrate (IRS-1), leading to its

ubiquination and proteosome degradation, thereby impairing insulin signaling and glucose uptake (Ueki et al. 2004). Treatment with 10,12 CLA has been demonstrated to decrease the protein levels of insulin receptor (IR)β (Poirier et al. 2006) and IRS-1 (Chung et al. 2005b, Poirier et al. 2006), key signaling proteins for insulin sensitivity. 10,12 CLA treatment decreased tyrosine phosphorylation of IRβ and IRS-1 in 3T3-L1 adipocytes (Poirier et al. 2006). However, 10,12 CLA treatment in human and 3T3-L1 adipocytes did not increase phosphorylation of Serine 307 on IRS-1 (Chung et al. 2005b, Poirier et al. 2006). CLA reduced the gene expression of GLUT4 and SLC2A5, which is a glucose/fructose transporter member, in WAT and 3T3-L1 adipocytes supplemented with 10,12 CLA (LaRosa et al. 2006). In cultures of newly differentiated human adipocytes 10,12, but not 9,11, CLA decreased GLUT4 gene and protein expression (Brown et al. 2004, Chung et al. 2005b, Kennedy et al. 2008). These data suggest that 10,12 CLA may impair insulin signaling by suppressing the levels of proteins involved in the signaling cascade.

The mRNA levels of adiponectin, a key adipokine associated with insulin sensitivity, decreases following supplementation with 10,12 CLA *in vivo (*Poirier et al. 2006, Liu et al. 2007) and *in vitro* (Ahn et al. 2006, Perez-Matute et al. 2007, Kennedy et al. 2009, Miller et al. 2008). Adiponectin is a known target gene of PPARγ (Iwaki et al. 2003). Therefore, suppression of adiponectin expression maybe due in part, to 10,12 CLA antagonizing PPARγ activity. Supplementation of rosiglitazone, a PPARγ agonist, prevented CLA suppression of adiponectin serum levels and CLA induced insulin resistance in mice (Liu et al. 2007). Although treatment of 3T3-L1 adipocytes with

triglitazone prevented 10,12 CLA suppression of TG levels and adiponectin oligomer assembly, it did not prevent 10,12 CLA suppression of adiponectin synthesis (Miller et al. 2008). These data suggest that CLA suppression of adiponectin levels maybe indirectly related to its TG lowering actions in adipocytes.

CLA stimulates lipolysis. Lipolysis is the process by which stored TG is mobilized, releasing free fatty acids (FFA) and glycerol for use by metabolically-active tissues. Typically, when energy demand is increased, lipolysis is increased via cAMP-mediated signaling (**Fig. 6**). LaRosa et al. (2006) demonstrated that C57BL/6J mice fed 10,12 CLA for 3 d had increased mRNA levels of HSL, a key enzyme involved in liberating FFA from TG stores.   However, HSL levels decreased following chronic (17 d) treatment. Consistent with these data, acute treatment with CLA mixture or 10,12 CLA alone increased lipolysis in 3T3-L1 (Park et al. 1997, Evans et al. 2002, Moon et al. 2006) and newly differentiated human adipocytes (Chung et al. 2005a).

In contrast, *in vivo* studies have demonstrated that chronic supplementation with a mixture of CLA has no effect on lipolysis (Xu et al. 2003, Simon et al. 2005). Chronic treatment with 1-200 μmol/L mixed isomer of CLA reduced glycerol release in isolated rat adipocytes (Perez-Matute et al. 2008). Similarly, chronic treatment with 10,12 CLA decreased HSL gene expression (Brown et al. 2003) and protein levels (Chung et al. 2005a). Feeding hamsters a high fat diet supplemented with 0.5% (w/w) 10,12 CLA for 3 wk had no effect on WAT HSL or TG lipase expression or body weight (Lasa et al. 2008). Consistent with these data, FFA levels have been reported to be lower in the

serum of OLETF rats supplemented with 1.0% (w/w) CLA mixture compared to controls (Rahman et al. 2001). However, this reduction of FFA may be due to increased uptake of FFA in liver or muscle. For example, supplementation with a CLA mixture or 10,12 CLA alone increased lipid accumulation in the liver of mice (Andreoli et al. 2008, Oikawa et al. 2008) and hamsters (Tarling et al. 2008, Navarro et al. 2009). Thus, CLA may acutely induce lipolysis in WAT, liberating FFA for uptake in metabolically-active tissue (i.e., liver and muscle). Lack of a chronic effect may be due to depleted TG stores in WAT. This potential scenario, however, could lead to ectopic lipid accumulation similar to lipodystrophy syndromes.

CLA increases inflammation. WAT not only functions to store TG, but produces a number of pro-inflammatory cytokines (i.e., interleukin [(IL)]-1β, IL-6, TNFα, and interferon [(IFN)]-γ) that cause insulin resistance and suppress lipid synthesis in adipocytes. Induction of these inflammatory genes is driven by MAPKs and transcription factors such as NFκB, which have been reported to antagonize PPARγ, thereby altering adipocyte function (**Fig. 7**). Indeed, TNFα stimulates lipolysis and inhibits lipogenesis (Kawakami et al. 1987). TNFα induces delipidation in adipocytes by suppression of glycerol 3-phosphate dehydrogenase (GPDH) and reduction of lipid droplet mass (Petruschke et al. 1993). Similarly, IL-1β and IFNγ were observed to induce delipidation of human adipocytes (Simons et al. 2007). Treatment with 10,12 CLA increases the expression or secretion of TNFα, IL-1β, IL-6, and IL-8 (Brown et al. 2004, Chung et al. 2005b, Poirier et al. 2006, LaRosa et al. 2006, Kennedy et al. 2009), which are known to

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antagonize PPARγ activity and insulin sensitivity, thereby causing delipidation (Chung et al. 2006, Kennedy et al. 2008, Lui et al. 2007, Purushotham et al. 2007). Similarly, 10,12 CLA decreased GPDH mRNA levels (Brown et al. 2004) and attenuated the TG content of newly differentiated human adipocytes (Brown et al. 2004, Kennedy et al. 2008) and 3T3-L1 adipocytes (LaRosa et al. 2007).

In humans, 10,12 CLA supplementation increase the levels of inflammatory prostaglandins (PG)s and cytokines (Steck et al. 2007, Riserus et al. 2002, Raff et al. 2008). For example, women supplemented with 5.5 g/d of a CLA mixture for 16 wk had higher levels of C-reactive protein in serum and 8-iso-PGF2α in urine (Tholstrup et al. 2008).   The expression of cyclooxygenase 2 (COX-2), an enzyme involved in the synthesis of PGs, was elevated in cultures of newly differentiated human adipocytes treated with 10,12 CLA (Kennedy et al. 2009). Furthermore, 10,12 CLA increased PGF2α secretion from human adipocytes (Kennedy et al. 2009). Inflammatory PGs such as $PGF_{2\alpha}$ have been reported to inhibit adipogenesis through phosphorylation of PPARγ (Liu et al. 2007, Reginato et al. 1998).

10,12 CLA induction of inflammatory genes appears to be dependent on the activation of MEK/ERK (Brown et al. 2004) and NFκB (Chung et al. 2005b, Poirier et al. 2006) signaling in adipocytes. Inflammatory signals or oxidative stress can induce translocation of NFκB (p65, p50) to the nucleus. NFκB can then bind directly to PPARγ or co-activators, or prevent co-repressor dispersion, thereby blocking DNA binding of the PPARγ/RXR heterodimer to target genes, thereby inhibiting adipogenesis (Takada et al.

2005, reviewed in Ye et al. 2008). Activation of ERK and NFκB were reported to be involved in 10,12 CLA suppression of adipogenic genes and insulin-stimulated glucose uptake (Brown et al. 2004, Chung et al. 2005b). Taken together, these data suggest that 10,12 CLA increases the production of inflammatory PGs and adipocytokines that antagonize PPARγ activity, leading to decreased adipogenesis or delipidation.

CLA regulation of apoptosis

CLA induces (pre)adipocyte apoptosis. 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 (**Fig. 8**). In rat mammary cancer cells, a 16-128 μM CLA mixture prevented rat mammary cancer cell growth through apoptosis and decreased DNA synthesis (IP et al. 1999). A number of *in vivo* and *in vitro* studies have reported apoptosis in adipocytes supplemented with a CLA mixture or 10,12 CLA alone (Evans et al. 2000, Miner et al. 2001, Hargrave et al. 2004, Moon et al. 2006, LaRosa et al. 2006). For example, supplementation of C57BL/6J mice with 1% (w/w) CLA mixture reduced BFM and increased apoptosis and TNFα gene expression in WAT (Tsuboyama-Kasaoka et al. 2000). TNFα gene expression and secretion has also been reported to be increased following supplementation of mice with 10,12 CLA alone (House et al. 2005, Poirier et al. 2006). TNFα is a major signaling cytokine involved in apoptosis (Wang et al. 1996) and adipocytes function (Cawthorn et al. 2007). *In vitro* studies have demonstrated that TNFα gene expression was acutely increased by 10,12 CLA, but secretion was not affected in cultures of newly

differentiated human adipocytes (Brown et al. 2004, Chung et al. 2005b). 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 (Liu et al. (2007).

CLA may induce apoptosis by activating ER stress and the integrated stress response (ISR). Microarray analysis demonstrated that treatment of mice with 1% (w/w) 10,12 CLA and 3T3-L1 adipocytes with 100 μmol/L 10,12 CLA increased the mRNA levels of genes involved in the ISR such as activating transcription factor 3 (ATF3), C/EBP homologous protein (CHOP), pseudokinase Tribbles 3/SKIP 3 (TRIB3), X-box binding protein (XBP-1), and growth arrest and DNA damage inducible protein (GADD34) (LaRosa et al. 2007). CHOP is known to possess apoptotic characteristics and activation of this protein can lead to induction of GADD34 and TRIB3 (Ohoka et al. 2005, Szegezdi et al. 2006). Notably, the ISR by CLA preceded the induction of inflammatory genes such as IL-6 and IL-8 in adipocytes (LaRosa et al. 2007). In mammary tumor cells, 10,12 CLA treatment (20-40 μM) increased CHOP expression and ER stress leading to apoptosis (Ou et al. 2008), further supporting CLA inducing apoptosis in WAT. Collectively, these data suggest that CLA may increase apoptosis in WAT, thereby decreasing adipocytes number via activating ER stress and the ISR, depending on the dose and isomer used. In vivo studies are needed demonstrating that these apoptotic effects of CLA are specific to WAT, and not found in other tissues.

Conclusion and Implications

Supplementation with a mixture of CLA isomers or 10,12 CLA alone consistently reduces adiposity in animal models, especially in rodents, but only in some humans. Potential reasons for these species differences include 1) the CLA isomers used (i.e., 10,12, but not 9,11, CLA reduces adiposity or TG content of WAT), 2) the dosage used (i. e., rodents consume ~20 times more CLA/kg body weight compared to human studies), and 3) age, body weight, body fat, or metabolic status of the animals or subjects. Potential mechanisms responsible for these anti-obesity properties of 10,12 CLA include 1) decreasing energy intake by suppressing appetite (Cao et al. 2007, So et al. 2009) or increasing energy expenditure via activation of AMPK and UCP in WAT, muscle, and liver tissue (West et al. 2000, Nagao et al. 2003, Terpstra et al. 2003, House et al. 2005, Choi et al. 2007, Ribot et al. 2007, Close et al. 2007, Wendel et al. 2007, So et al. 2008, Ferramosca et al. 2008), or LBM (Bhattacharya et al. 2005, Banu et al. 2006, 2007, 2008), 2) decreasing lipogenesis (Brown et al. 2001, Brown et al. 2003, Brown et al. 2004, Chung et al. 2005a), or increasing lipolysis (Park et al. 1997, Chung et al. 2005a, LaRosa et al. 2006), 3) decreasing adipogenesis (Park et al. 1997, Evans et al. 2000, Brown et al. 2003, Brown et al. 2004, House et al. 2005, Xu et al. 2003, LaRosa et al. 2006, Martin et al. 2008, Kennedy 2008, Platt et al. 2008) or increasing delipidation (Kang et al. 2003, Brown et al. 2004, LaRosa et al. 2006, Kennedy et al. 2009), or 4) increasing adipocyte stress, inflammation, and/or insulin resistance (Riseruet al. al. 2002a,b, Moloney et al. 2004, Chung et al. 2005a,b, Poirier et al. 2006, LaRosa et al. 2006, LaRosa et al. 2007, Kennedy et al. 2009), leading to apoptosis (Evans et al. 2000,

Miner et al. 2001, Tsuboyama-Kasaoka et al. 2000, Moons et al. 2006, LaRosa et al. 2006, LaRosa et al. 2007, Lui et al. 2007).

Based on these data, we propose the following working model (**Fig. 9**) depicting how CLA decreases WAT mass. We speculate that 10,12 CLA either binds to a cell surface FA or G protein coupled receptor or passively diffuses into the adipocyte, activating a stress signal that increases intracellular calcium ($[Ca^{2+}]_i$) and reactive oxygen species (ROS), which in turn activates NFκB and MAPKs and increase FFA, PG, and adipocytokine release that together antagonize PPARγ activity, leading to insulin resistance, delipidation, and possibly apoptosis. These events result in ectopic FFA accumulation in blood, liver, and muscle, thereby enhancing FFA availability for oxidation at the expense of causing FFA-induced insulin resistance in these tissues. In the absence of enhancing energy expenditure to oxidize these elevated levels of FFAs, hyperlipidemia, hyperglycemia, and lipodystrophy, ensue. Future studies are needed to identify these potential upstream candidates activated by CLA that may cause this proposed stress cascade in adipocytes. Elucidating this potential mechanism will provide valuable information on the efficacy, specificity, and potential side effects of CLA isomers as dietary strategies for weight loss or maintenance. Lack of such knowledge is an important problem, because until it becomes available, the effective and safe use of CLA supplements to control obesity cannot be recommended.

Chapters III-V will address the central hypothesis for this research that 10,12 CLA elevates the levels of $[Ca^{2+}]_i$ and ROS, which lead to the activation of

calcium/calmodulin-dependent protein kinase II (CAMKII), MEK/ERK, and NFκB-induced inflammation, resulting in the suppression of PPARγ activity and target gene expression, insulin resistance, and delipidation in primary cultures of human adipocytes.

To test this hypothesis, the following three specific aims were investigated using primary cultures of newly differentiated human adipocytes.

1. Determine the mechanism by which 10,12 CLA suppresses the expression of PPARγ and its target genes (Chapter III).

2. Identify upstream mechanism(s) by which 10,12 CLA induces inflammation, and insulin resistance (Chapter IV).

3. Determine the extent to which resveratrol prevents 10,12 CLA induced cellular stress, inflammation, insulin resistance and delipidation (Chapter V).

Figure 2.1 10,12 CLA regulation of energy balance.
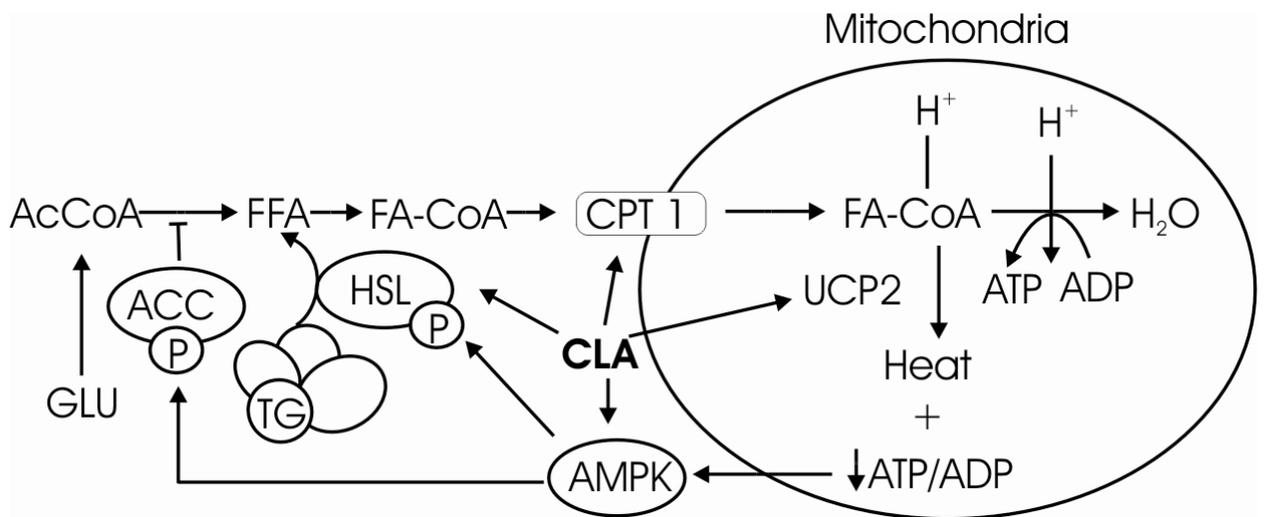
Figure 2.2. 10,12 CLA increases energy expenditure.
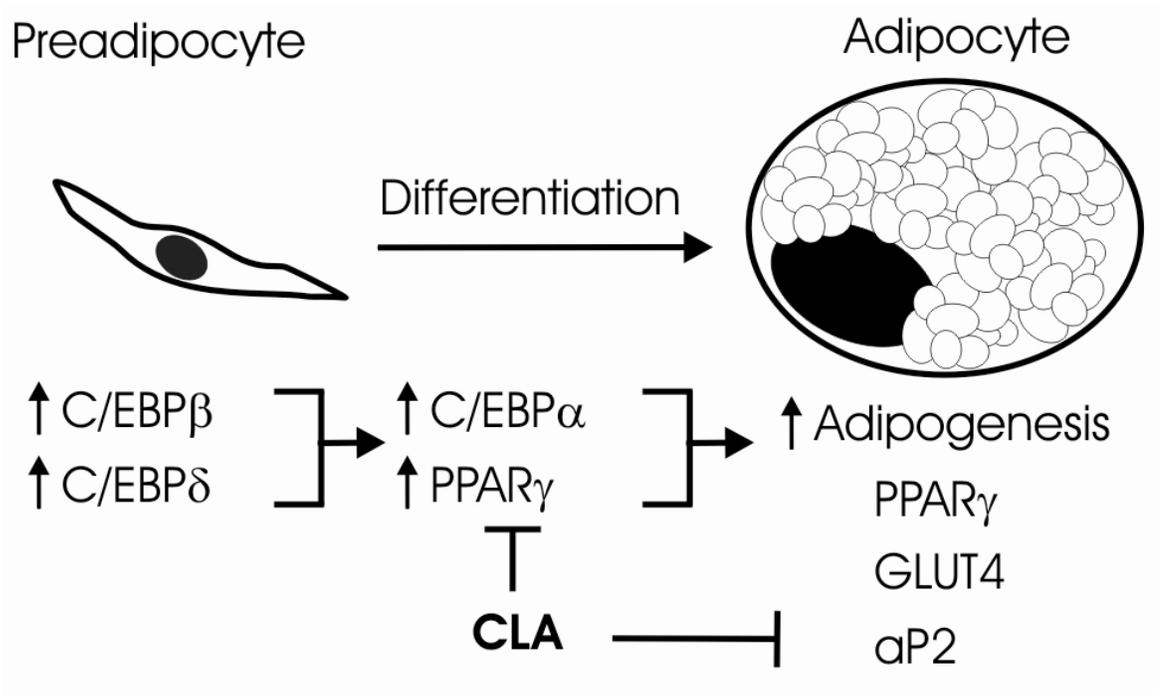
Figure 2.3. 10,12 CLA inhibits adipogenesis.
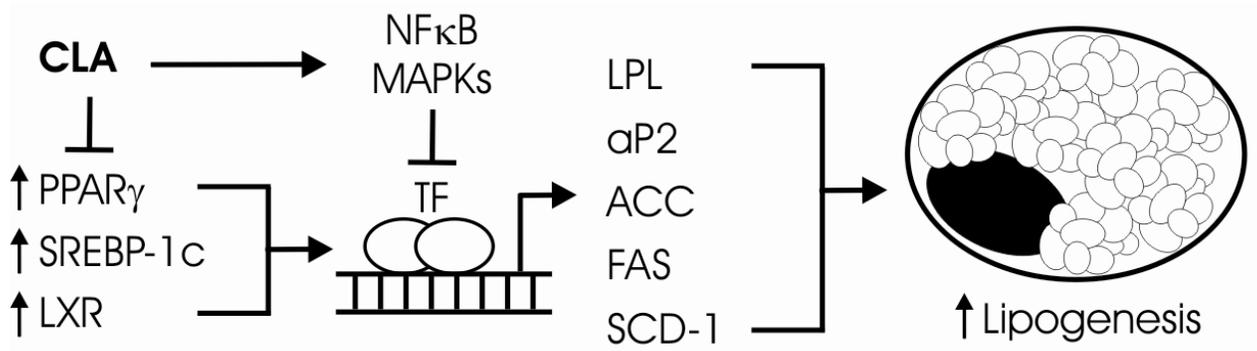
Figure 2.4. 10,12 CLA suppresses lipogenesis.
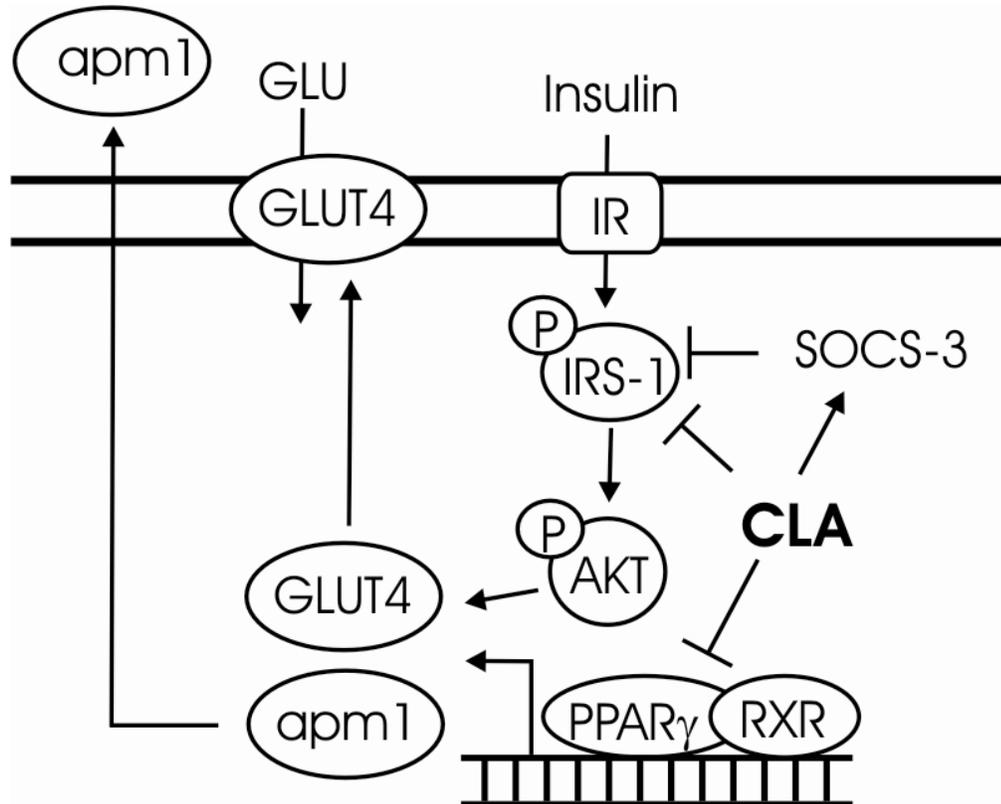
Figure 2.5. 10,12 CLA inhibits insulin signaling.
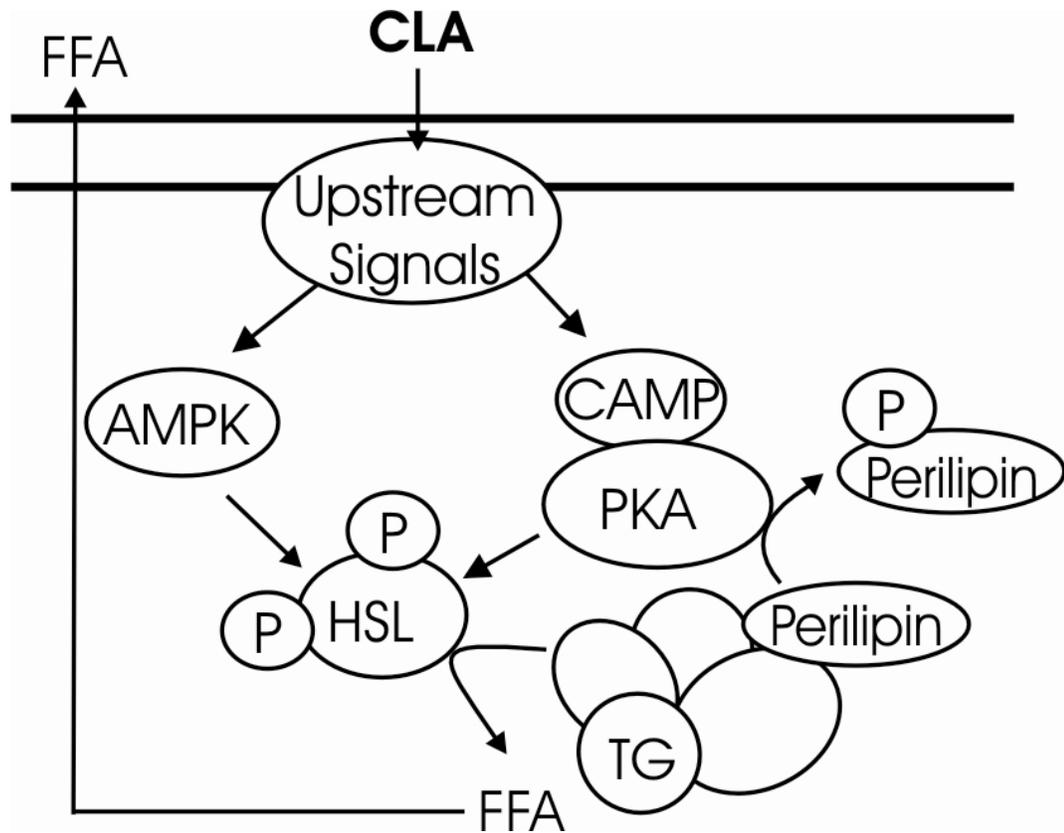
Figure 2.6. 10,12 CLA stimulates lipolysis.
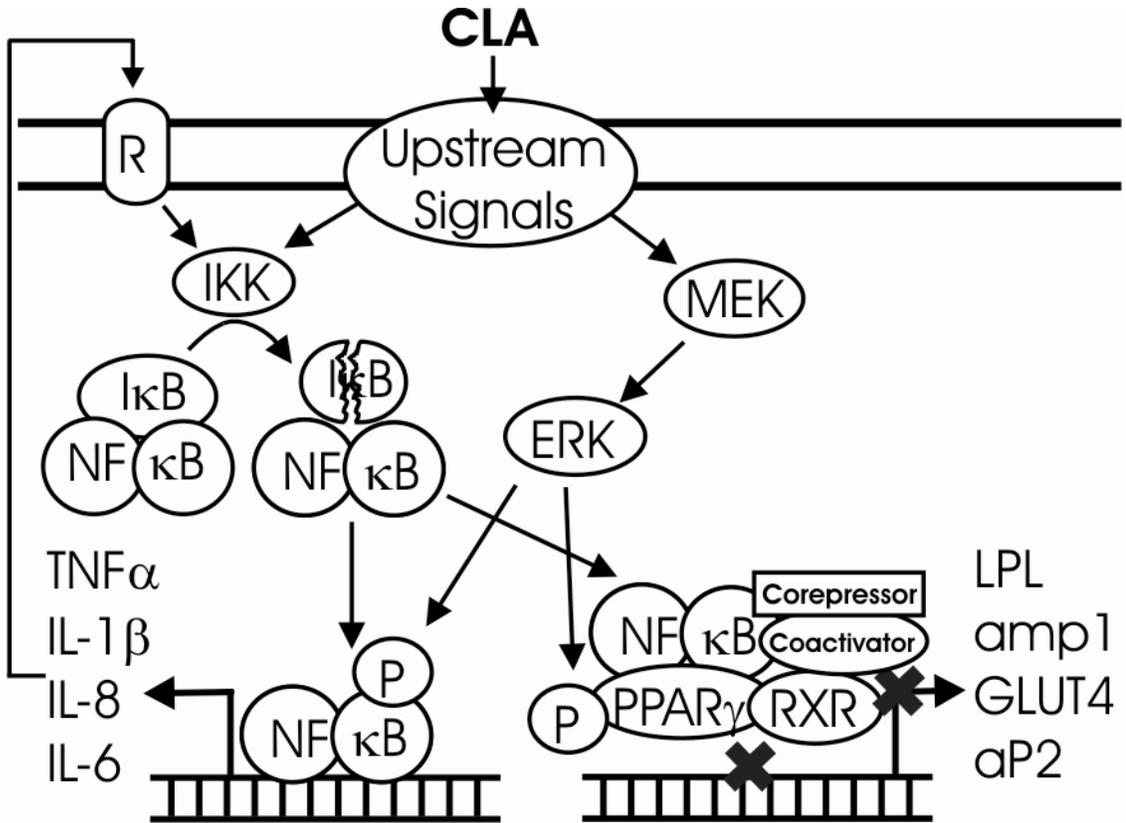
Figure 2.7. 10,12 CLA increases inflammation.
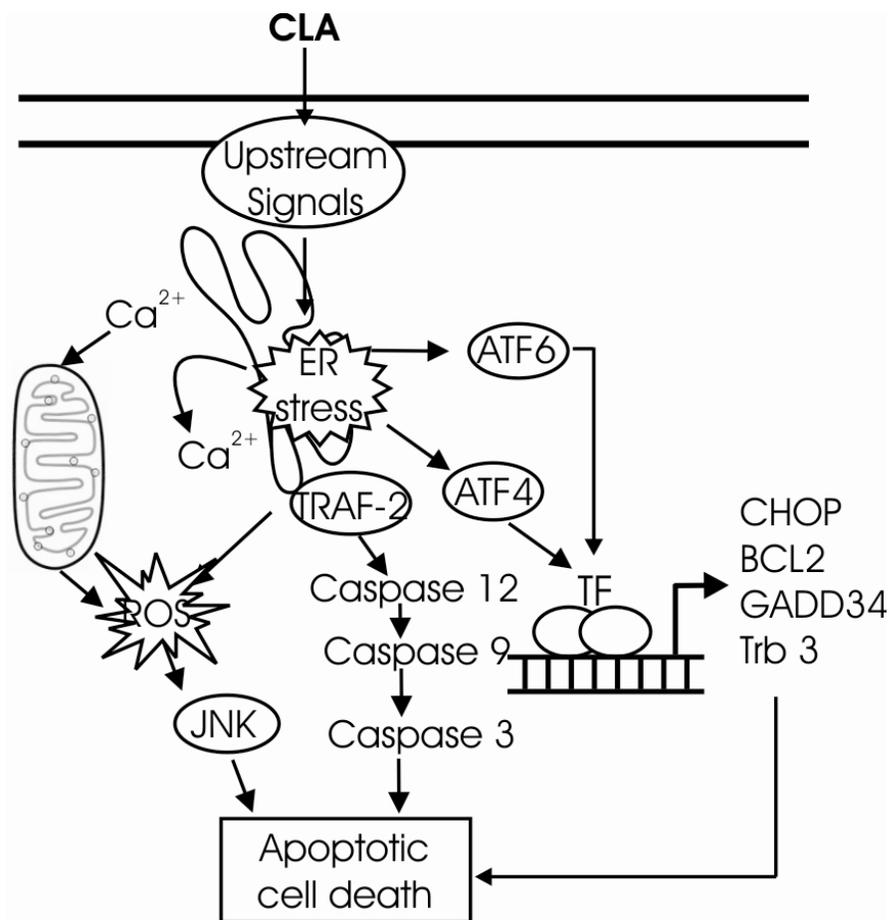
Figure 2.8. 10,12 CLA induces apoptosis.
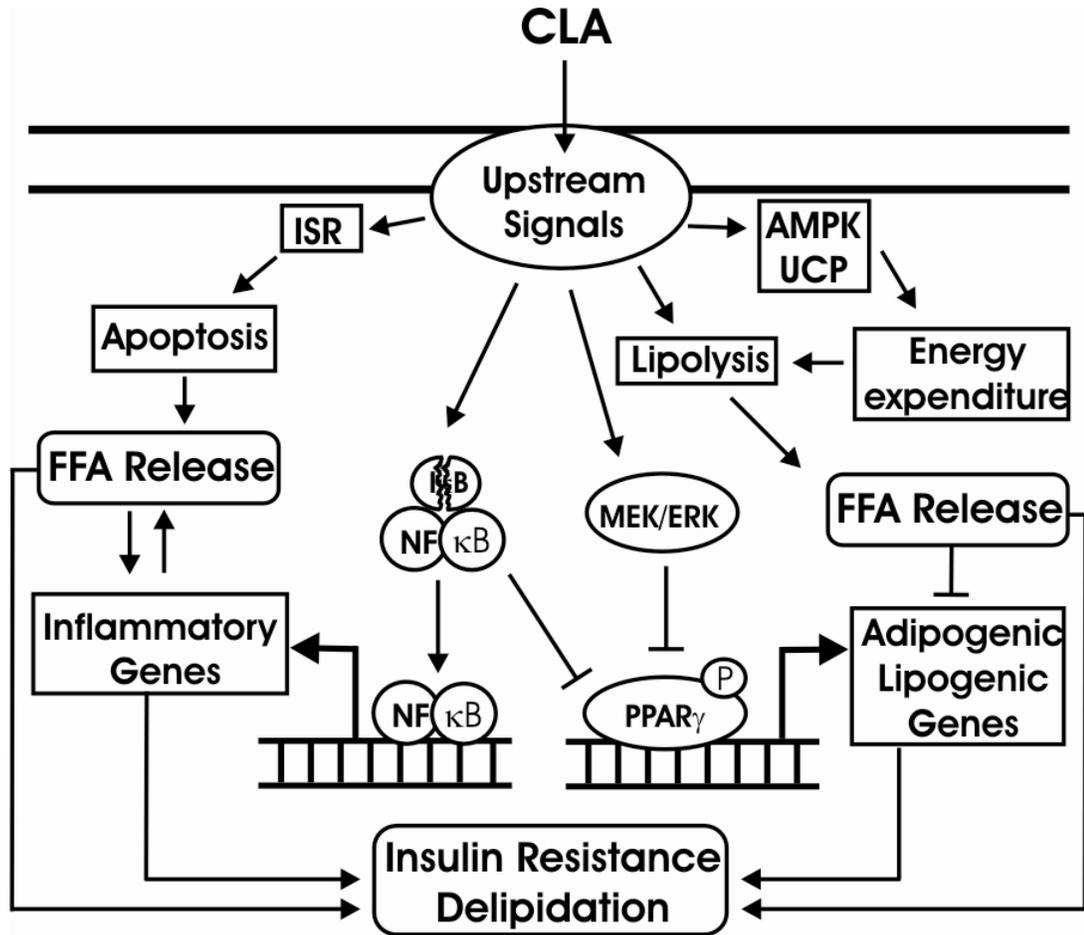
Figure 2.9. Working Model-10,12 CLA mechanism of action in adipocytes.

CHAPTER III

TRANS-10, CIS-12 CONJUGATED LINOLEIC ACID ANTAGONIZES LIGAND -DEPENDENT PPARγ ACTIVITY IN PRIMARY CULTURES OF HUMAN ADIPOCYTES

The following chapter was previously published in the Journal of Nutrition volume 138, pages 455 to 461, in 2008. The coauthors of the article were Soonkyu Chung, Kathy LaPoint, Oluwatoyin Fabiyi, and Michael McIntosh. Permission from the publisher to use the article in its entirety can be found in Appendix A. References from this article can be found in the Reference section.

Abstract

We previously demonstrated that trans-10, cis-12 (10,12) conjugated linoleic acid (CLA) causes human adipocyte delipidation, insulin resistance, and inflammation in part by attenuating peroxisome proliferator activated receptor (PPAR) γ target gene expression. We hypothesized that CLA antagonizes the activity of PPARγ in an isomer-specific manner. 10,12 CLA, but not cis-9, trans-11 (9,11) CLA, suppressed ligand-stimulated activation of a peroxisome proliferator response element (PPRE)-luciferase reporter. This decrease in activation of PPARγ by 10,12 CLA was accompanied by an increase in PPARγ and extracellular-signal related kinase (ERK)1/2 phosphorylation, followed by decreased PPARγ protein levels. To investigate if 10,12 CLA-mediated delipidation was preventable with a PPARγ ligand (BRL), cultures were treated for 1 wk with 10,12 CLA or 10,12 CLA + BRL and adipogenic gene and protein expression, glucose uptake, and triglyceride (TG) were measured. BRL co-supplementation completely prevented 10,12 CLA suppression of adipocyte fatty acid binding protein (aP2), lipoprotein lipase (LPL), and perilipin mRNA levels without preventing reductions in PPARγ or insulin-dependent glucose transporter 4 (GLUT4) expression, glucose uptake, or TG. Lastly, the impact of CLA withdrawal in the absence or presence of BRL for 2 wk was investigated. CLA withdrawal did not rescue CLA-mediated reductions in adipogenic gene and protein expression. In contrast, BRL supplementation for 2 wk following CLA withdrawal rescued mRNA levels of PPARγ target genes. However, the levels of PPARγ and GLUT4 protein and TG were only partially rescued by BRL.

Collectively, we demonstrate for the first time that 10,12 CLA antagonizes ligand-dependent PPARγ activity, possibly via PPARγ phosphorylation by ERK.

Introduction

Dysfunction of adipose tissue can result in insulin resistance and lipodystrophy. One major regulator in the development and function of adipose tissue is peroxisome proliferation-activated receptor (PPAR)γ, which induces the expression of a host of adipogenic genes such as lipoprotein lipase (LPL), insulin-stimulated glucose transporter 4 (GLUT4), perilipin (PLIN), and adipocyte fatty acid binding protein (aP2). Mutations of PPARγ in humans are associated with insulin resistance and lipodystrophy (Barroso et al. 1999, Savage et al. 2003). PPARγ null cells exhibit impaired adipogenesis (Rosen et al. 1999) and dominant negative mutations in PPARγ inhibit adipogenesis (Gurnell et al. 2000). Thus, PPARγ activity is essential in adipose tissue for glucose uptake and TG accumulation.

Regulation of PPARγ occurs through a variety of proposed mechanisms including 1) covalent modification by phosphorylation, 2) ligand binding, and 3) heterodimerization with the retinoic acid receptor (RXR) (Mandrup et al. 1997, Hamm et al. 1999). Phosphorylation of PPARγ by activation of the mitogen-activated-protein kinase (MAPK) pathway has been reported to inhibit adipogenesis (Diradourian et al. 2005). It has been demonstrated that phosphorylation of serine 112 of PPARγ2 results in its ubiquination and proteosome degradation (Hu et al. 1996). Activation of PPARγ by natural (i.e., polyunsaturated fatty acids, PUFAs) or synthetic ligands such as

Thiazolidinediones (TZDs) initiates heterodimerization with RXR followed by their binding to PPREs in the promoters of adipogenic target genes. The TZDs are hypoglycemics that activate PPARγ, leading to upregulation of adipogenic genes, thereby enhancing insulin sensitivity. Natural ligands of PPARγ2 such as cis- PUFAs or prostaglandins (PG)s such as PGJ2 (Shiraki et al. 2006) have a relatively low affinity for PPARγ compared to TZDs. In contrast, saturated fatty acids and certain trans PUFAs such as conjugated linoleic acid (CLA) have been reported to impair insulin sensitivity, possibly by decreasing the expression of PPARγ and many of its downstream target genes (Brown et al. 2003, Brown et al. 2004, Kang et al. 2003, Granlund et al. 2003).

CLA consists of dienoic isomers of linoleic acid, including trans-10, cis-12 CLA and cis-9, trans-11 CLA. CLA decreases body fat mass in animals (House et al. 2005) and some humans (Larsen et al. 2003). Our group has demonstrated that trans-10, cis-12 CLA decreases adipogenic gene expression and the triglyceride (TG) content of human (pre)adipocytes (Brown et al. 2003, Brown et al. 2004). We have also demonstrated that activation of mitogen-activated protein kinase kinase/extracellular signal-related kinase (MEK/ERK) (Brown et al. 2004) and nuclear factor κB (NFκB) (Chung et al. 2005) signaling by trans-10, cis-12 CLA were essential for its suppression of adipogenic gene expression and delipidation in human adipocytes. A number of side effects have been associated with trans-10, cis-12 CLA supplementation in humans such as insulin resistance, hyperglycemia, and dyslipidemia (Riserus et al. 2002, Riserus et al. 2004). Dyslipidemia, insulin resistance, and hyperglycemia are similar characteristics found in humans with mutations in PPARγ. Two recent reports by Belury's group (Lui et al.

2007, Purushotham et al. 2007) show that the PPARγ agonist Rosiglitazone prevents or attenuates inflammation, lipodystrophy, and insulin resistance in mice fed a crude mixture of CLA isomers containing equal amounts of cis-9, trans-11 CLA and trans-10, cis-12 CLA. However, the isomer-specific mechanism by which CLA suppresses the expression of PPARγ and its target genes in human adipocytes remains to be elucidated. To address this issue, we examined the impact of CLA on PPARγ in the absence and presence of the PPARγ ligand Rosiglitazone (BRL).

Materials and Methods

Materials

All cell culture ware were purchased from Fisher Scientific (Norcross, GA). Western lightning chemiluminescence substrate was purchased from Perkin Elmer Life Science (Boston, MA). One-step reverse transcription-polymerase chain reaction (RT-PCR) kit used in semi-quantitative mRNA analysis was purchased from Qiagen, Inc (Valencia, CA). Immunoblotting buffers, precast gels and gene-specific primers were purchased from Invitrogen (Carlsbad, CA), and ribosomal 18S competimer technology internal standards and DNA-free were purchased from Ambion (Austin, TX). Polyclonal GLUT4 antibody was a gift from Drs. S. Cushman and X. Chen (NIDDK, NIH, Bethesda, MD). aP2 antibody was a gift from Dr. D. Bernlohr (University of Minnesota). Monoclonal antibodies for PPARγ (sc7273) and polyclonal antibodies for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc20357) and β-actin (sc1616) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho (Thr-

202/204) and total ERK antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Cy3- and FITC-conjugated IgG were purchased from Jackson Immunoresearch (West Grove, PA). Fetal bovine serum (FBS) was purchased from Cambrex/BioWhittaker (Walkersville, MD). BRL was a gift from Glaxo Smith Kline. Isomers of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA). The Nucleofactor and Dual Glo luciferase kits were obtained from Amaxa (Cologne, Germany) and Promega (Madison, WI), respectively. All other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue (WAT) was obtained from non-diabetic females, between the ages of 20-50 years old with a body mass index (BMI) $\leq$ 30 during abdominoplasty with consent from the Institutional Review Board at the University of North Carolina at Greensboro. Tissue was digested using collagenase and stromal vascular (SV) cells were isolated as previously described (Brown et al. 2004). Experimental treatment of cultures containing ~50% preadipocytes and ~50% adipocytes occurred on day 12 of differentiation. Each experiment was done in duplicate and repeated at least three times using a mixture of cells from 2-3 subjects unless otherwise indicated.

Preparation of fatty acids

Both isomers of CLA were complexed to fatty acid-free (≥98%) bovine serum albumin (BSA) at a 4:1 molar ratio using 1 mmol/L BSA stocks.

Immunoblotting

Immunoblotting was conducted as we previously described (Brown et al. 2004). To resolve PPARγ phospho-proteins, total cell extracts (75 ug protein) were subjected to 10% SDS-PAGE (acrylamide:bisacrylamide 100:1 w/w) containing 4 M urea and to electrophoresis at 80 V for 20 h as we previously described (Chung et al. 2006). Separated proteins were subsequently transferred to PVDF membranes and immunoblotted with a monoclonal PPARγ antibody. For determining the phosphorylation status of PPARγ, a portion of the cell extracts from BSA vehicle and CLA treatment were incubated with 20 U of calf intestinal phosphatase (Cip) for 30 min at 37°C and for 15 min at 55°C. Subsequently, the samples were subjected to SDS-PAGE containing urea as described above.

Immunostaining of PPARγ

Cells were cultured on coverslips for immunofluorescence microscopy and stained as described previously (Brown et al. 2004) except for the permeablization step. Fixed cells were permeabilized with 0.1% Triton X-100 for 1 min on ice. Monoclonal anti-PPARγ (1:10) were incubated overnight at 4ºC. Fluorescent images were captured with a SPOT digital camera mounted on an Olympus BX60 fluorescence microscope.

Transient transfections of human adipocytes

For measuring PPARγ activity, primary human adipocytes were transiently transfected with the multimerized PPAR-responsive luciferase (luc) reporter construct pTK-PPRE3x-luc (Kliewer et al. 1992) using the Amaxa Nucleofactor as previously described (Chung et al. 2006). On day 6 of differentiation, 1 x 10^6 cells from a 60 mm plate were trypsinized and resuspended in 100 μL of nucleofector solution (Amaxa) and mixed with 2 μg of pTK-PPRE3x-luc and 25 ng pRL-CMV for each sample. Electroporation was performed using the V-33 nucleofector program (Amaxa). Cells were replated in 96-well plates after 10 min recovery in calcium-free RPMI media. Firefly luciferase activity was measured using the Dual-Glo luciferase kit and normalized to *Renilla* luciferase activity from the co-transfected control pRL-CMV vector. All luciferase data are presented as a ratio of firefly luciferase to Renilla luciferase activity. We consistently obtained ~75% transfection efficiency revealed by parallel transfections with a green fluorescent protein reporter construct. Both adipocytes and non-adipocytes were transfectable using this protocol based on aP2 immunostaining and DAPI nuclear staining.

RNA analysis

Following treatment, cultures were harvested for total RNA using Tri-Reagent according to manufacturer's protocol. Contaminating DNA was removed with DNAase (DNA-free, Ambion). 1 ug of RNA from each sample were used for semi-quantitative RT-PCR using the One-Step RT-PCR kit (Qiagen) as previously described in (Brown et

al. 2003). The gene specific primer pairs used were previously described (Brown et al. 2003).

Lipid staining

Lipid staining of cultures of human adipocytes was conducted as previously described (Brown et al. 2003) using Oil-Red-O (ORO).

[^{3}H] 2-deoxy-glucose uptake

Newly-differentiated cultures of adipocytes were incubated with BSA vehicle, 30 umol/L cis-9, trans-11 CLA, 30 umol/L trans-10, cis-12 CLA, 30 umol/L trans-10, cis-12 CLA + 1 umol/L BRL, or 1 umol/L BRL in adipocyte media for 2 d. Then, for an additional 2 d, cultures were incubated in 1 ml of serum-free basal DMEM containing 1,000 mg/liter D-(+)-glucose with or without 20 pmol/L of human insulin with BSA vehicle, 30 umol/L cis-9, trans-11 CLA, 30 umol/L trans-10, cis-12 CLA, 30 umol/L trans-10, cis-12 CLA + 1 umol/L BRL, or 1 umol/L BRL in adipocyte media for another 2 d. Following the experimental treatments, insulin-stimulated uptake of [3H]-2-deoxy-glucose was measured following a 90 min incubation in the presence of 100 nmol/L human insulin as described previously (Chung et al. 2005).

Statistical analysis

Statistical analyses were performed for data in Figure 3.1 testing the main effects of BRL and CLA and the interaction of the two (BRL x CLA) using two-way ANOVA

(JMP Version 6.03, SAS Institute; Cary, NC). Analyses for statistically significant differences for data in Figure 3.4C were conducted using one-way ANOVA. Student's t tests were used to compute individual pairwise comparisons of least square means (P<0.05). Data are expressed as the means ± S.E.

Results

Trans-10, cis-12 CLA decreases the activity and increases phosphorylation of PPARγ.

To determine the extent to which CLA decreased PPARγ activity, basal and ligand-induced activation of PPARγ activity were examined. There were no significant differences in basal levels of PPARγ activity due to CLA treatment in the absence of BRL (Fig. 3.1). However, PPARγ activity in BRL-stimulated cultures (+BRL) was lower in cultures treated with 30 umol/L trans-10, cis-12 CLA compared to control and 30 umol/L cis-9, trans-11 CLA-treated cultures. The extent to which trans-10, cis-12 CLA decreased PPARγ activity (~40%) was comparable to that of PPARγ antagonist GW9662, which inhibited ligand-induced PPARγ activity without impacting basal activity (data not shown).

Given the inverse relationship between PPARγ activity and its phosphorylation status (Diradourian et al. 2005), we wanted to determine the kinetics of PPARγ phosphorylation during treatment with trans-10, cis-12 CLA. Trans-10, cis-12 CLA caused a band shift in PPARγ1/2 after 24 h treatment (Fig. 3.2A). Intriguingly, robust ERK1/2 phosphorylation at 24 h accompanied the PPARγ1/2 band shift, consistent with

ERK1/2's role as a donor of phosphate groups to nuclear PPARγ1/2, and with our published data demonstrating that ERK1/2 is required for CLA's suppression of adipogenic gene expression and glucose uptake (Brown et al. 2004). However, because a PPARγ band shift could be due to processes other than phosphorylation (e.g., by acetylation, methylation, or sumylation), calf intestinal alkaline phosphatase (Cip) was added to the cell extracts to remove phosphorylated groups. Trans-10, cis-12 CLA-induced band shifts of PPARγ1/2 were either lowered or attenuated by phosphatase treatment (Fig. 3.2B). Taken together, these data suggest that trans-10, cis-12 CLA promotes PPARγ and ERK phosphorylation, which contributes, at least in part, to CLA's isomer-specific reduction of PPARγ activity.

Trans-10, cis-12 CLA decreases the protein levels of PPARγ.

We previously demonstrated that a physiological level (e.g., 30 umol/L) of trans-10, cis-12 CLA decreased the mRNA levels of PPARγ and several of its target genes in differentiating cultures of human SV cells (Brown et al. 2003), and in the newly-differentiated cultures of human adipocytes (Brown et al. 2004). However, the isomer-specific impact of CLA on PPARγ protein levels in human adipocytes is unknown. PPARγ2 protein levels were decreased after 4 d and undetectable after 6 d of treatment with trans-10, cis-12 CLA compared to the BSA vehicle or cis-9, trans-11 CLA-treated cultures (Fig. 3.3A). Consistent with these data, newly-differentiated cultures treated with 30 umol/L trans-10, cis-12 CLA for 4 d had dramatically less nuclear PPARγ staining compared to BSA vehicle-treated cultures (Fig. 3.3B). These data demonstrate

that trans-10, cis-12 CLA decreases PPARγ protein levels in an isomer-specific manner in newly-differentiated human adipocytes.

Chronic effects of a trans-10, cis-12 CLA in the presence of a PPARγ ligand.

To further evaluate the antagonistic effects of trans-10, cis-12 CLA on PPARγ activity, we examined the extent to which co-supplementation with the PPARγ agonist BRL could prevent trans-10, cis-12 CLA suppression of adipogenic genes and proteins, glucose uptake, and TG accumulation. Trans-10, cis-12 CLA decreased the mRNA (Fig. 3.4A) and protein (Fig. 3.4B) levels of PPARγ, aP2, LPL, and GLUT4 compared to BSA vehicle controls or cis-9, trans-11 CLA. Although BRL co-supplementation prevented CLA-mediated reductions in aP2, LPL, and PLIN gene expression, it did not prevent CLA suppression of PPARγ2 or GLUT4 mRNA levels (Fig. 3.4A). Consistent with these data, BRL co-supplementation prevented CLA suppression of aP2 protein expression, but did not prevent CLA suppression of PPARγ or GLUT4 protein levels (Fig. 3.4B).

CLA isomer-specific reduction of insulin-stimulated glucose uptake (Fig. 3.4C) or TG accumulation (Fig. 3.4D) was not prevented by co-supplementation with BRL. Collectively, these data demonstrate that trans-10, cis-12 CLA chronically suppresses adipogenic gene and protein expression, glucose uptake, and TG content, which are only partially prevented by a PPARγ ligand.

Effects of withdrawal from trans-10, cis-12 CLA in presence of a PPARγ ligand.

Next, we wanted to determine if the delipidating effects of CLA could be rescued by CLA withdrawal in the absence or presence of a PPARγ ligand. Surprisingly,

withdrawal of trans-10, cis-12 CLA treatment for 2 wk did not restore the mRNA levels of LPL, PLIN, or GLUT4 gene (Group1, Fig. 3.5A) or the protein levels of PPARγ or GLUT4 (Group 1, Fig. 3.5B). Interestingly, the pattern of gene and protein expression in Group 1 was almost identical to that of the cultures treated for 1 wk with trans-10, cis-12 CLA (Fig. 3.4), indicating the effects of CLA were sustained over 2 wk. Consistent with these gene and protein data, cultures treated with trans-10, cis-12 CLA had less stainable TG 2 wk after withdrawal (Group 1, Fig. 3.5C) compared to controls.

BRL supplementation for 2 wk following CLA withdrawal rescued PPARγ, aP2, and LPL gene expression compared to BSA vehicle- or cis-9, trans-11 CLA-treated cultures, while PLIN and GLUT4 were partially rescued (Group 2, Fig. 3.5A). BRL supplementation for 2 wk following CLA withdrawal reversed or attenuated trans-10, cis-12 CLA suppression of aP2 and GLUT4 protein levels, respectively, compared to cultures not receiving BRL for 2 wk (Group 1). Although CLA-treated cultures supplemented for 2 wk with BRL (Group 2) had more PPARγ protein compared to those not receiving BRL for 2 wk (Group 1), PPARγ protein levels did not return to the levels of the BSA vehicle- or cis-9, trans-11 CLA-treated cultures. Similarly, supplementation of cultures with BRL for 2 wk during CLA withdrawal (Group 2) marginally increased TG content of cultures treated with trans-10, cis-12 CLA compared to cultures not receiving BRL during withdrawal (Group 1). Interestingly, BRL was only effective in preventing delipidation when it was co-supplemented with CLA and then supplemented for another 2 wk following CLA withdrawal (Group 2, Fig. 3.5C). Taken together, these data demonstrate that trans-10, cis-12 CLA-mediated delipidation persists after CLA

withdrawal, and is relatively refractory to supplementation with a PPARγ ligand unless the ligand is supplemented during and after CLA treatment.
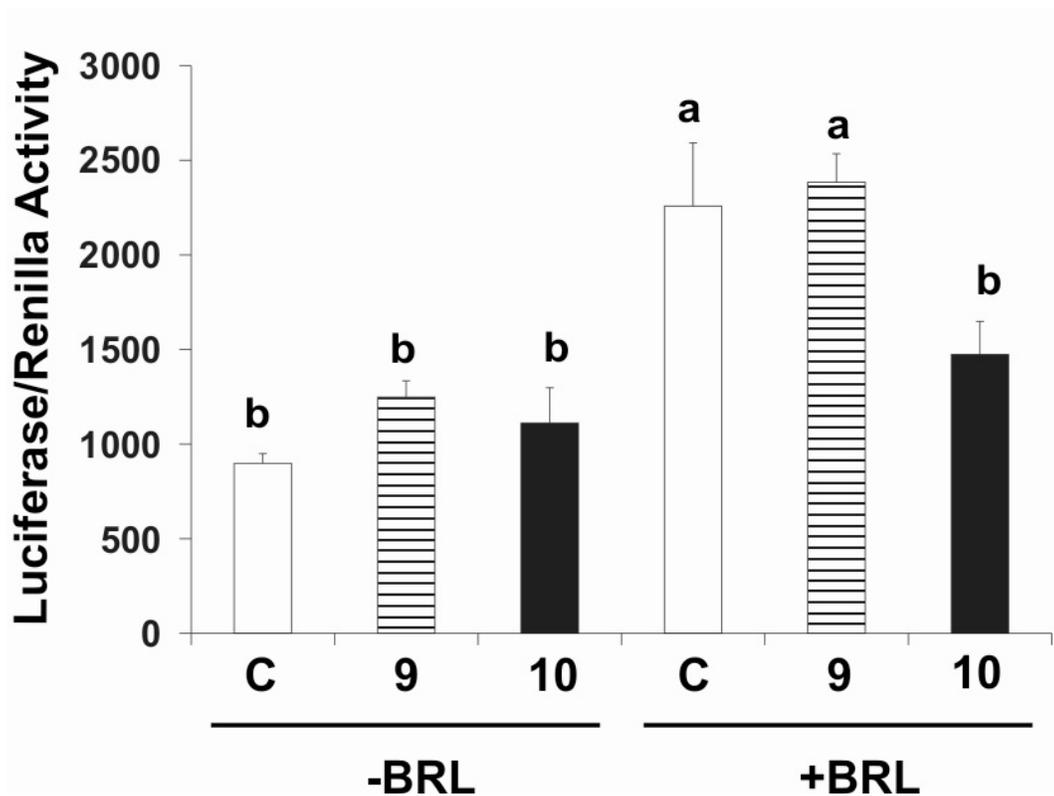
Figure 3.1.   10,12 CLA blocks ligand-induced activation of PPARγ. Cultures of newly-differentiated human adipocytes were transfected on day 6 with pTK-PPRE3x-luc and pRL-CMV. Twenty four hours later, transfected cells were treated with DMSO vehicle control (C), 30 umol/L trans-10, cis-12 CLA, or 30 umol/L cis-9, trans-11 CLA in the absence or presence of 0.1 umol/L BRL for 24 h.   (± SEM, n=3).
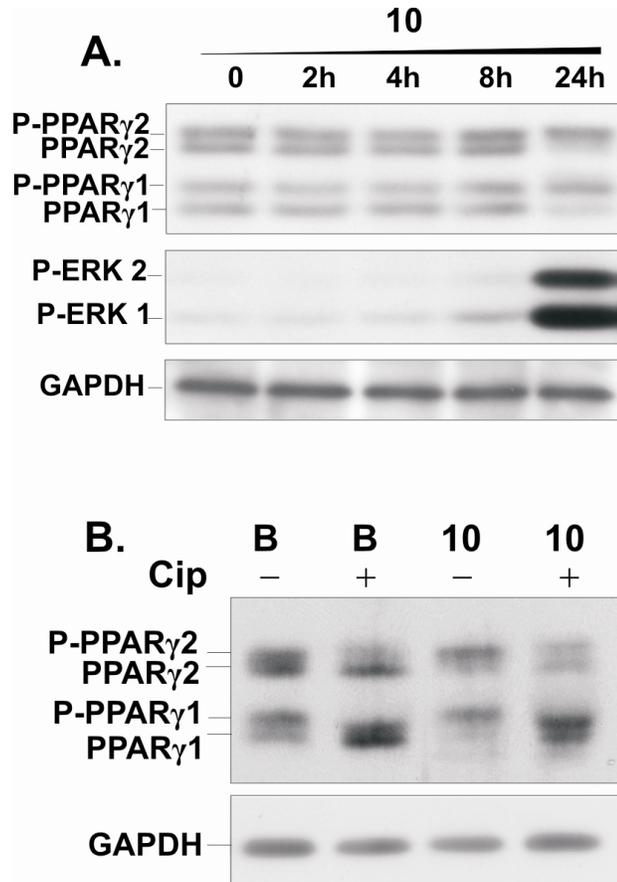
Figure 3.2. 10,12 CLA increases PPARγ phosphorylation. A: Cultures of newly-differentiated human adipocytes were serum-starved for 24 h and then treated without (0) or with 30 umol/L trans-10, cis-12 CLA (10) for 2, 4, 8, or 24 h. Subsequently, cell extracts were harvested, proteins separated by SDS-PAGE-urea, and immunoblotted for the phosphorylated and unphosphorylated forms of PPARγ1/2, ERK1/2, and GAPDH (load control). B: Cultures were treated for 24 h with BSA vehicle (B) or 30 umol/L trans-10, cis-12 CLA (10). A portion of the cell extracts from BSA vehicle and CLA treatment were incubated with calf intestinal phosphatase (Cip). Proteins were separated with SDS-PAGE-urea, and probed with antibodies targeting PPARγ1/2 and GAPDH. Data in Panels A and B are representative of 2 independent experiments.
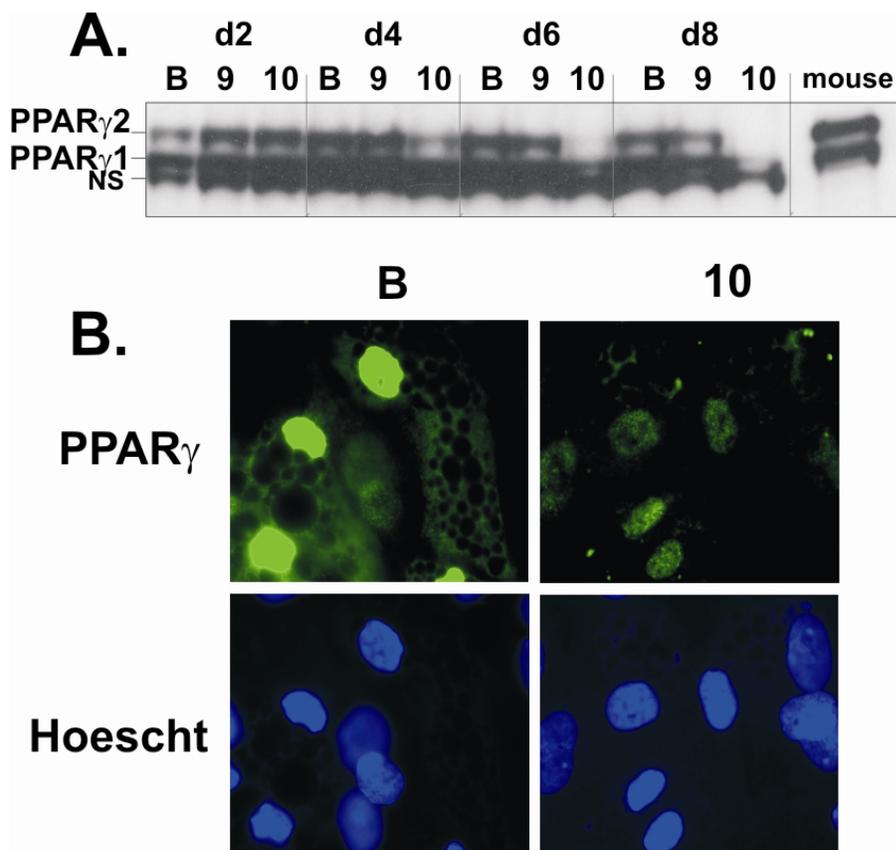
Figure 3.3. 10,12 CLA decreases PPARγ protein levels. A: Cultures of newly-differentiated human adipocytes were treated with BSA vehicle (B), 30 umol/L cis-9, trans-11 CLA (9), or 30 umol/L trans-10, cis-12 CLA (10) for 2, 4, 6, or 8 d. Cells extracts were immunoblotted for PPARγ. To identify PPARγ1/2 in cultures of human adipocytes, cell extracts from 3T3-L1 adipocytes (mouse) were isolated and immunoblotted for PPARγ. A third band was identified in human adipocytes and labeled as nonspecific (NS). B: Cultures were treated with BSA vehicle (B) or 30 umol/L trans-10, cis-12 CLA (10) for 4 d. PPARγ was detected using immunofluoresence microscopy. Data are representative of 2 independent experiments.
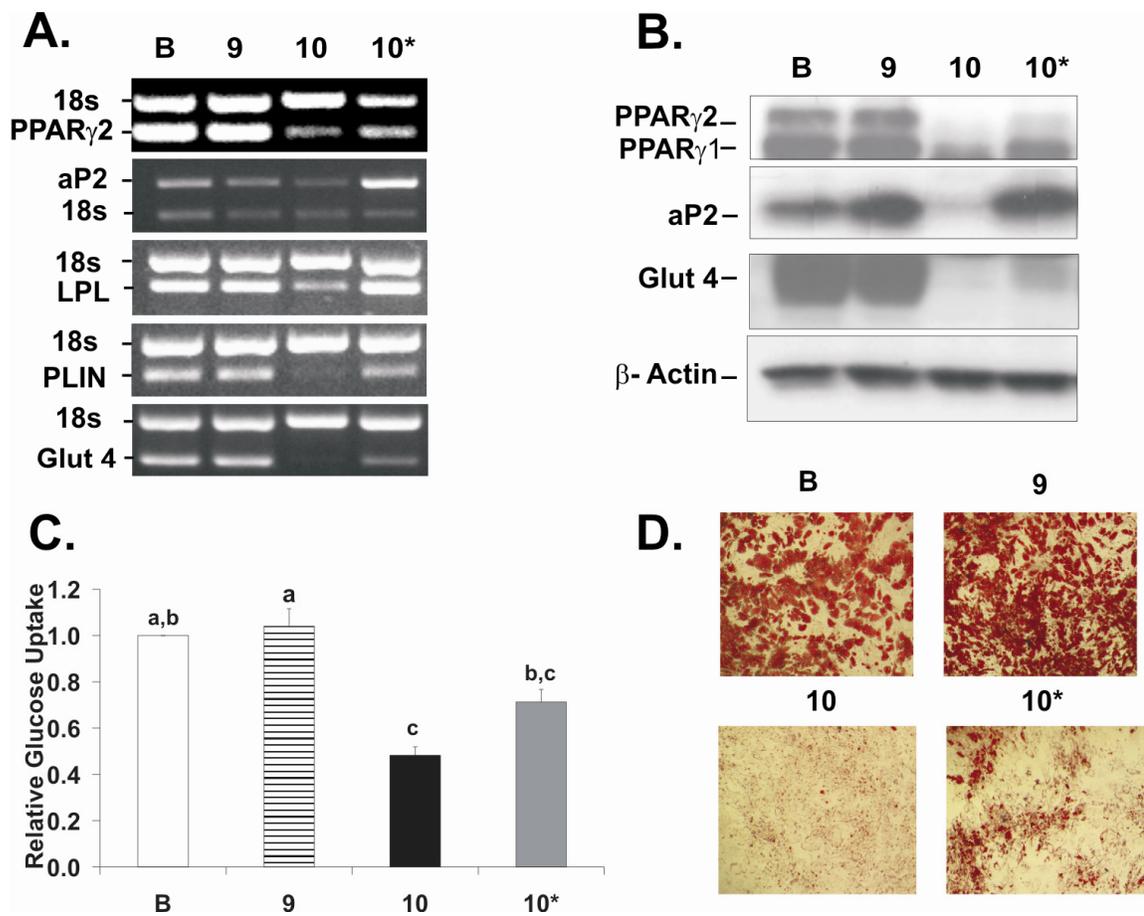
Figure 3.4. 10,12 CLA antagonizes ligand-activated PPARγ expression, glucose uptake, and TG accumulation. A: Cultures of newly-differentiated human adipocytes were treated for 1 wk with either BSA vehicle (B), 30 umol/L cis-9, trans-11 CLA (9), 30 umol/L trans-10, cis-12 CLA (10), or 30 umol/L trans-10, cis-12 CLA + 1 umol/L BRL (10*) and then harvested. RNA was isolated and the mRNA levels of PPARγ2, adipocyte-specific fatty acid binding protein (aP2), lipoprotein lipase (LPL), perilipin (PLIN), and insulin-sensitive glucose transporter 4 (Glut 4) were measured using semi-quantitative RT-PCR. 18S rRNA was used as an internal control. B: Cultures were treated as in Panel A for 1 wk and then cell extracts were isolated and immunoblotted for PPARγ, aP2, Glut4, and β-actin. C: Cultures were treated as in Panel A for 4 d and then insulin-stimulated uptake of [3H]-2-deoxy-glucose was measured. Means ($\pm$ SEM; n=6). D: Cultures were treated as in Panel A for 1 wk and then stained with oil Red O and phase-contrast photomicrographs were taken using an Olympus inverted microscope with a 10X objective. Data in A, B, and D are representative of 2-3 independent experiments.

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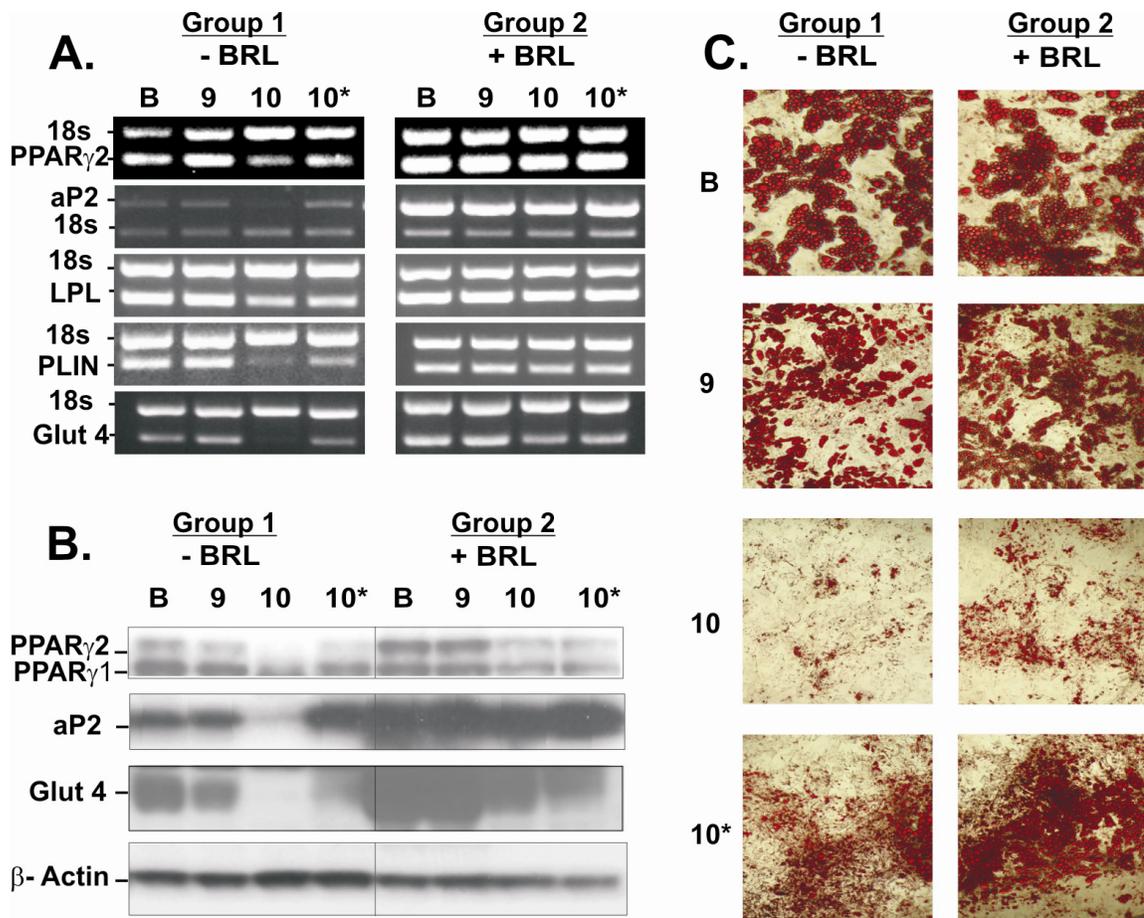
Figure 3.5. Effects of withdrawal of 10,12 CLA in the presence of a PPARγ ligand.
Cultures of newly-differentiated human adipocytes were treated for 1 wk with either BSA vehicle (B), 30 umol/L cis-9, trans-11 CLA (9), 30 umol/L trans-10, cis-12 CLA (10), or 30 umol/L trans-10, cis-12 CLA + 1 umol/L BRL (10*) and then had their treatments withdrawn for 2 wk (Group 1, -BRL), or were treated with 1umol/L BRL for 2 wk during CLA withdrawal (Group 2, +BRL). A: RNA was isolated and the mRNA levels of PPARγ2, aP2, LPL, PLIN, and Glut4 were measured using semi-quantitative RT-PCR. 18S rRNA was used as an internal control. B: Cell extracts were isolated and immunoblotted for PPARγ aP2, Glut4, and β-actin. C: Cultures were stained with Oil Red O and phase-contrast photomicrographs were taken using an Olympus inverted microscope with a 10X objective. Data are representative of 2-3 independent experiments.
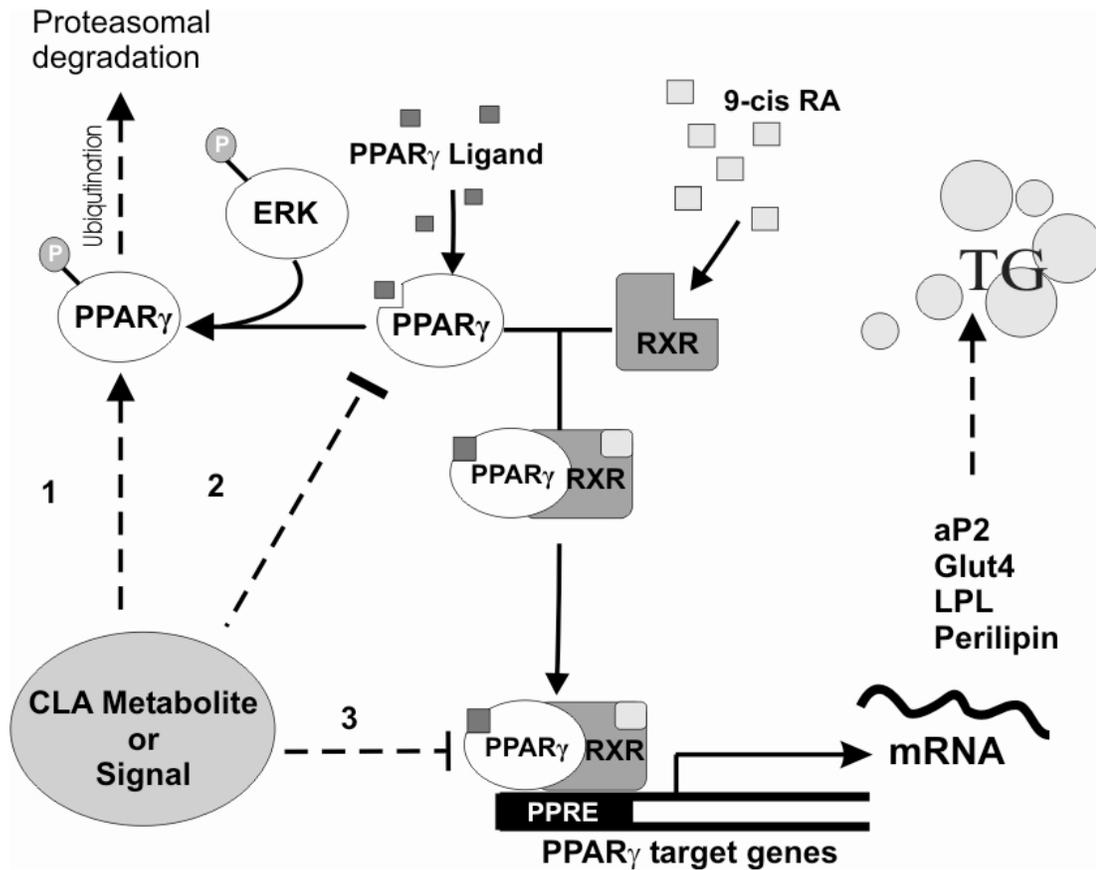
Figure 3.6. Working Model. CLA, metabolites, or signals suppress PPARγ activity by 1) phosphorylating PPARγ via activation of NFκB and ERK1/2, 2) inhibiting ligand activation and/or heterodimer formation with RXR, or 3) impairing transcriptional activation of target genes, thereby decreasing TG synthesis.

Discussion

The PPARγ agonist Rosiglitazone has been demonstrated to prevent or attenuate inflammation, lipodystrophy, and insulin resistance in mice fed a crude mixture of CLA isomers (e.g., primarily cis-9, trans-11 CLA and trans-10, cis-12 CLA) (19, 20). These data suggest an antagonism between one or both CLA isomers and PPARγ. However, the isomer-specific mechanism by which CLA suppresses the activity of PPARγ in human adipocytes remains unknown. We demonstrate in this article that trans-10, cis-12, but not cis-9, trans-11, CLA attenuates ligand-induced activation of PPARγ (Fig. 3.1), possibly via phosphorylation of PPARγ by ERK1/2 (Fig. 3.2). Inactivation of PPARγ leads to suppression of protein and mRNA levels of PPARγ and several of its target genes in newly-differentiated human adipocytes (Figs. 3.3, 3.4, 3.5). BRL co-supplementation did not prevent insulin resistance caused by trans-10, cis-12 CLA (Fig. 3.4). Furthermore, we show that trans-10, cis-12 CLA-mediated suppression of TG accumulation does not return to control levels following CLA withdrawal or by supplementation with a PPARγ agonist following CLA treatment (Fig. 3.5). Only BRL co-supplementation followed by 2 wk of BRL supplementation restored the TG content of trans-10, cis-12 CLA-treated cultures to control levels. Taken together, these data provide further support for the concept that CLA's anti-adipogenic effects in humans are 1) due to the trans-10, cis-12 isomer and not the cis-9, trans-11 isomer, and 2) directly linked to the suppression of PPARγ activity, adipogenic gene and protein expression, insulin-stimulated glucose uptake and TG content, which appears to be due in part to an antagonism of ligand-mediated activation of PPARγ.

Potential mechanisms explaining the isomer-specific attenuation of PPARγ activity by CLA are shown in our working model in Fig. 3.6. We propose that trans-10, cis-12 CLA, a metabolite, or a signal activated by CLA suppresses PPARγ activity by 1) phosphorylating PPARγ via activation ERK1/2, 2) inhibiting ligand activation and heterodimer formation with RXR, or 3) impairing DNA binding of the PPRE to target genes, thereby decreasing adipogenic gene transcription, insulin-stimulated glucose uptake, and TG synthesis.

PPARγ phosphorylation. Support for the first two mechanisms comes from our discovery that trans-10, cis-12 CLA suppresses adipogenic gene expression and metabolism through activation of ERK1/2 (Brown et al. 2004) and NFκB (Chung et al. 2006). Reports demonstrating that NFκB (Jiang et al. 2001, Ruan et al. 2002, Ruan et al. 2003, Suzawa et al. 2003, Nie et al. 2005) and MAPK (Adams et al. 1997, Camp et al. 1997, De Mora et al. 1997) activation hinders PPARγ DNA binding affinity or transcriptional activation provides a potential mechanism by which trans-10, cis-12 CLA suppresses the expression of PPARγ target genes, leading to delipidation. ERK1/2 activates NFκB (Chen et al. 2004) and inactivates PPARγ (Shao et al. 1998), resulting in its ubiquination and proteosome degradation (Burns et al. 2007). Further support comes from studies showing that PPARγ agonists attenuate cytokine-mediated inflammation by suppressing NFκB and/or MAPK signaling (Su et al. 1999, Staus et al. 2000, Chen et al. 2004, Engleman et al. 2005). Clearly, the activity of PPARγ is regulated by its phosphorylation status via phosphatases and kinases (Burns et al. 2007). Indeed, the phosphorylation of PPARγ at a consensus MAPK site within its A/B domain (e.g., serine

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112) by ERK1/2 or JNK reduces its transcriptional activation potential, leading to insulin resistance and/or decreased adipogenesis (Hu et al. 1996, Adams et al. 1997, Camp et al. 1997, De Mora et al. 1997, Shao et al. 1998, Engleman et al. 2005, Zhang et al. 1996). Interestingly, we previously reported that the MEK/ERK inhibitor U0126 blocked trans-10, cis-12 CLA suppression of adipogenic genes and glucose and fatty acid uptake (Brown et al. 2004). Consistent with these data, we found that trans-10, cis-12 CLA simultaneously increased the phosphorylation of ERK1/2 and PPARγ (Fig. 3.2A). Based on these data, our working hypothesis is that trans-10, cis-12 CLA antagonizes PPARγ's activity acutely and PPARγ expression chronically in adipocytes via NFκB and ERK1/2 activation, leading to decreased glucose and fatty acid uptake and TG synthesis.

Ligand binding. CLA may also compete with endogenous (i.e., unsaturated fatty acids) or exogenous (i.e., Rosiglitazone-BRL) ligands for activation of PPARγ. Low affinity PPARγ ligands such as PUFAs increase PPARγ activity and target gene expression (Kahn et al. 2003). Several CLA isomers, including cis-9, trans-11 CLA, have been shown to be ligands for PPARγ (Granlund et al. 2003, Yu et al. 2002) or its partner RXR (Kahn et al. 2003). Consistent with the reported antagonism between PPARγ and inflammation, cis-9, trans-11 CLA has been shown to suppress NFκB activation and inflammatory cytokine production by lipopolysaccharide (LPS) in dendritic cells (Loscher et al. 2005) and in WAT of obese mice (Moloney et al. 2007). However, we found that co-supplementation of trans-10, cis-12 CLA-treated cultures with up to 30 umol/L cis-9, trans-11 CLA did not reverse insulin resistance or adipogenic gene expression (data not shown). In contrast, Granlund et al. 2003 demonstrated that

both cis-9, trans-11 CLA and trans-10, cis-12 CLA decreased the activity of a Darglitazone-stimulated, LXRα-PPRE-LUC reporter in a dose-dependent manner up to 25 umol/L in COS-1 cells and 3T3-L1 cells (Granlund et al. 2003). We have also demonstrated in 3T3-L1 that both CLA isomers antagonize ligand-induced activation of PPARγ (Brown et al. 2003). Alternatively, CLA phosphorylation of PPARγ in the A/B domain could reduce PPARγ affinity for ligand and/or cofactor recruitment (Burns et al. 2007).

Transcriptional activation. Another possible mechanism by which CLA reduces PPARγ activity is by impairing DNA binding of the PPARγ/RXR heterodimer itself to the PPRE in target genes, thereby decreasing transcriptional activation. Conceptually, this would lead to decreased lipogenesis and TG accumulation. However, chromatin immunoprecipation studies are needed to support this speculative mode of action of CLA.

One possible explanation for the long-term effects of CLA following withdrawal could be that CLA accumulates within the phospholipid and neutral lipid fractions of the cell, as we have previously shown for both isomers (Brown et al. 2003). Thus, CLA could continue to antagonize PPARγ/RXR activity following withdrawal, thereby impacting endogenous ligand production, phosphorylation, and/or directly interfering with their transcriptional activation.

In summary, although co-supplementation with BRL, a high affinity ligand for PPARγ, generally prevented or attenuated trans-10, cis-12 CLA suppression of adipogenic gene and protein expression and TG content, it did not prevent CLA's

suppression of a PPARγ reporter construct or insulin-stimulated glucose uptake. Furthermore, BRL supplementation for 2 wk after CLA withdrawal did not completely rescue its anti-adipogenic and TG-lowering effects. Taken together, these data suggest that trans-10, cis-12 CLA may decrease PPARγ activity acutely by increasing PPARγ phosphorylation via ERK1/2 and chronically by decreasing PPARγ transcription, thereby decreasing the amount PPARγ available for ligand binding, leading to the suppression of insulin-stimulated glucose uptake and TG accumulation.

CHAPTER IV

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

Abstract

We previously demonstrated that trans-10, cis-12 (10,12) conjugated linoleic acid (CLA) caused inflammation and insulin resistance in cultures of human adipocytes by activating nuclear factor κB (NFκB) and extracellular signal related kinase (ERK) signaling. However, the upstream mechanism by which CLA activates ERK and NFκB is unclear. In this study, we tested the hypothesis that intracellular calcium $[Ca^{2+}]_i$ is an upstream mediator of this effect. We demonstrated that the increase in $[Ca^{2+}]_i$ mediated by 10,12 CLA was attenuated by BAPTA, an intracellular calcium chelator, and by TMB-8, a chemical inhibitor of calcium release from endoplasmic reticulum (ER), and D609, a phospholipase C (PLC) inhibitor, but not by the extracellular calcium chelator EGTA. These data suggest that 10,12 CLA increases $[Ca^{2+}]_i$ levels by stimulating calcium release from the ER. Moreover, BAPTA, TMB-8, and D609 also attenuated 10,12 CLA-mediated production of reactive oxygen species (ROS), activation of ERK1/2 and cJun-NH$_2$-terminal kinase (JNK), or induction of inflammatory and stress response gene expression. Consistent with these data, 10,12 CLA-mediated binding of NFκB to the promoters of interleukin (IL)-8 and cyclooxygenase (COX)-2 was attenuated by TMB-8.

KN-62, a calcium-calmodulin kinase (CaMK) inhibitor, also suppressed 10,12 CLA-mediated ROS production and ERK1/2 and JNK activation. Additionally, KN-62 attenuated 10,12 CLA induction of inflammatory and stress response genes, increase in prostaglandin $F_{2\alpha}$, and suppression of peroxisome proliferator activated receptor gamma protein levels and insulin-stimulated glucose uptake. Collectively, these data suggest that 10,12 CLA-mediated ROS production, MAPK activation, inflammatory gene induction and insulin resistance are dependent on calcium release from the ER or CaMK activity in human adipocytes.

Introduction

Obesity and its co-morbidities are the most prevalent metabolic diseases in the U.S., affecting over 30% of the adult population (CDC 2007). Conjugated linoleic acid (CLA), dienoic isomers of linoleic acid, is one potential treatment for obesity. Numerous animal (House et al. 2005) and human studies (Whigham et al. 2007) have demonstrated that supplementation with trans-10, cis-12 (10,12) CLA alone, or in a mixture with the cis-9, trans-11 (9,11) isomer, reduces body weight and fat deposition. 10,12 CLA has been shown to suppress the mRNA and protein levels of peroxisome proliferator activated receptor gamma (PPARγ, the master regulator of adipogenesis, in murine 3T3-L1 adipocytes (Granlund et al. 2003) and cultures of newly-differentiated human adipocytes (Brown et al. 2004).

Our research group has reported that 10,12 CLA, but not 9,11 CLA, inhibited human preadipocyte differentiation (Brown et al. 2003) and caused delipidation of newly-

differentiated human adipocytes (Brown et al. 2004). Isomer-specific delipidation of adipocytes by CLA was due largely to a decrease in adipogenic/lipogenic gene expression, uptake of glucose and fatty acids, and triglyceride (TG) synthesis as opposed to an increase in oxidation (Brown et al. 2004, Chung et al. 2005). Interestingly, 10,12 CLA suppression of glucose and fatty acid uptake was dependent on activation of mitogen-activated protein kinase/extracellular kinase signal-regulated kinase (MEK/ERK) and nuclear factor κB (NFκB) signaling, as well as robust secretion or expression of the proinflammatory cytokines interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)α (Brown et al. 2004, Chung et al. 2005, Chung et al. 2005). Consistent with these in vitro data, CLA supplementation in humans is associated with hyperglycemia, dyslipidemia, insulin resistance, and elevated levels of inflammatory prostaglandin (PG)s and cytokines (Basu et al. 2003, Riserus et al. 2002, Riserus et al. 2002, Tholstrup et al. 2007). However, the upstream mechanism(s) by which 10,12 CLA induces inflammation, insulin resistance, and adipocyte delipidation remain unclear.

One possible upstream mediator of CLA-induced inflammation and insulin resistance is intracellular calcium ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ is a vital second messenger for the activation of proteins involved in adipocyte proliferation, differentiation, and metabolism (Zemel et al. 1998). Elevated levels of $[Ca^{2+}]_i$ have been reported to activate NFκB (Martin et al. 2006, Jeong et al. 2005, Gewirtz et al. 2000), ERK1/2 (Martin et al. 2006, Jeong et al. 2005, Melien et al. 2002), and phospholipase A2 (PLA2) (Balsinde et al. 1999, Qui et al. 1993), leading to cytokine or PG production. Calmodulin (CaM), a major calcium-dependent protein, plays an important role in inflammation and

metabolism. CaM activates a number of protein kinases, such as CaMK I, II, and IV (Means et al. 2000, Colomer et al. 2007). Relevant to this study, CaMK II has been shown to inhibit adipocyte differentiation in response to inflammatory PGs such as PGF2α (Miller et al. 1996).

Based on its critical role in cell signaling, enzyme activation, and adipocyte differentiation, we hypothesized that $[Ca^{2+}]_i$ is an upstream mediator of 10,12 CLA-induced inflammation and insulin resistance in human adipocytes. In this study, we demonstrated for the first time that CLA increases $[Ca^{2+}]_i$ levels, leading to the production of reactive oxygen species (ROS), NFκB, cJun-NH2-terminal kinase (JNK), ERK1/2, and ultimately, the induction of inflammatory genes and PGs, and insulin resistance in human adipocytes.

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). The polyclonal antibody for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc20357) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho (P) (Thr183/185) SAPK/JNK and anti-P (Thr-202/204) and total ERK1/2 antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Hyclone fetal bovine serum (FBS) was purchased from Fisher Scientific. Isomers

of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA). Dichlorofluorescein (DCF), Fluo-3 acetoxymethyl ester (Fluo-3 AM), and 1,2-bis (2-aminophenoxy)ethane-N, N, N', N'-tetraacetic acid (BAPTA)-AM were purchased from Molecular Probes (Eugene, OR). 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxy-benzoate (TMB-8) and 1-[N,O-bis-(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) were purchased from Calbiochem-EMD Biosciences, Inc (La Jolla, CA). Ethylene glycol-bis(β-aminoethylester)-N,N,N',N'-tetracetic acid (EGTA) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and chemicals were purchased from Sigma Chemical Co. unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue (WAT) was obtained with consent from the Institutional Review Board at the University of North Carolina at Greensboro, during abdominoplasty of non-diabetic females between the ages of 20-50 years old with a body mass index ≤ 30. Tissue was digested using collagenase; stromal vascular cells were

isolated as previously described (Brown et al. 2004). Cultures containing newly-differentiated human adipocytes were treated on day 6 of the differentiation program. Each independent experiment was repeated at least twice using a mixture of cells from 2-3 subjects, unless otherwise indicated.

Culturing of Human Simpson̄Golabī̄Behmel Syndrome (SGBS) cells

SGBS cells were generously provided by Dr. Martin Wabitsch at the University of Ulm, Germany. They were grown to confluence in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham's supplemented with 10% bovine serum, 33 uM biotin, 17 uM pantothenate, 100 ug/ml streptomycin and 62.5 ug/ml penicillin. To induce differentiation, SGBS cells were washed repeatedly with PBS buffer and then cultured in serum-free medium supplemented with 10 nM insulin, 200 pM triiodothyronine, 1 uM cortisol, 2 uM BRL 49653, 0.115 mg/ml 1-methyl-3-isobutylxanthine (IBMX), 0.25 mmol/L dexamethasone (DEX) and 0.01 mg/ml human transferrin for 4 d. After 4 d, the medium was replaced with the differentiation medium lacking BRL 49653, IBMX, and DEX. These cells were used for the ChIP experiments on day 6 of differentiation.

Measuring ROS

For the DCF assay, primary human adipocytes were seeded in 96-well plates and differentiated for 6 d. On day 6, medium was changed to serum- and phenol red-free medium for 24 h. After 24 h, cells were preloaded with 5 uM DCF 37° C for 1 h and then treated with various treatments for 3 h. Cells were then washed once with HBSS and fluorescence was immediately measured in a plate reader with an excitation/emission wavelength of 485/528 nm. DCF values were calculated after normalizing background fluorescence levels of DCF.

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

[Ca^{2+}]$_i$ levels were measured using Fluo-3 AM. Briefly, cells were preloaded with 5 uM Fluo-3 AM and an anionic detergent, 10% Pluronic F-127, at 25°C for 30 min in the dark. Cells were then washed with a buffer consisting of HBSS, CaCl2, and probenecid, which prevents Fluo-3 AM leakage from cells. Baseline fluorescence was measured using a Synergy Multi-detection Microplate Reader (Bio-Tek Inc, Winooski, VT). Cells were then treated in the absence or presence of CLA isomers and ionomycin. Fluorescence was monitored at 10 sec intervals for 5 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/F0) were plotted over time. For simplicity, single representative experiments are shown.

Immunoblotting

Immunoblotting was performed using 4-12% NuPage precasted gels (Invitrogen) as previously described (Chung et al. 2005).

Chromatin Immunoprecipitation (ChIP) assay

ChIP experiments were performed essentially as described previously (Nielsen et al. 2006). Briefly, SGBS cells were grown and differentiated on 10 cm NUNC dishes and treated with BSA, CLA, or TMB. After treatment, cells were crosslinked, harvested in lysis buffer (i.e., 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0, and 1x Complete proteinase inhibitor cocktail), sonicated, and DNA

concentration was determined by A260 measurement. Samples were diluted to equal concentrations and preincubated for 3 h with 2 µg p65 antibody (sc-372 Santa Cruz) and 40 µg BSA in a total volume of 400 µl lysis buffer. 20 µl Protein A beads were washed 3 times in lysis buffer, diluted in lysis buffer to a total volume of 100 µl, and incubated for 2 h with 10 µg BSA. Following preincubation, prepared beads were added to the chromatin samples and incubated overnight. Beads were washed as previously described and DNA was purified by phenol-chloroform extraction. Immunoprecipitated DNA and 5% input DNA were analyzed using real-time PCR.

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 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.

Measurement of PGF2α levels

PGF2α levels were measured according to a standard protocol provided by Cayman Chemicals for their PGF2α enzyme immunoassay (EIA) kit. Medium was collected from cultures and assayed in duplicate at multiple dilutions.

[3H]-2-Deoxy-glucose uptake

Newly-differentiated cultures of human adipocytes were incubated in serum-free basal DMEM containing 1,000 mg/liter D-(+)-glucose with or without 20 pmol/L of human insulin. Cultures were treated with BSA vehicle, 50 uM 10,12 CLA, or 50 uM 10,12 CLA + 10 uM KN-62 for 48 h. Following treatment, insulin-stimulated uptake of [3H]-2-deoxy-glucose was measured following a 90 min incubation with 100 nmol/L human insulin as described previously (Brown et al. 2004).

Statistical analyses

Unless otherwise indicated, data are expressed as the means + SE Data were analyzed using one-way analysis of variance. Student's t tests were used for pairwise comparisons of least square means ($p < 0.05$). All analyses were performed using JMP IN, Version 4.04 Software (SAS Institute, Cary, NC).

Results

10,12 CLA increases $[Ca^{2+}]_i$.

To examine the effect of CLA isomers on $[Ca^{2+}]_i$ levels, we used the calcium-sensitive fluorescent dye Fluo-3 AM. Ionomycin, an ionophore reported to raise $[Ca^{2+}]_i$ levels by binding extracellular calcium and permeating the plasma membrane, was used as a positive control (Fig. 4.1). Consistent with our hypothesis, 30 uM 10,12 CLA increased $[Ca^{2+}]_i$ acutely (within sec) and chronically (over 5 min), comparable to the level of calcium increase in ionomycin-treated cells. In contrast, 30 uM 9,11 CLA only modestly increased $[Ca^{2+}]_i$ levels. These data demonstrate that 10,12 CLA induces an acute and sustained increase of $[Ca^{2+}]_i$ levels in an isomer-specific manner, but do not indicate the source of $[Ca^{2+}]_i$.

Calcium chelators and inhibitors of calcium action block 10,12 CLA increase of $[Ca^{2+}]_i$.

To determine the mechanism by which 10,12 CLA increased $[Ca^{2+}]_i$, cells were first treated with the $[Ca^{2+}]_i$ chelator BAPTA-AM. Preincubation of Fluo-3-loaded adipocytes with BAPTA blocked the increase of $[Ca^{2+}]_i$ by ionomycin (Fig. 4.2A) and 30 uM 10,12 CLA in a dose-dependent manner (Fig. 4.2B).

Next, TMB-8 was used to determine the degree to which 10,12 CLA mobilized calcium from the ER. As shown in Fig. 4.2C, TMB-8 attenuated the increase in $[Ca^{2+}]_i$ mediated by thapsigargin, a positive control that promotes the release of calcium from the ER by inhibiting Ca2+-ATPase. TMB-8 was similarly effective in attenuating 10,12

CLA elevation of $[Ca^{2+}]_i$ (Fig. 4.2D), suggesting that 10,12 CLA also stimulates calcium release from the ER.

Finally, cells were treated with the extracellular calcium chelator EGTA to determine whether 10,12 CLA induces the influx of extracellular calcium. As expected, EGTA suppressed ionomycin increase of $[Ca^{2+}]_i$ (Fig. 4.2E). However, EGTA only slightly attenuated 10,12 CLA increase of $[Ca^{2+}]_i$, suggesting that extracellular calcium plays a minimal role in 10,12 CLA regulation of $[Ca^{2+}]_i$ (Fig. 4.2F). Collectively, these data suggest that 10,12 CLA increases $[Ca^{2+}]_i$ to a large extent from intracellular stores, and in particular, by stimulating calcium release from the ER.

10,12 CLA production of ROS and activation of ERK1/2 and JNK are dependent on $[Ca^{2+}]_i$ and CaMK.

To determine the extent to which CLA production of ROS and activation of MAPK were dependent on $[Ca^{2+}]_i$, we investigated the effects of calcium chelators and inhibitors on ROS, ERK1/2, JNK, and ATF3 in CLA-treated cultures. 10,12 CLA (50 uM) increased the production of ROS within 3 h in an isomer-specific manner (Fig. 4.3; 9,11 CLA data not shown), which was blocked by BAPTA, TMB-8, and KN-62, a CaMK II inhibitor.

10,12 CLA increased the phosphorylation of ERK1/2 within 12 h in an isomer-specific manner (Fig. 4.4A), which was attenuated by BAPTA, TMB-8, and KN-62 (Fig. 4.4A). Phosphorylation of ERK1/2 by thapsigargin was also attenuated by TMB-8 (data not shown), confirming that calcium release from the ER increases ERK1/2 activation.

Consistent with these data, 10,12 CLA activation of JNK (Fig. 4.4B-D) was also attenuated by BAPTA, TMB-8, and KN-62 (Fig. 4.4D), while KN-62 and TMB-8 attenuated 10,12 CLA's increase of ATF3 protein levels.   These data suggest that ROS, ERK1/2, JNK and ATF3 are downstream targets of 10,12 CLA-mediated $[Ca^{2+}]_i$ signaling and calcium-dependent CaMK.

10,12 CLA regulation of NFκB is dependent on $[Ca^{2+}]_i$.

To investigate the role of calcium in mediating 10,12 CLA activation of NFκB, cultures of human SGBS adipocytes were pretreated with 100 uM TMB-8 for 1 h and then treated over time (3-12 h) with 30 uM 10,12 CLA. NFκB binding to inflammatory genes was subsequently analyzed using ChIP. Ten hours of treatment with 10,12 CLA increased NFκB binding to the IL-8 and COX-2 promoters, which was blocked by TMB-8 (Fig. 4.5). These data suggest that 10,12 CLA induction of NFκB DNA binding to inflammatory genes is mediated, in part, by calcium release from the ER.

10,12 CLA-mediated increase of $[Ca^{2+}]_i$ is linked to inflammatory gene expression.

The integrated stress response (ISR) has been recently shown to play a role in 10,12 CLA induction of inflammation (LaRosa et al. 2007). To determine the effect of CLA on the expression of ISR and inflammatory genes, cultures were treated with 9,11 CLA or 10,12 CLA for 6, 12, 24, or 48 h. Both 30 uM or 100 uM 10,12 CLA increased the expression of ISR genes (i.e., ATF3, CHOP, GADD34) within 6-12 h (Fig. 4.6), and

inflammatory genes (i.e., IL-6, IL-8, IL-1β, COX-2) by 12 h (Fig. 4.7) in an isomer-specific manner.

To determine the importance of $[Ca^{2+}]_i$ in mediating CLA induction of ISR and inflammatory genes, cultures were first pretreated with BAPTA or TMB-8, and then treated with 30 uM 10,12 CLA for 12 h. BAPTA completely blocked 10,12 CLA induction of IL-8, IL-6, CHOP, and GADD34 (Fig. 4.8). BAPTA reduced COX-2 gene expression by 70%, while decreasing ATF3 gene expression by 44% (Fig. 4.8). Treatment of cultures with TMB-8 suppressed the 10,12 CLA-induced expression of IL-8, IL-6, COX-2, CHOP, and GADD34 (Fig. 4.8), while only modestly reducing ATF3 mRNA levels. To determine the role of CaMK in mediating 10,12 CLA induction of ISR and inflammatory genes, cultures where pretreated with KN-62. KN-62 attenuated or blocked induction of IL-8, IL-6, COX-2, CHOP, GADD34, and ATF3 gene expression (Fig. 4.8). These data suggest that 10,12 CLA induction of ISR and inflammatory genes is linked to CaMK activation and the release of calcium from the ER.

Phospholipase C (PLC) activity plays a role in 10,12 CLA-mediated increase in markers of inflammation.

We investigated the role of PLC, a membrane-bound, signal transducing protein involved in the release of calcium from intracellular stores (Camina et al. 1999), using the PLC inhibitor D609. D609 attenuated 10,12 CLA increase of $[Ca^{2+}]_i$ (Fig. 4.9A), ROS (Fig. 4.9B), and ISR and inflammatory gene expression (Fig. 4.9C). These data suggest

that PLC activity plays a role in 10,12 CLA mobilization of calcium, increase of ROS, and ISR and inflammatory gene expression.

10,12 CLA activates the inflammatory PG pathway independently of $[Ca^{2+}]_i$.

PGF2α has been shown to suppress adipogenesis (Miller et al. 1996), and is associated with inflammation and insulin resistance in humans consuming CLA supplements (Basu et al. 2000, Riserus et al. 2002, Riserus et al. 2002). Consistent with these findings, 10,12 CLA increased COX-2 gene expression (Fig. 4.8), which was decreased by BAPTA, TMB-8, and KN-62. For these reasons, we hypothesized that $[Ca^{2+}]_i$ plays a role in 10,12 CLA induction of the inflammatory PG pathway. 10,12 CLA activation of PLA2 occurred within 12 h (Fig. 4.10A) and was isomer-specific (Fig. 4.10B). However, neither BAPTA nor TMB-8 suppressed 10,12 CLA-mediated phosphorylation of PLA2 (Fig. 4.10C) or increased levels of PGF2α after 24 h of treatment (Fig. 4.10D), and KN-62 only had a modest effect. Collectively, these data show that chronic 10,12 CLA treatment robustly increases inflammatory PGF2α production in human adipocytes in vitro as it does in humans in vivo, and that this effect appears to be relatively independent of $[Ca^{2+}]_i$ levels or CaMK activity.

10, 12 CLA-mediated insulin resistance is prevented by KN-62.

KN-62 blocked 10,12 CLA suppression of PPARγ protein levels after 48 h of treatment (Fig. 4.11A), while TMB-8 had no effect. Similarly, KN-62 blocked 10,12 CLA induction of SOCS-3 gene expression (Fig. 4.11B) and attenuation of insulin-

stimulated glucose uptake after 48 h of treatment (Fig. 4.11C), but TMB-8 had no effect (data not shown). These data demonstrate that chronic treatment with 10,12 CLA causes insulin resistance in human adipocytes, which is dependent on CaMK activation, but not directly on calcium release from the ER.
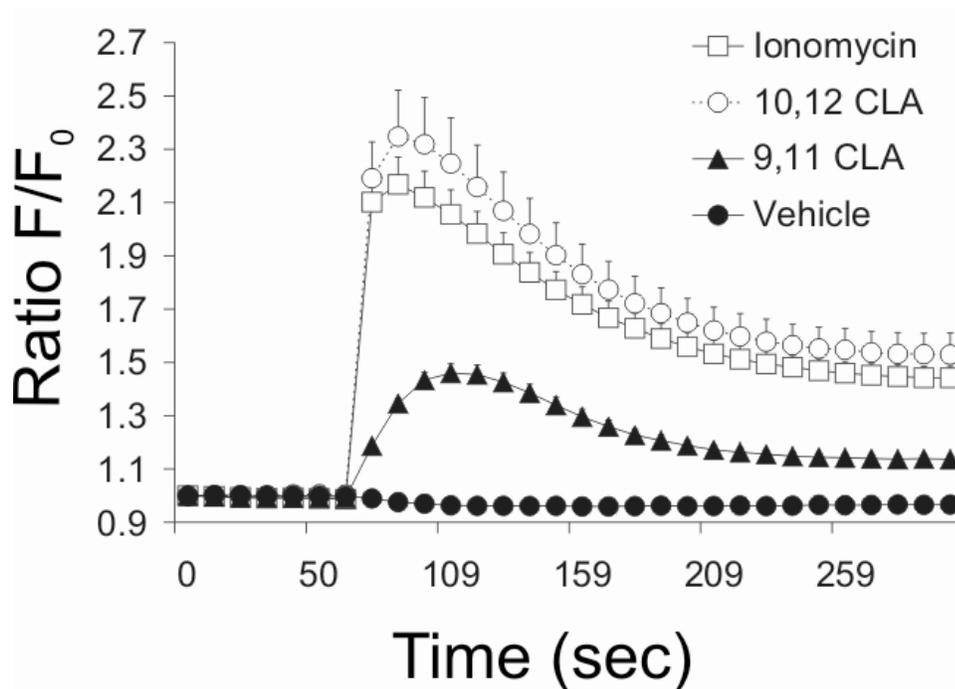
Figure 4.1. 10,12 CLA increases [Ca^{2+}]$_i$. Cultures of newly differentiated human adipocytes were preloaded with 5 uM Fluo-3 AM. Cultures were subsequently injected with BSA vehicle, 1ug/ml ionomycin (positive control), 30 uM 10,12 CLA, 30 uM 9,11 CLA, or vehicle (BSA). Emitted fluorescence intensities were collected over time using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means (± SE; n=3) are representative of three independent experiments.
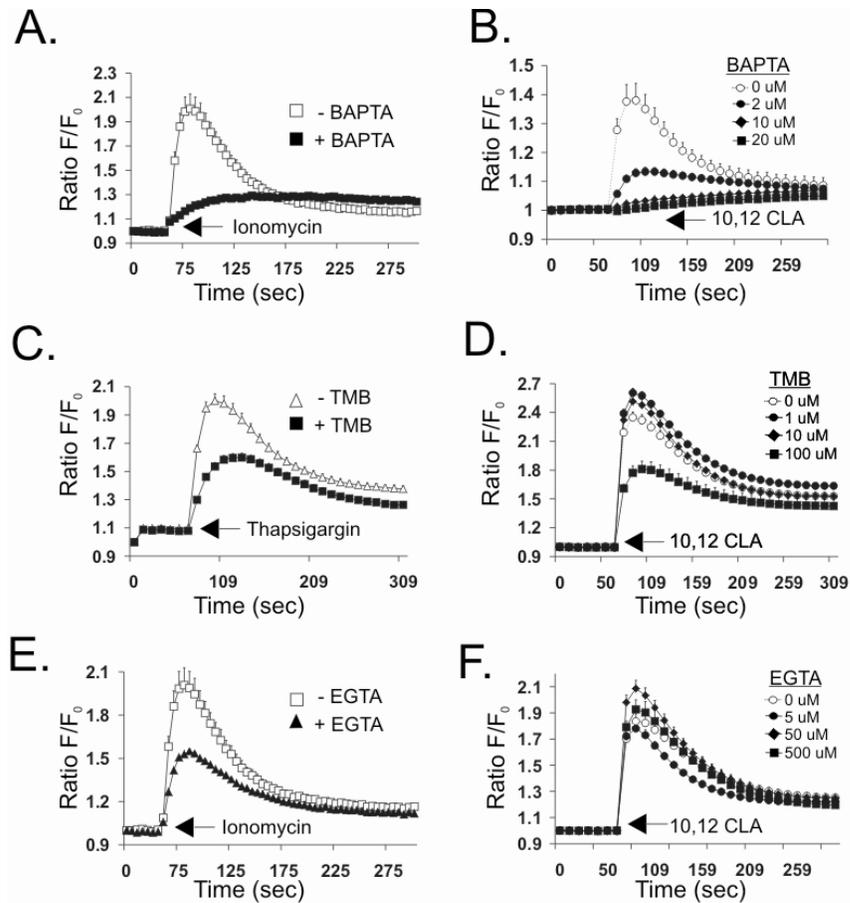
Figure 4.2. 10,12 CLA increase of $[Ca^{2+}]_i$ is attenuated by BAPTA or TMB-8, but not EGTA.   Cultures of newly-differentiated human adipocytes were preloaded with 5 uM Fluo-3 AM. A: Cultures were injected with 1 ug/ml ionomycin (positive control) in the absence or presence of 20 uM BAPTA pretreatment. B: Cultures were injected with 30 uM 10,12 CLA in the absence or presence of 2, 10, or 20 uM BAPTA pretreatment. C: Cultures were injected with thapsigargin (positive control) in the absence or presence of 100 uM TMB pretreatment. D: Cultures were injected with 30 uM 10,12 CLA in the absence or presence of 1, 10, or 100 uM TMB pretreatment. E: Cultures were injected with 1ug/ml ionomycin in the absence or presence of 500 uM EGTA pretreatment. F: Cultures were injected with 30 uM 10,12 CLA in the absence or presence of 5, 50, or 500 uM EGTA pretreatment. Emitted fluorescence intensities were collected over time using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means ($\pm$ SE; n=3) are representative of three independent experiments.
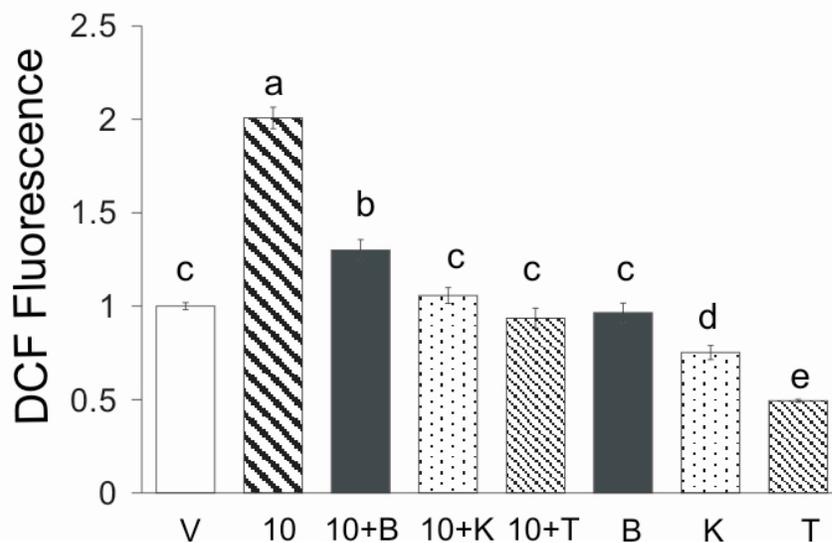
Figure 4.3. 10,12 CLA increase of ROS is attenuated by BAPTA, TMB-8, and KN-62. Cultures of newly-differentiated human adipocytes were serum-starved ~24 h and then preloaded with DCF for 1 h. Cultures were pretreated for 1 h with 2 uM BAPTA (B), 10 uM KN-62 (K), or 100uM TMB-8 (T) and then treated with BSA or 10, 12 CLA for 3 h. Emitted fluorescence intensities were measured using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means (+SE; n=12) are representative of three independent experiments.
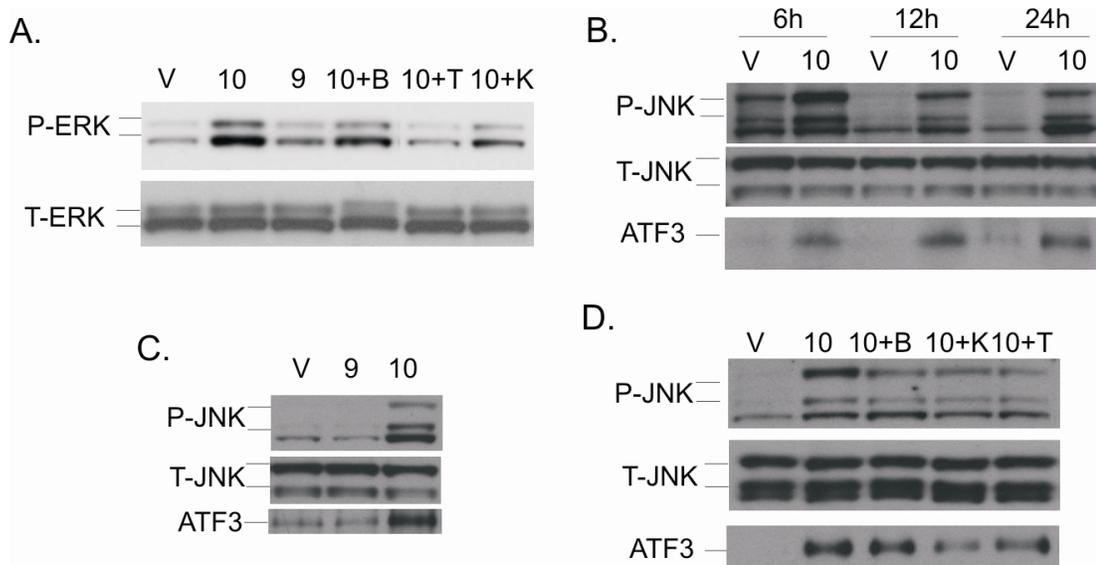
Figure 4.4. 10,12 CLA activation of ERK1/2 and JNK are attenuated by BAPTA, TMB-8, and KN-62. A: Cultures of newly differentiated human adipocytes were treated for 12 h with BSA vehicle (V), 50 uM 10,12 CLA alone (10), 50 uM 9,11 CLA alone (9), or 50 uM 10,12 CLA in the presence of 2 uM BAPTA (10+B), 10 uM KN-62 (10+K), or 100 uM TMB-8 (10+T). Proteins were then harvested, subjected to electrophoresis, and immunoblotted using a p-ERK and total ERK. B: Cultures were treated with BSA vehicle or 50 uM 10,12 CLA for 6, 12, 24, or 48 h and then immunoblotted for p-JNK, total JNK and ATF3. C: Cultures were treated for 12 h with BSA vehicle (V) 50 uM 9,11CLA, or 50 uM 10,12 CLA (10) and immunoblotted as in B. D: Cultures were treated for 12 h with BSA vehicle (V) or 50 uM 10,12 CLA (10) alone or 10,12 CLA in the presence of 2 uM BAPTA (10+B), 10 uM KN-62 (10+K), or 100 uM TMB-8 (10+T) and immunoblotted as in B. Data in all panels are representative of three independent experiments.
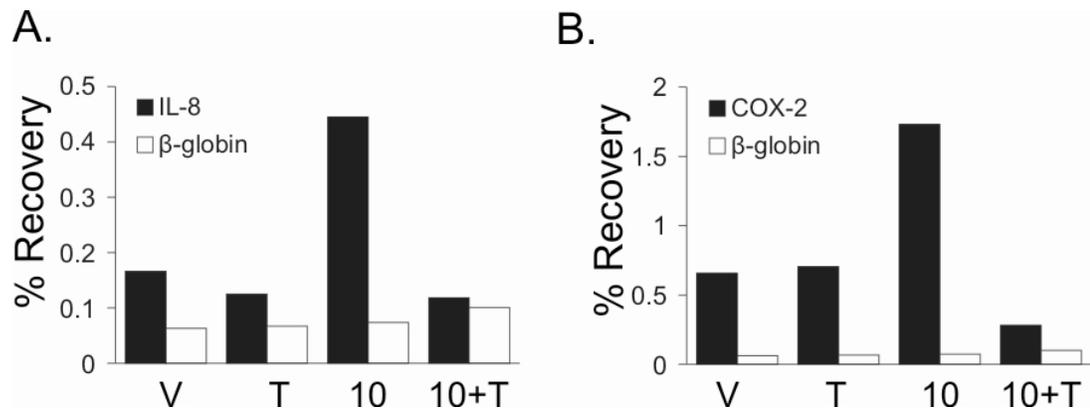
Figure 4.5. 10,12 CLA-mediated activation of NFκB-DNA binding is attenuated by TMB-8. A,B: Cultures of newly differentiated SGBS cells were treated for 640 min with BSA vehicle (V), 30 uM 10,12 CLA (10) in the absence or presence of 100uM TMB-8 (T). ChIP assays quantified DNA binding of NFκB to the IL-8 (A) and COX-2 (B) proximal promoters using real-time PCR with primers positioned at NFκB response elements (black bars). Primers positioned at the β-globin promoter (white bars) were used as "no binding" control. Results are shown as percent recovery relative to input. Results are representative of at least three independent experiments.
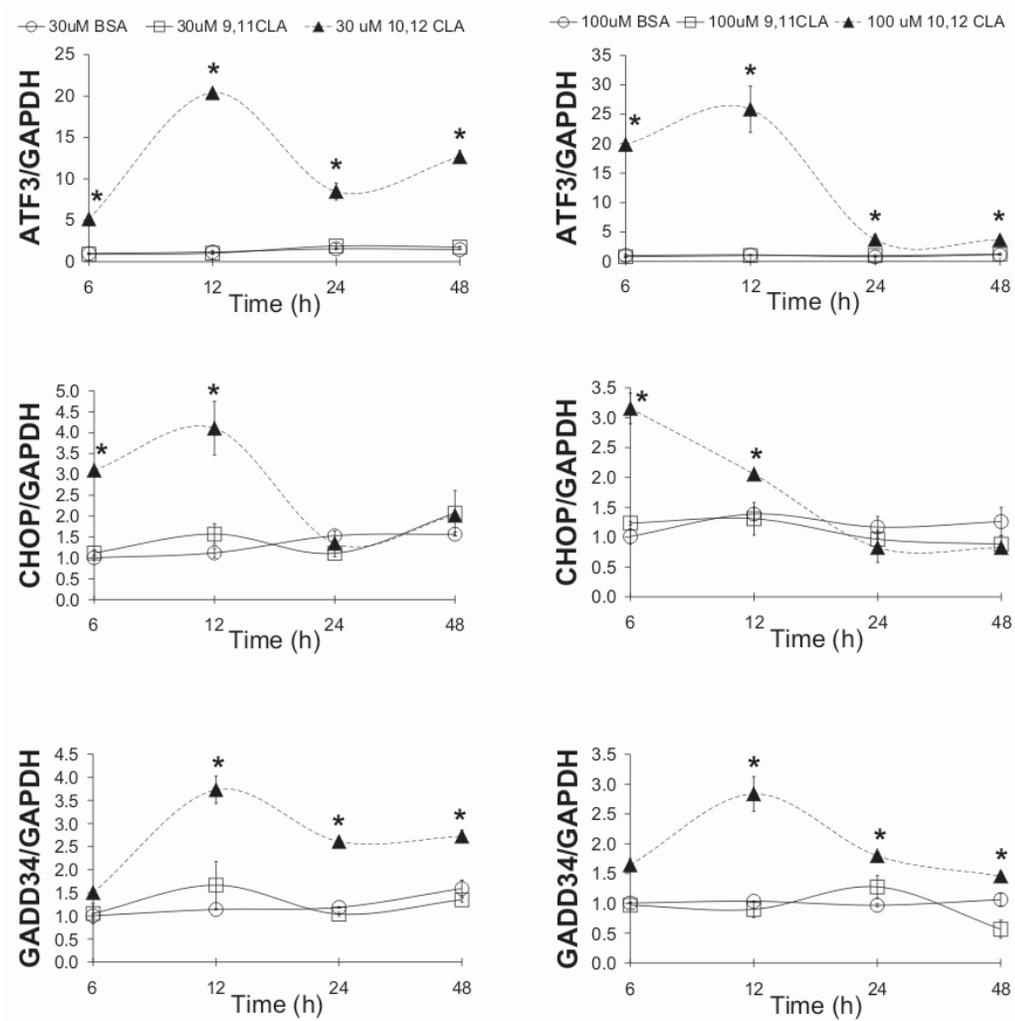
Figure 4.6. Time course of CLA-mediated increase in stress-related gene expression. Cultures of newly-differentiated human adipocytes were serum-starved for ~24 h and then treated for 6, 12, 24, or 48 h with BSA vehicle (○), 30 uM cis-9, trans-11 CLA (□), or 30 uM trans-10, cis-12 CLA (▲), and then harvested. RNA was isolated and the mRNA levels of ATF3, CHOP, GADD34, and GAPDH (load control) were measured using real time qPCR. Means (+SE; n=2) with asterisks (*) differ significantly (p<0.05) from the BSA controls at each time point, and are representative of at least two independent experiments.
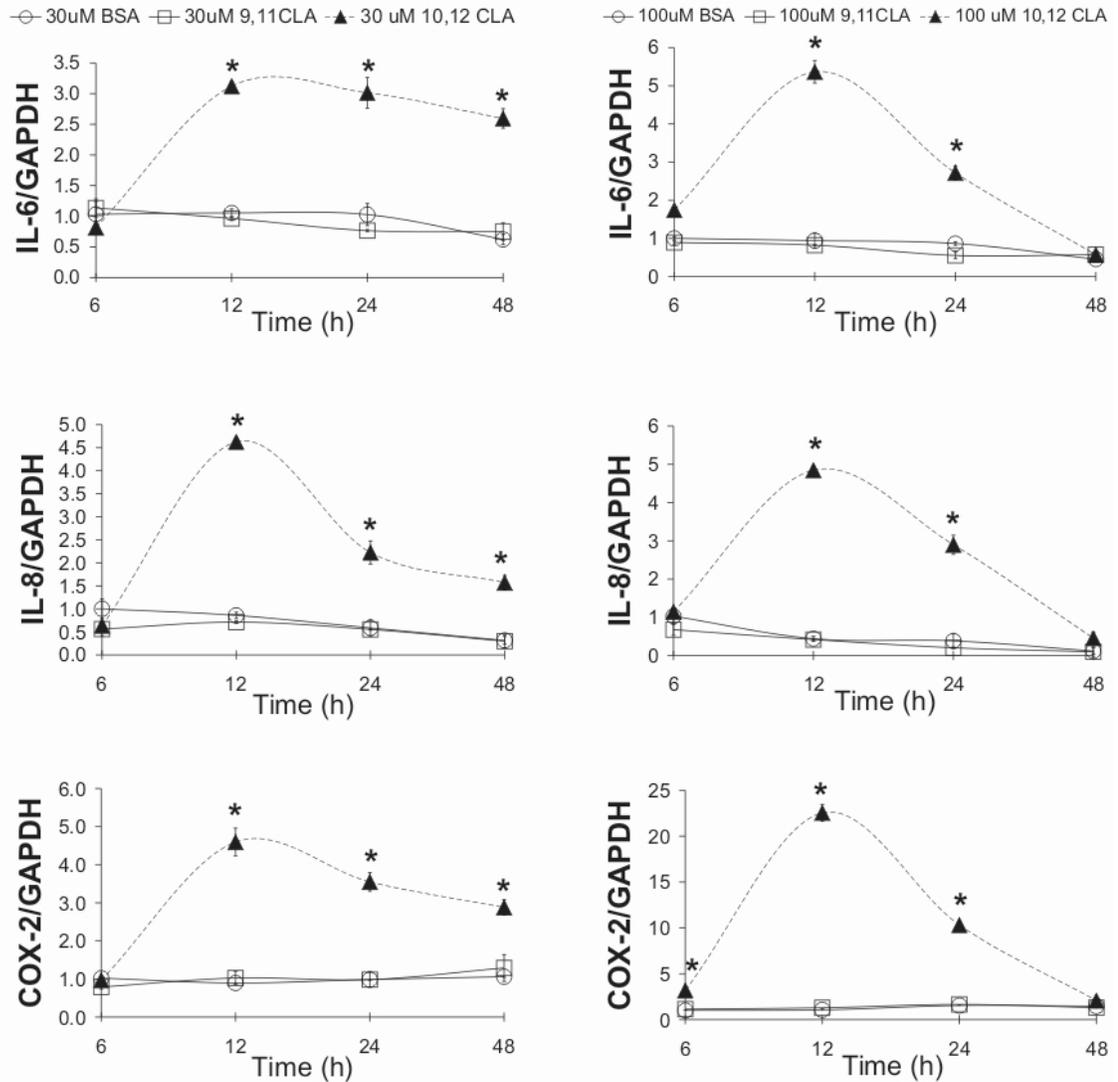
Figure 4.7. Time course of CLA-induced inflammatory gene expression. Cultures of newly-differentiated human adipocytes were serum-starved for ~24 h and then treated for 6, 12, 24, or 48 h with BSA vehicle (○), 30 uM cis-9, trans-11 CLA (□), or 30 uM trans-10, cis-12 CLA (▲), and then harvested. RNA was isolated and the mRNA levels of interleukin (IL)-6, IL-8, COX-2, and GAPDH were measured using real time qPCR. Means (+SE; n=2) with asterisks (*) differ significantly (p<0.05) from the BSA controls at each time point, and are representative of at least two independent experiments.
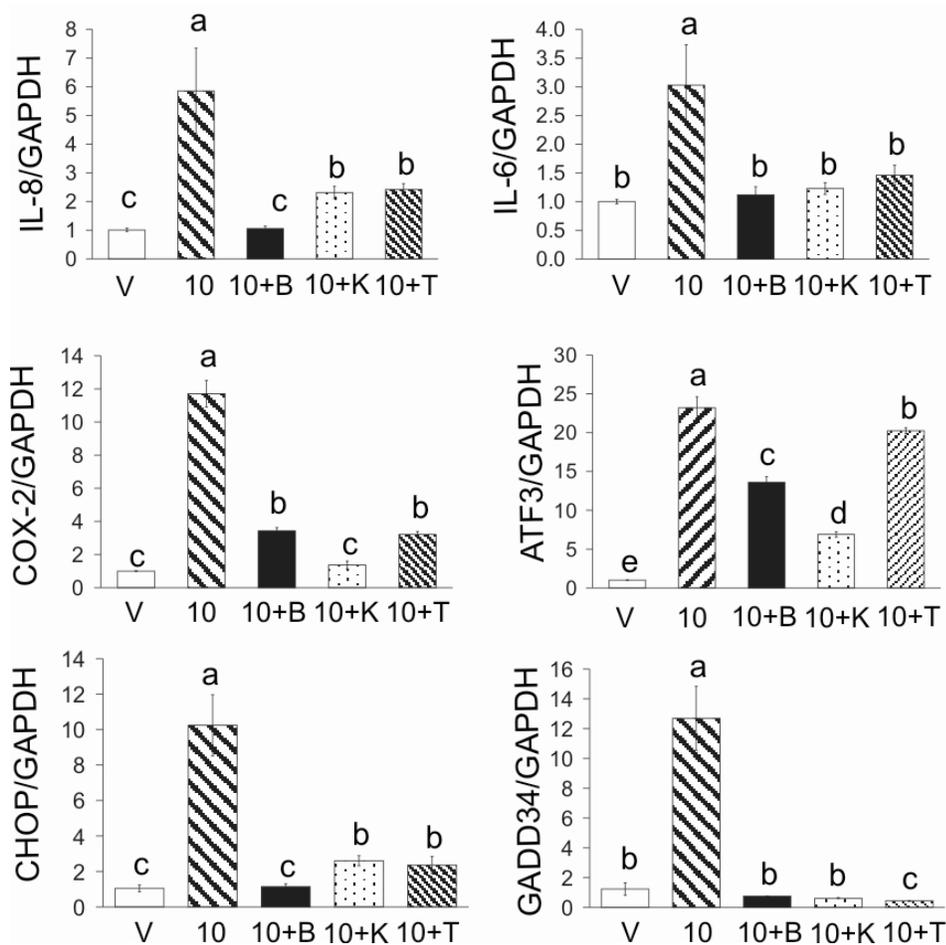
Figure 4.8. 10,12 CLA-induced expression of inflammatory and stress-related genes are attenuated by BAPTA, TMB-8, and KN-62. Cultures of newly differentiated human adipocytes were treated for 12 h with BSA vehicle (V), 50 uM 10,12 CLA alone (10), or 10,12 CLA in the presence of 2 uM BAPTA (10+B), 10 uM KN-62 (10+K), or 100 uM TMB-8 (10+T). Subsequently, RNA was subsequently isolated and the mRNA levels of IL-8, IL-6, COX-2, ATF-3, CHOP, GADD34, and GAPDH were measured by real time qPCR. Data are normalized to the vehicle controls. Means (+ SE; n=2) that do not share a common lower case letter differ (P<0.05). Data are representative of three independent experiments.
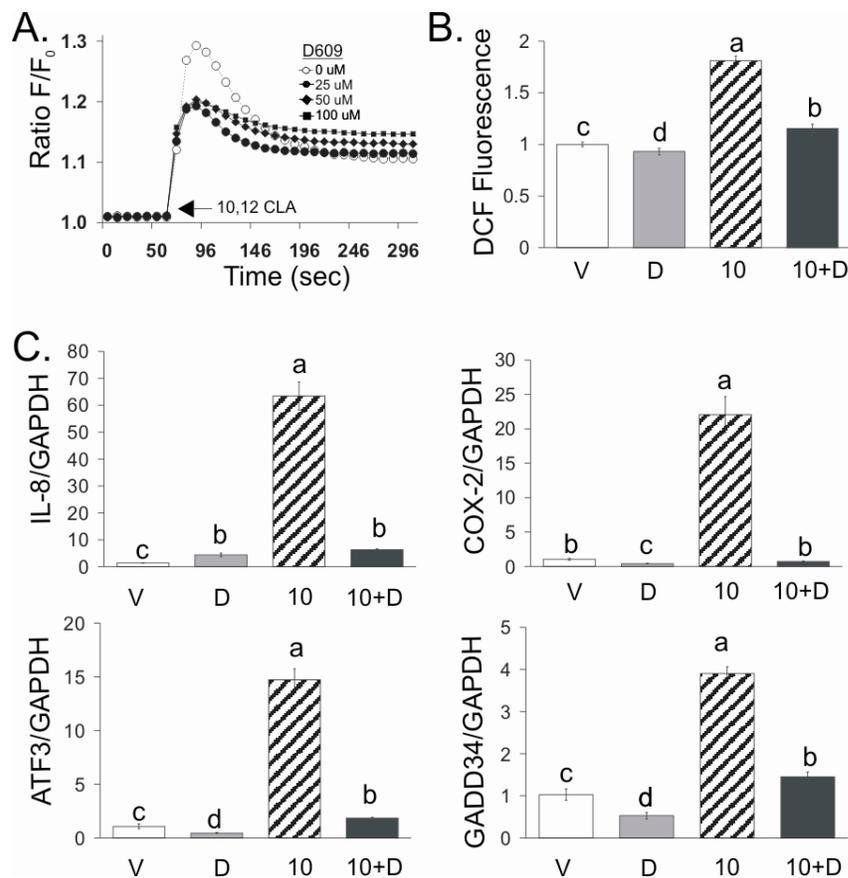
Figure 4.9. 10,12 CLA increase of $[Ca^{2+}]_i$ and ROS levels and expression of inflammatory and stress-related genes are dependent on PLC. A: Cultures of newly differentiated human adipocytes were preloaded with 5 uM Fluo-3 AM. Cultures were injected with 50 uM 10,12 CLA in the absence or presence of 25, 50, or 100 uM D609 pretreatment. Emitted fluorescence intensities were collected over time using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means (+SE; n=3) are representative of two independent experiments. B: Cultures of newly differentiated human adipocytes were preloaded with DCF for 30 min. Cultures were then pretreated with 50uM D609 (D) followed by 3 h treatment with BSA (V) or 50 uM 10,12 CLA (10). Emitted fluorescence intensities were measured using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means (+ SE; n = 3-12) are representative of three independent experiments. C: Cultures were pretreated for 30 min with 50 uM D609 (D) followed by 12 h treatment with BSA vehicle (V) or 50 uM 10,12 CLA (10). RNA was subsequently isolated and mRNA levels of IL-8, COX-2, ATF-3, GADD34, and GAPDH were measured by real time qPCR. Data are normalized to the vehicle controls. Means (+ SE; n=2) that do not share a common lower case letter differ (P<0.05). Data are representative of three independent experiments.
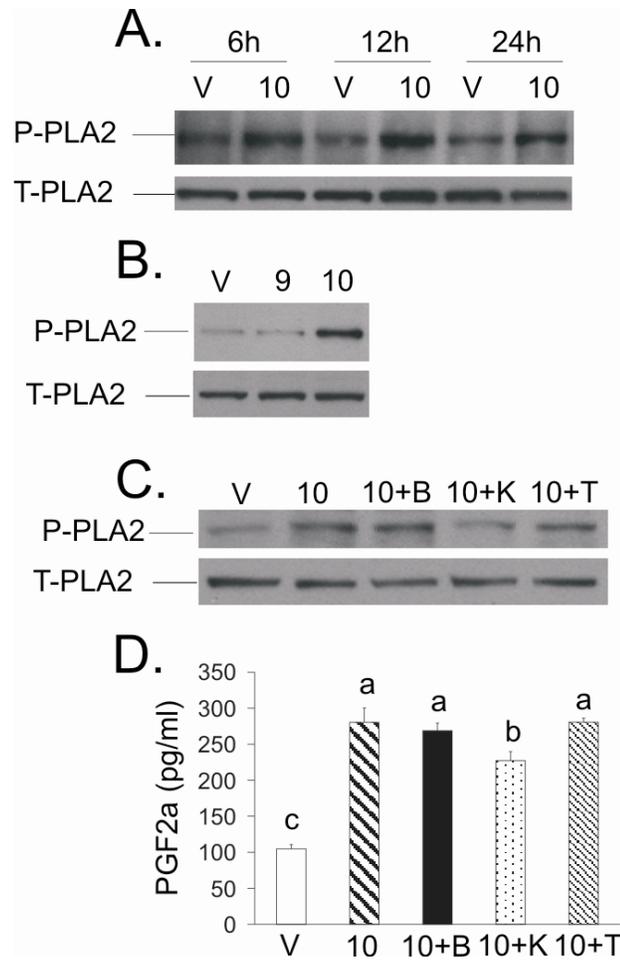
Figure 4.10.   10,12 CLA activation of the inflammatory PG pathway is not dependent on $[Ca^{2+}]_i$.   A: Cultures of newly-differentiated human adipocytes were treated with BSA vehicle (V) or 30 uM 10,12 CLA (10) for 6, 12, or 24 h. Cells were harvested and immunoblotted for p-PLA2 and total PLA2.   Data are representative of three experiments.   B: Cultures were treated for 12 h with BSA vehicle (V), 30 uM 9,11 CLA (9) or 30 uM 10,12 CLA (10), and immunoblotted as in A. Data are representative of three experiments.   C: Cultures were treated for 12 h with BSA vehicle (V), 50 uM 10,12 CLA alone (10), or 10,12 CLA in presence of 2 uM BAPTA (10+B), 10 uM KN-62 (10+K), or 100 uM TMB-8 (10+T), and immunoblotted as in A. Data are representative of three experiments. D: Cultures were treated for 24 h with BSA vehicle (V), 50 uM 10,12 CLA alone (10), or 10,12 CLA in the presence of 2 uM BAPTA (10+B), 10 uM KN-62 (10+K), or 100 uM TMB-8 (10+T).   Conditioned media were subsequently collected and PGF2α levels were measured using a commercially available EIA kit. Means (+ SE; n=3) not sharing a common lower case letter differ (P<0.05).
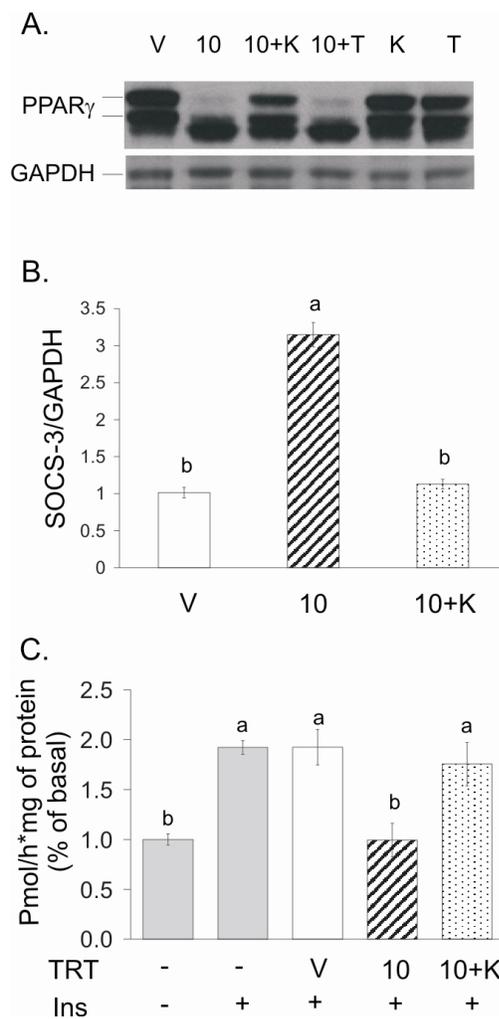
Figure 4.11. 10,12 CLA-mediated insulin resistance is attenuated by KN-62. A: Cultures of newly-differentiated human adipocytes were treated for 48 h with BSA vehicle (V), 50 uM 10,12 CLA (10), 10,12 CLA + 10 uM KN-62 (10+K), 10,12 CLA + 100uM TMB (10+T), 10uM KN-62 (K) or 100uM TMB-8 (T). Cultures were harvested and immunoblotted for PPARγ and GAPDH. B: Cultures were treated for 48 h with BSA vehicle (V), 50 uM 10,12 CLA alone (10), or 10,12 CLA in the presence of 10 uM KN-62 (10+K). Cells were harvested for RNA and SOCS-3 and GAPDH were measured by qPCR. C: Cultures were treated for 48 h with BSA vehicle (V), 50 uM 10,12 CLA alone (10), or 10,12 CLA in the presence of 10 uM KN-62 (10+K). Uptake of basal or insulin-stimulated [3H]-2-deoxy-glucose was subsequently measured in cultures treated without (-) or with (+) insulin. Means (+ SE; n=3) that do not share a common lower case letter differ (P<0.05). Data are representative of three independent experiments.

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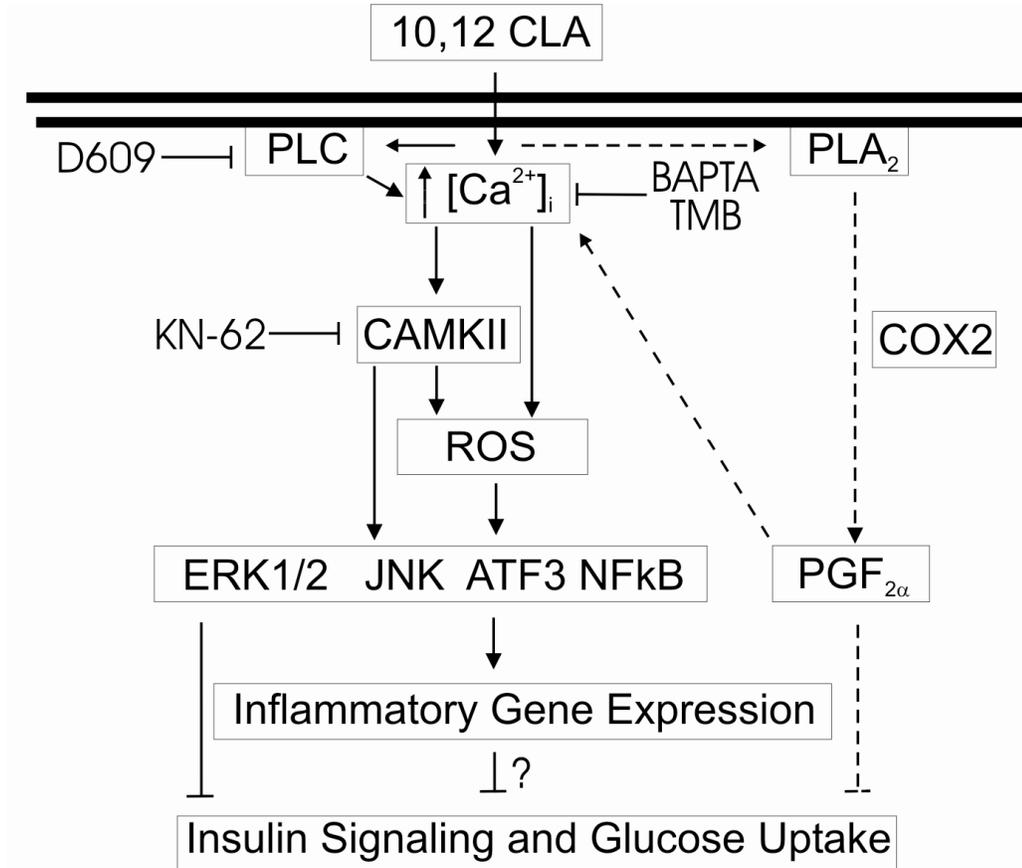
Figure 4.12. Working model: 10,12 CLA-mediated oxidative stress, inflammation, and insulin resistance are regulated, in part, by $[Ca^{2+}]_i$. Acutely, 10,12 CLA induces an rapid and robust elevation of $[Ca^{2+}]_i$. levels, which are dependent on PLC activity and mobilization of calcium from the ER. This increase activates ROS and CAMK II, which in turn activate MAPK and NFκB that trigger ISR and inflammatory gene expression. Independent of calcium, 10,12 CLA activates the inflammatory PG pathway involving PLA2 and PGF2α within 12-24 h of treatment, respectively. Chronically, these CLA-induced signals antagonize PPARγ, causing insulin resistance after 48 h of treatment.

Discussion

[Ca^{2+}]$_i$. regulates a myriad of vital cell processes by activating diverse signaling cascades, many of which require calcium-binding proteins like CaM. Calcium-activated CaM regulates the activity of a variety of enzymes including CaMKs and phosphorylases involved in inflammatory and stress responses (Colomer et al. 2007). For these reasons, influx of calcium from extracellular sources (e.g., plasma membrane ion channels) and efflux from intracellular stores (e.g., ER, mitochondria) are tightly regulated.

The purpose of this study was to determine the role of [Ca^{2+}]$_i$. in mediating 10,12 CLA-induced ROS production, inflammatory gene and protein expression, and insulin resistance in human adipocytes. To our knowledge, data presented in this article are the first to demonstrate that 10,12 CLA rapidly (1 min) increases [Ca^{2+}]$_i$. levels in human adipocytes in an isomer- and site-specific manner (Figs. 4.1-4.2). Futhermore, blocking [Ca^{2+}]$_i$. accumulation and inhibiting PLC or CaMK activity prevented 10,12 CLA-mediated 1) production of ROS within 3 h (Fig. 4.3), 2) activation of ERK1/2, JNK, or NFκB within 6-10 h (Figs. 4.4-4.5), and 3) induction of ISR/ER stress or inflammatory genes within 6-12 h (Fig. 4.6-4.9). Chronically (48 h), inhibiting CaMK activity suppressed 10,12 CLA-mediated insulin resistance (Fig. 4.11). Taken together, these data provide support for our working model shown in Fig. 4.12, hypothesizing that 10,12 CLA's impact on calcium signaling and markers of inflammation and insulin resistance occurs in two response phases. The initial response to CLA begins with a rapid and robust release of calcium from the ER that leads to ROS production, MAPK and NFκB activation, and ISR and inflammatory gene expression. The second and more chronic

response begins with PLA2 activation and PGF2α production that is independent of calcium. This causes another phase of calcium signaling that further activates CaMK II, leading to the suppression of PPARγ and the development of insulin resistance.

In support of our findings, calcium and ROS have been implicated in the activation of NFκB and inflammation (Pahl et al. 1996). Likewise, we found that blocking calcium signaling with BAPTA, TMB, or KN62 prevented CLA-mediated ROS production (Fig. 4.3), suggesting an increase in $[Ca^{2+}]_i$. levels precedes ROS production in human adipocytes. Furthermore, these compounds attenuated 10,12 CLA-mediated activation of MAPKs (Fig. 4.4) and NFκB (Fig. 4.5), and the expression of ISR/ER stress and inflammatory genes (Fig. 4.8). The PLC inhibitor D609 attenuated 10,12 CLA increase in $[Ca^{2+}]_i$. levels, ROS, and inflammatory gene expression (Fig. 4.9). Collectively, these data demonstrate the important role of $[Ca^{2+}]_i$. in mediating the inflammatory and stress responses to 10,12 CLA in cultures of human adipocytes.

The prevention of CLA-mediated insulin resistance by KN-62, but not TMB-8, suggests that 10,12 CLA increased $[Ca^{2+}]_i$. levels from sources other than just the ER. Although KN-62 was initially identified as a calcium-CaMK II inhibitor, this inhibitor has also been reported to block voltage-sensitive channels (Tokumitsu et al. 1990, Tornquist et al. 1996, Cui et al. 1996, Marley et al. 1996). Thus, it seemed possible that 10,12 CLA increases Ca2+ influx across the plasma membrane. However, KN-62 (data not shown) and the extracellular calcium chelator EGTA did not dramatically affect 10,12 CLA's increase of $[Ca^{2+}]_i$. (Fig. 4.2), suggesting that extracellular calcium influx is not a major source of CLA-induced $[Ca^{2+}]_i$.. In agreement with other studies, the

phosphatidylcholine-specific (PC)-PLC inhibitor D609 (Fig. 4.9a), attenuated 10,12 CLA-mediated increase in $[Ca^{2+}]_i$. levels. Activation of PC-PLC has been reported to increase $[Ca^{2+}]_i$. levels via conversion of diacylglycerol (DAG) by DAG kinase to phosphatidic acid, which mobilizes calcium from inositol 1,4,5-triphosphate-independent calcium pools (Camina et al. 1999, Andrei et al. 2004, Suh et al. 2008). Therefore, it appears that 10,12 CLA stimulates an efflux of calcium from intracellular stores like the ER, rather than an influx of calcium from extacellular sources.

The mitochondria can also store and release calcium, potentially providing another source of $[Ca^{2+}]_i$. for signaling (Butow et al. 2004). Mitochondrial dysfunction or stress, similar to ER stress, can adversely affect the mitochondria's capacity for calcium storage or regulated release, thereby increasing $[Ca^{2+}]_i$. levels. For example, treating cells with a mitochondria uncoupler increases $[Ca^{2+}]_i$. levels, thereby activating ERK1/2 (Luo et al. 1997). Similarly, impairing mitochondrial respiration increases $[Ca^{2+}]_i$. levels and activates CaMK IV (Arnould et al. 2002). We investigated whether 10,12 CLA could affect mitochondria function by examining two indicators of altered mitochondrial membrane potential or integrity (e.g., the JC-1 assay and cytochrome C release from mitochondria, respectively). Neither marker of mitochondrial dysfunction was affected by 10,12 CLA (data not shown), suggesting that acute 10,12 CLA treatment does not directly cause mitochondrial dysfunction.

CaMK II has been reported to phosphorylate ERK1/2 in response to G protein-coupled receptor (GPCR) agonists, depolarizing stimuli, or ionophores that lead to increased free $[Ca^{2+}]_i$. levels (Borodinsky et al. 2002, Ginnan et al. 2004, Huang et al.

2004, Lu et al. 2005). Similarly, we previously demonstrated that the GPCR antagonist pertussis toxin blocked 10,12 CLA-mediated activation of ERK1/2 (Brown et al. 2004). In this study, we demonstrated the KN-62 also attenuates 10,12 CLA phosphorylation of ERK1/2 and JNK (Fig. 4.4). KN-93, another CaMK inhibitor, has also been shown to attenuate LPS activation of ERK1/2, JNK, NFκB, and AP-1 activation (Cuschieri et al. 2005). Collectively, these data support our hypothesis that CLA's activation of ERK1/2 and JNK are closely linked to $[Ca^{2+}]_i$. and CaMK activity.

We provide data demonstrating that the CaMK II inhibitor KN-62 prevents 10,12 CLA-mediated insulin resistance (Fig. 4.11). Elevated $[Ca^{2+}]_i$. levels have been linked to insulin resistance in insulin-sensitive cells (Draznin et al. 1987, Draznin et al. 1988, Begum et al. 1993), including rat adipocytes (Draznin et al. 1989). In vivo, BAPTA was shown to reverse insulin resistance in rats fed a high fat diet (Jang et al. 2002). Unfortunately, we could not use BAPTA for our glucose uptake studies due to its toxicity when used chronically (48 h). Future studies using gene silencing techniques may be able to define the specific role of CaMK signaling and insulin sensitivity in CLA-treated cultures of human adipocytes.

10,12 CLA delipidation of murine adipocytes in vivo and in vitro has recently been linked to the ISR pathway (LaRosa et al. 2007). These authors demonstrated that 10,12 CLA, but not 9,11 CLA, induced markers of the ISR pathway in mouse and 3T3-L1 adipocytes. They also suggest that the ISR precedes the later induction of inflammatory gene expression, and contributes to delipidation by 10,12 CLA. Our data in cultures of human adipoctyes are similar to these published data in murine adipocytes.

10,12 CLA has also been shown to cause atypical ER stress in a murine mammary carcinogenesis model (Ou et al. 2008). Similarly, the rapid (1 min) increase in $[Ca^{2+}]_i$. levels by 10,12 CLA appears to increase ROS production (3 h) and activate specific MAPKs (6 h) and ISR/ER stress response genes (6-12 h) relatively early, which, in turn, lead to chronic (48 h) activation of CaMK and subsequent insulin resistance in cultures of human adipocytes. However, the specific mechanism by which 10,12 CLA increases $[Ca^{2+}]_i$. and ISR/ER stress, and the extent to which these early upstream signals are responsible for adipocyte delipidation, are still unknown.

CHAPTER V

CONJUGATED LINOLEIC ACID-MEDIATED INFLAMMATION AND INSULIN RESISTANCE IN HUMAN ADIPOCYTES ARE ATTENUATED BY RESVERATROL

The following chapter was previously published in the Journal of Lipid Research volume 50, pages 225 to 232, in 2009. The coauthors of the article were Angel Overman, Kathleen LaPoint, Robin Hopkins, Tiffany West, Chia-Chi Chuang, Kristina Martinez, Doris Bell, and Michael McIntosh. Permission from the publisher to use the article in its entirety can be found in Appendix A. References from this article can be found in the Reference section.

Abstract

Inflammation plays a role in trans-10, cis-12 (10,12) conjugated linoleic acid (CLA)-mediated delipidation and insulin resistance in adipocytes. Given the anti-inflammatory role of resveratrol (RSV), we hypothesized that RSV would attenuate inflammation and insulin resistance caused by 10,12 CLA in human adipocytes. RSV blocked 10,12 CLA induction of the inflammatory response by preventing activation of extracellular signal related kinase (ERK) and induction of inflammatory gene expression (i.e., IL-6, IL-8, IL-1β) within 12 h. Similarly, RSV suppressed 10,12 CLA-mediated activation of the inflammatory prostaglandin (PG) pathway involving phospholipase A$_2$, cyclooxygenase-2 (COX-2), and PGF$_{2\alpha}$. In addition, RSV attenuated 10,12 CLA increase of intracellular calcium and reactive oxygen species (ROS) associated with cellular stress, and activation of stress-related proteins (i.e., ATF3, JNK) within 12 h. 10,12 CLA-mediated insulin resistance and suppression of fatty acid uptake and triglyceride content were attenuated by RSV. Lastly, 10,12 CLA-mediated decrease of peroxisome proliferator activated receptor (PPAR)γ protein levels and activation of a peroxisome proliferator response element (PPRE) reporter were prevented by RSV. RSV increased the basal activity of the PPRE, suggesting RSV increases PPARγ activity. Collectively, these data demonstrate for the first time that RSV prevents 10,12 CLA-mediated insulin resistance and delipidation in human adipocytes by attenuating inflammation and cellular stress and increasing PPARγ activity.

Introduction

Feeding a mixture of conjugated linoleic acid (CLA) isomers (i.e., trans-10, cis-12 [10,12] CLA and cis-9, trans-11 [9,11] CLA) reduces adiposity in animals (House et al. 2005) and some humans (Whigman et al. 2007). The triglyceride (TG) lowering properties of CLA appear to be due exclusively to the 10,12 isomer (Park et al. 1999, Brown et al. 2001, Brown et al. 2003), and involve decreased uptake and metabolism of glucose (Brown et al. 2004), and increased lipolysis (Chung et al. 2005) in adipocytes.

These antiobesity properties of 10,12 CLA are dependent on the activation of mitogen-activated protein kinase kinase/extracellular signal-related kinase (MEK/ERK) (Brown et al. 2004) and nuclear factor κB (NFκB) (Chung et al. 2005, Poirier et al. 2006) in adipocytes. These signaling pathways induced by 10,12 CLA are linked to the induction and secretion of cytokines (Chung et al. 2005, Poirier et al. 2006), which are known to antagonize peroxisome proliferator activated receptor (PPAR)γ target gene expression and insulin sensitivity (Suzawa et al. 2003, Adams et al. 1997, Chung et al. 2006, Kennedy et al. 2008, Lui et al. 2007, Purushotham et al. 2007). Consistent with these data, 10,12 CLA supplementation of humans is associated with hyperglycemia, insulin resistance, elevated levels of inflammatory prostaglandins (PG)s and cytokines, and dyslipidemia (Riserus et al. 2002, Riserus et al. 2002, Tholstrup et al. 2007).

Recently, supplementation of mice and 3T3-L1 adipocytes with 10,12 CLA has been shown to activate the integrated stress response (ISR) pathway (LaRosa et al. 2007), which is linked to inflammation, insulin resistance, and endoplasmic reticulum (ER) stress (Gregor et al. 2007). Cellular stress can be caused by a relatively disproportional

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influx of macronutrients that adversely affect organelle function, including the mitochondria and ER. This cellular stress increases the release of calcium and reactive oxygen species (ROS), which leads to inflammation and/or insulin resistance (Gregor et al. 2007). These stressors can impair the adipocyte's ability to synthesize and/or store fatty acid (FA)s as TG, causing lipids to accumulate in non-adipocytes (e.g., hepatocytes, myotubes) and resulting in disorders like steatosis and insulin resistance, respectively (Lui et al. 2007). Therefore, it is possible that 10,12 CLA, a trans conjugated FA not normally abundant in the diet, causes cellular stress in adipocytes, initiating a signaling cascade that adversely affects adipocyte function. These issues raise concern about the safe and effective use of supplements containing 10,12 CLA as dietary strategy for weight loss.

Resveratrol (RSV), a phenolic phytochemical found in grapes, berries, and peanuts, has been shown to inhibit tumor necrosis factor (TNF)α-induced inflammatory gene expression and secretion in 3T3-L1 adipocytes (Ahn et al. 2007). In mice, supplementing a high saturated FA diet with RSV increased lifespan, motor activity, AMP kinase, and insulin sensitivity (Baur et al. 2006) and reduced adipocyte size and metabolic disease (Lagouge et al. 2006) compared to a high saturated FA diet alone. These and other studies (Saiko et al. 2008) suggest that low micromolar blood levels of RSV protect against inflammatory- and stress-related diseases. However, the mechanism(s) by which RSV protects against the development of these diseases are not fully understood, and its impact on CLA-mediated inflammation is unknown.

Therefore, we wanted to determine the extent to which RSV prevented some of the side effects (i.e., inflammation, cellular stress, insulin resistance) associated with CLA supplementation. To begin to answer this question, we examined the isomer-specific influence of CLA in the absence and presence of RSV on 1) the induction or activation of genes, proteins, PGs, ROS, and intracellular calcium levels $[Ca^{+2}]_i$ associated with inflammation and cellular stress, 2) insulin-resistance, 3) FA uptake and TG content, and 4) the protein levels and activity of PPARγ.

Materials and Methods

Materials

All cell culture ware were purchased from Fisher Scientific (Norcross, GA). Western lightning chemiluminescence substrate was purchased from Perkin Elmer Life Science (Boston, MA). Immunoblotting buffers and precast gels were purchased from Invitrogen (Carlsbad, CA). DNA-free was purchased from Ambion (Austin, TX). Gene-specific primers were purchased from Applied Biosystems (Forest City, CA). Polyclonal antibodies for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc20357), activating transcription factor (ATF) 3, and β-actin (sc1616) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-P (Ser505) phospholipase A_2 (PLA2), anti-P (Thr183/185) c-JunNH$_2$-terminal kinase (JNK), and anti-P (Thr-202/204) and total ERK1/2 antibodies were purchased from Cell Signaling Technologies (Beverly, MA). PGF$_{2\alpha}$ levels were measured in conditioned media using an Enzyme Immunoassay (EIA) kit from Caymen Chemicals (Ann Arbor, MI). Fetal bovine serum (FBS) was purchased

from Hyclone (Logan, UT). The thiazolidinedione (TZD) BRL (Rosiglitazone) was a generous gift from Dr. Per Sauerberg, Novo Nordisk, Denmark. Isomers of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA) or Natural ASA (Hovdebygda, Norway). The Nucleofactor and Dual Glo luciferase kits were obtained from Amaxa (Cologne, Germany) and Promega (Madison, WI), respectively. Dichlorofluorescein (DCF) and Fluo-3 acetoxymethyl ester (Fluo-3 AM) were purchased from Molecular Probes (Eugene, OR). All other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue (WAT) was obtained from non-diabetic females, between the ages of 20-50 years old with a body mass index (BMI) $\leq$ 30 during abdominoplasty. Consent was obtained from the Institutional Review Board at the University of North Carolina at Greensboro. Tissue was digested using collagenase and stromal vascular (SV) cells were isolated as previously described (Brown et al. 2004). Cultures containing ~50% preadipocytes and ~50% adipocytes were treated between day 6-12 of differentiation. Each experiment was repeated at least twice at different times using a mixture of cells from 2-3 subjects unless otherwise indicated.

Preparation of FAs

Both isomers of CLA were complexed to FA-free ($\geq$98%) bovine serum albumin (BSA; Sigma #A-7030) at a 4:1 molar ratio using 1 mmol/L BSA stocks. This specific

type of BSA has a relatively low inflammatory capacity compared to at least 10 different types of BSA we have tested in our lab.

Immunoblotting

Immunoblotting was conducted as we previously described (Brown et al. 2004).

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

$[Ca^{2+}]_i$ levels were measured using Fluo-3 AM. Briefly, cells were preloaded with 5 uM Fluo-3 AM and 10% Pluronic F-127, an anionic detergent at 25°C for 30 min in the dark. Cells were then washed with a buffer consisting of HBSS, $CaCl_2$, and probenecid, which prevents Fluo-3 leakage from cells, and baseline fluorescence was measured using a Synergy Multi-detection Microplate Reader (Bio-Tek Inc, Winooski, VT). Cells were then treated in the absence or presence of CLA or BSA vehicle in the absence and presence of RSV. Fluorescence was monitored for 10 sec intervals for 5 min. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Changes in the ratio of calcium-dependent fluorescence over prestimulus background fluorescence (F/F0) are plotted over time in single representative experiments.

Measuring ROS levels

For the DCF assay, primary human adipocytes were seeded in 96-well plates and differentiated for 6 days. On day 6, media was changed to serum- and phenol red-free media for 24 h. Following 24 h, cells were treated with various treatments for 3 h. Cells

were then spiked and incubated with 5uM DCF and placed at 37° C for 1 h. Cells were

then washed once with HBSS and fluorescence was immediately measured in a plate

reader with an excitation/emission wavelength of 485/528nm.   DCF values were

calculated after normalizing background fluorescence levels of DCF.

Transient transfections of human adipocytes

For measuring PPARγ activity, primary human adipocytes were transiently

transfected with the multimerized PPRE-responsive luciferase (luc) reporter construct

pTK-PPRE3x-luc (Kliewer et al. 1992) using the Amaxa Nucleofactor as previously

described (Chung et al. 2005). On day 6 of differentiation, 1×10^6 cells from a 60 mm

plate were trypsinized and resuspended in 100 μL of nucleofector solution (Amaxa) and

mixed with 2 μg of pTK-PPRE3x-luc and 25 ng pRL-CMV for each sample.

Electroporation was performed using the V-33 nucleofector program (Amaxa). Cells

were replated in 96-well plates after 10 min recovery in calcium-free RPMI media.

Firefly luciferase activity was measured using the Dual-Glo luciferase kit and normalized

to *Renilla* luciferase activity from the co-transfected control pRL-CMV vector. All

luciferase data are presented as a ratio of firefly luciferase to Renilla luciferase activity.

RNA isolation and real time PCR

Total RNA was isolated from the cultures using Tri Reagent purchased from

Molecular Research Center (Cincinnati, OH) according to manufacturer's protocol. For

real time qPCR, 2.0 ug total RNA was converted into first strand cDNA using Applied

Biosystems High-Capacity cDNA Archive Kit. qPCR was performed in an Applied Biosystems 7500 FAST Real Time PCR System using Taqman Gene Expression Assays. To account for possible variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample, and data were normalized accordingly.

Lipid staining

Lipid staining was conducted using Oil-Red-O (ORO) as previously described (Brown et al. 2003). The TG levels were measured using a modified, commercially-available TG assay as previously described (Brown et al. 2003). The protein content was determined using the BioRad's BCA assay.

2-[^{3}H]deoxy-glucose and [^{14}C]oleic acid uptake

Following the experimental treatments for 48 h, insulin-stimulated uptake of 2-[^{3}H]deoxy-glucose and [^{14}C]oleic acid were measured following a 90 min incubation in the presence of 100 nmol/L human insulin as described previously (Brown et al. 2004).

Statistical analysis

Statistical analyses were performed for data in Figures 5.1D, 5.2B, 5.3D, 5.4A,B, and 5.5B,C by testing the main effects of CLA (BSA, CLA) and RSV (- or + RSV) and their interaction (CLA x RSV) using two-way ANOVA (JMP Version 6.03, SAS Institute; Cary, NC). For data in Figure 5.2C, a two way ANOVA of the main effects

Treatment (BSA, 9,11 CLA, 10,12 CLA, 10,12 CLA+RSV) and Time (6, 12, 24, 48 h) and their interactions for each dosage was conducted. A one-way ANOVA was conducted for data in Figures 5.1C, 5.3B, and 5.6B,C. Student's t tests were used to compute individual pairwise comparisons of least square means (P<0.05). Data are expressed as means ± S.E.

Results

RSV blocks 10,12 CLA induction of inflammation and stress-related signaling.

A preliminary dose response study showed that 50 uM RSV most-effectively decreased the activation of ERK1/2 (Fig. 5.1A) and JNK (data not shown), and expression of inflammation genes (Fig. 5.1C) caused by 10,12 CLA without reducing cell viability. Thus, we examined the extent to which 50 uM RSV prevented inflammation caused by 50 uM 10,12 CLA in human adipocytes. RSV attenuated 10,12 CLA activation of ERK1/2 (Fig. 5.1B) and induction of IL-6, IL-8, and IL-1β gene expression (Fig. 5.1D) within 12 h. Similarly, RSV blocked 10,12 CLA induction of the inflammatory PG pathway (Fig. 5.2A = PLA$_2$, 5.2B = COX-2, 5.2C = PGF$_{2\alpha}$). Next, we examined the influence of RSV on [Ca^{+2}]$_i$ and ROS levels in CLA-treated cultures. RSV attenuated the rapid, 10,12 CLA increase in [Ca^{+2}]$_i$ and ROS (Fig. 5.3A = [Ca^{+2}]$_i$, 5.3B = ROS), events directly linked to cellular stress, inflammation, and insulin resistance (Gregor et al. 2007, Houstis et al. 2006). Similarly, RSV attenuated 10,12 CLA activation of the stress-related proteins ATF3 and JNK (Fig. 5.3C), and the mRNA levels of ATF3 (Fig. 5.3D). RSV alone increased the levels of ATF3 mRNA and protein. Taken together, these data

demonstrate that RSV attenuates 10,12 CLA-mediated inflammation and cellular stress in cultures of human adipocytes.

10,12 CLA suppression of insulin sensitivity, FA uptake, and TG content are prevented by RSV.

We previously demonstrated that 10,12 CLA causes insulin resistance, dependent on the activation of ERK1/2 (Brown et al. 2004) and NFκB (Chung et al. 2005). Given the insulin sensitizing effects reported for RSV in rodents (Baur et al. 2006, Lagouge et al. 2006, Sun et al. 2007), we speculated that RSV would improve insulin sensitivity in cultures of human adipocytes treated with 10,12 CLA. Indeed, 10,12 CLA treated-cultures co-supplemented with 50 uM RSV had higher levels of insulin-stimulated glucose uptake compared to cultures treated with 10,12 CLA alone (Fig. 5.4A). Consistent with these data, RSV blocked 10,12 CLA induction of the suppressor of cytokine synthesis (SOCS)-3, a protein that causes insulin resistance through serine phosphorylation of IRS-1. RSV also attenuated 10,12 CLA suppression of adiponectin and sirtuin (SIRT)1 mRNA levels compared to cultures treated with 10,12 CLA alone, suggesting RSV enhances glucose and/or FA metabolism in these cultures (Fig. 5.4B). Similarly, RSV co-supplementation attenuated delipidation and suppression of FA uptake by 10,12 CLA (Fig. 5.5A-C). Thus, RSV may enhance insulin-stimulated glucose and FA uptake in CLA-treated cultures by upregulating genes that stimulate metabolism.

10,12 CLA suppression of PPARγ inhibited by RSV.

To determine the mechanism by which RSV improves insulin sensitivity and reduces delipidation, we examined the extent to which RSV enhanced the protein levels and activity of PPARγ, a transcription factor that enhances glucose and FA uptake and utilization. RSV prevented CLA-mediated decrease of PPARγ protein levels (Fig. 5.6A). To determine whether RSV blocks CLA-suppression of PPARγ activity, the ligand-induced activation of a PPRE-luciferase reporter construct was measured. 10,12 CLA-mediated suppression of the BRL-activated PPRE reporter was prevented by co-supplementation with RSV (Fig. 5.6B), suggesting that RSV prevents CLA suppression of ligand-stimulated PPARγ activity. Concordantly, RSV increased the activation of the PPRE reporter in a dose-dependent manner in the absence of BRL (Fig. 5.6C). Collectively, these data support our hypothesis that RSV enhances insulin sensitivity and TG content of 10,12 CLA-treated human adipocytes by increasing the activity and/or protein expression of PPARγ.
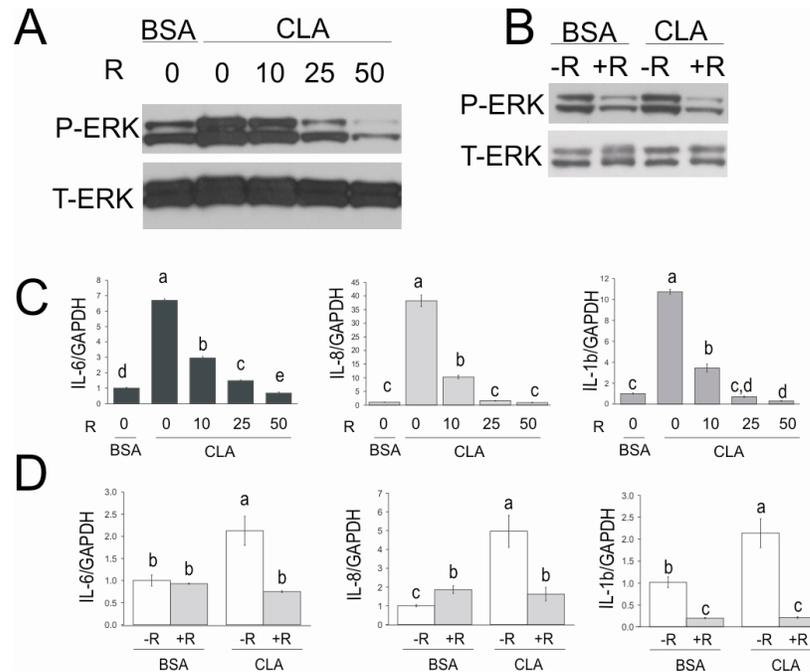
Figure 5.1. RSV attenuates 10,12 CLA activation of ERK1/2 and induction of cytokines. Cultures of newly-differentiated human adipocytes were serum-starved for ~24 h and then treated for 12 h with BSA vehicle or 50 uM 10,12 CLA in the absence (-) or presence (+) of 0, 10, 25, or 50 uM RSV (R). Subsequently, cultures were harvested for the determination of the protein levels of P-ERK1/2 and total (T)-ERK1/2 by immunoblot (A, B) or mRNA levels for IL-6, IL-8, and IL-1β by real time qPCR (C, D). A, B: Data are representative of one (A) or at least three (B) independent experiments. C: D: Data are representative of one (C) or at least three (D) independent experiments. Means ($\pm$ S.E.; n=2 for C, and n=3 for D) not sharing a lower case letter differ significantly (p<0.05). The RSV dose in B and D was 50 uM.
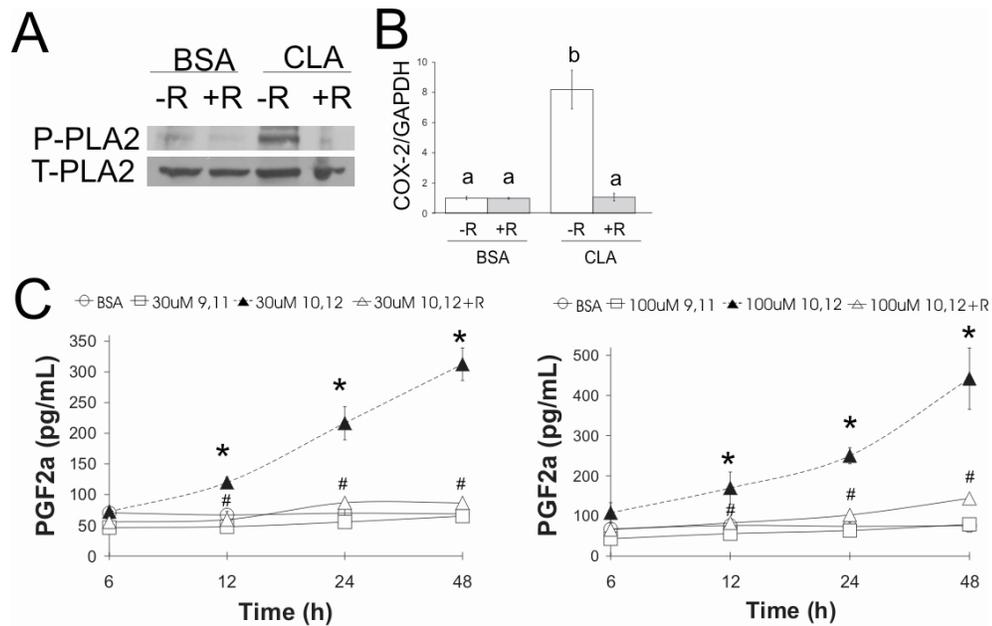
Figure 5.2. **RSV attenuates 10,12 CLA activation of the inflammatory PG pathway.** (A= PLA2, B= COX-2, C= PGF$_{2\alpha}$). Cultures of newly-differentiated human adipocytes were serum-starved for ~24 h and then treated for either 12 h (A and B) or 6, 12, 24, or 48 h (C) with BSA vehicle or 50 uM 10,12 CLA in the absence (-) or presence (+) of 50 uM RSV (R). Subsequently, cultures were harvested for the determination of the protein levels of P-PLA$_2$ and total (T)- PLA$_2$ by immunoblot (A), mRNA levels for cyclooxygenase 2 (COX-2) by real time qPCR (B), or the secreted levels of PGF$_{2\alpha}$ in conditioned media by enzyme immunoassay (C). A: Data are representative of at least three independent experiments. B: Means ($\pm$ S.E.; n=3) not sharing a lower case letter differ significantly (p<0.05), and are representative of at least three independent experiments. C: Means ($\pm$ S.E.; n=4) with asterisks (*) differ significantly (p<0.05) from the BSA controls at each time point, and are representative of at least two independent experiments. Means with number sign (#) are significantly lower than cultures treated with 10,12 CLA alone.
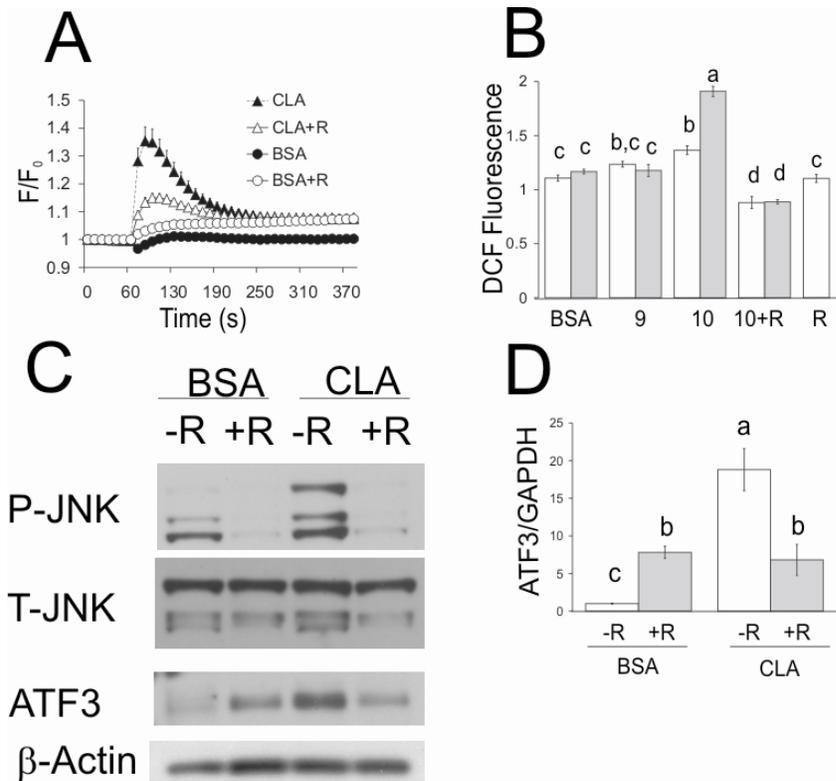
Figure 5.3. **RSV attenuates 10,12 CLA increase in intracellular calcium and indicators of cellular stress.** A: Cultures of newly differentiated human adipocytes were serum starved for ~24 h and then preloaded with 5 uM Fluo-3 AM. Subsequently, cultures were injected with 30 uM 10, 12 CLA (▲), 30 uM 10,12 CLA + 50 uM RSV (CLA+R, Δ), BSA vehicle (BSA, ●), or BSA+RSV (BSA+R, ○). Emitted fluorescence intensities were collected over time using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means ($\pm$S.E.; n=12) are representative of three independent experiments. B: Cultures of newly differentiated human adipocytes were serum-starved ~24 h and then treated with 50 (□) or 150 uM (■) BSA, 9, 11 CLA, 10, 12 CLA, 10, 12 CLA + 50 uM RSV (R) or 50 uM RSV alone for 3 h. Cultures were then loaded with DCF for 1 h and emitted fluorescence intensities were measured using a multi-detection microplate reader. Excitation wavelength was 485 nm and fluorescence was collected at 528 nm. Means ($\pm$ S.E.; n = 3-12) are representative of three independent experiments. C, D: Cultures of newly-differentiated human adipocytes were serum-starved for ~24 h and then treated for 12 h with BSA vehicle or 50 uM 10,12 CLA in the absence (-) or presence (+) of 50 uM RSV (R). Subsequently, cultures were harvested for the determination of the protein levels of P-JNK, total (T)-JNK, ATF3, and β-actin by immunoblot (C) or mRNA levels for ATF3 by real time qPCR (D). C: Data are representative of at least three independent experiments. D: Means ($\pm$ S.E.; n=3) not sharing a lower case letter differ significantly (p<0.05), and are representative of at least two independent experiments.

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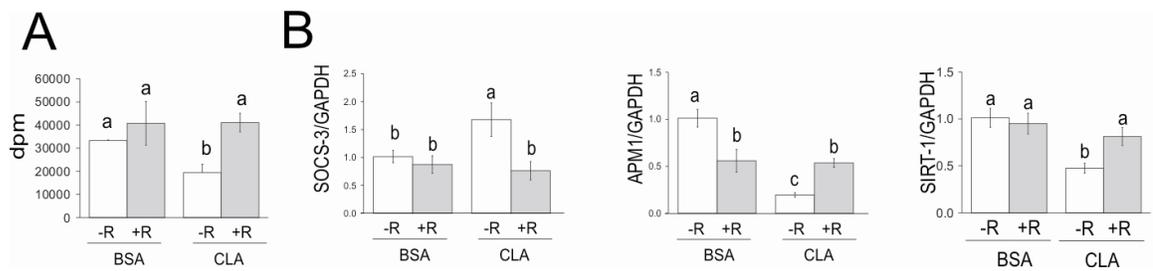
Figure 5.4. RSV blocks 10,12 CLA-mediated insulin resistance. Cultures of newly-differentiated human adipocytes were serum-starved for ~24 h and then treated for either 48 h (A) or 24 h (B) with BSA vehicle or 50 uM 10,12 CLA in the absence (-) or presence (+) of 50 uM RSV (R). A: Insulin-stimulated glucose uptake using 2-[^{3}H]deoxyglucose (2-DOG) was measured after a 90 min incubation in the presence of insulin. Means ($\pm$ S.E.; n=6) not sharing a lower case letter differ significantly (p<0.05). B: The mRNA levels of suppressor of cytokine synthesis 3 (SOCS-3), adiponectin (APM-1), and SIRT-1 were measured by real-time PCR. Means ($\pm$ S.E.; n=3) not sharing a lower case letter differ significantly (p<0.05), and are representative of at least two independent experiments.
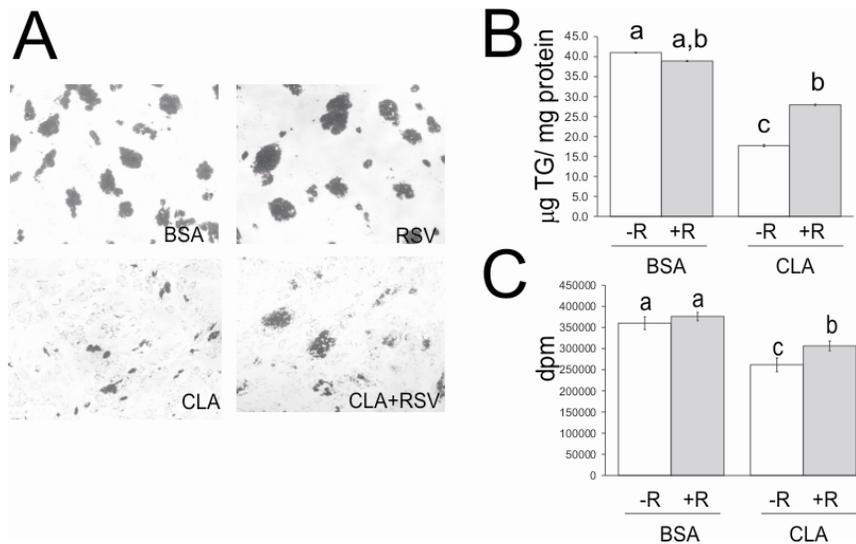
Figure 5.5. **RSV attenuates delipidation by 10,12 CLA.** Cultures of newly-differentiated human adipocytes were treated in adipocyte media for 7 d with BSA vehicle or 50 uM 10,12 CLA in the absence (-) or presence (+) of 50 uM RSV (R). Fresh media containing treatments were changed every 2 d. Cultures were then either stained with Oil Red O and phase-contrast photomicrographs were taken using an Olympus inverted microscope with a 10X objective (A) or their TG content was measured using a commercially available TG assay kit (B). A: Data are representative of two independent experiments. B: Means ($\pm$ S.E.; n=6) not sharing a lower case letter differ significantly (p<0.05). C: Cultures of newly-differentiated human adipocytes were serum-starved for ~24 h and then treated for either 48 h with BSA vehicle or 50 uM 10,12 CLA in the absence (-) or presence (+) of 50 uM RSV (R). [14C]oleic acid uptake was measured after 90 min incubation in the presence of insulin. Means ($\pm$ S.E.; n=3) not sharing a lower case letter differ significantly (p<0.05).
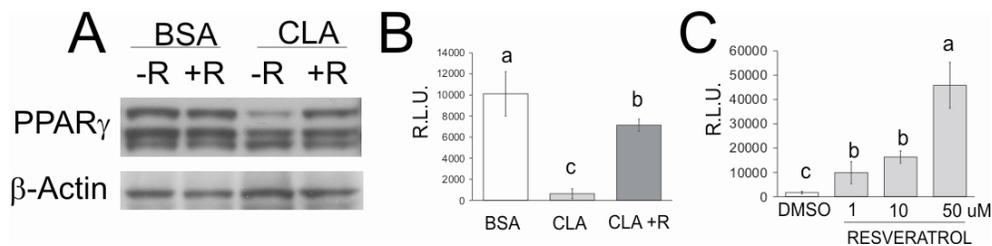
Figure 5.6. RSV inhibits 10,12 CLA suppression of PPARγ activity. Cultures of newly-differentiated human adipocytes were serum-starved for ~24 h and then treated for 24 h with BSA vehicle or 50 uM 10,12 CLA in the absence (-) or presence (+) of 50 uM RSV (R). Subsequently, cultures were harvested for the determination of PPARγ and β-actin protein levels by immunoblot (A). B: Cultures of newly-differentiated human adipocytes were transfected on day 6 with pTK-PPRE3x-luc and pRL-CMV. Transfected cells were treated with BSA vehicle, 50 uM 10,12 CLA or 50 uM 10,12 CLA + 50 uM RSV (R) for 24 h. Subsequently, cultures were treated with 0.1 uM BRL for 28 h, and then the luciferase activation of the reporter was measured using a luminometer ($\pm$ S.E., n=3). C: Transfected cells were treated with 1, 10, or 50 uM R for 24 h, and then the luciferase measured as in B ($\pm$ S.E., n=3). Means ($\pm$ S.E.; n=3) not sharing a lower case letter differ significantly (p<0.05). Data in A-C are representative of at least two independent experiments.

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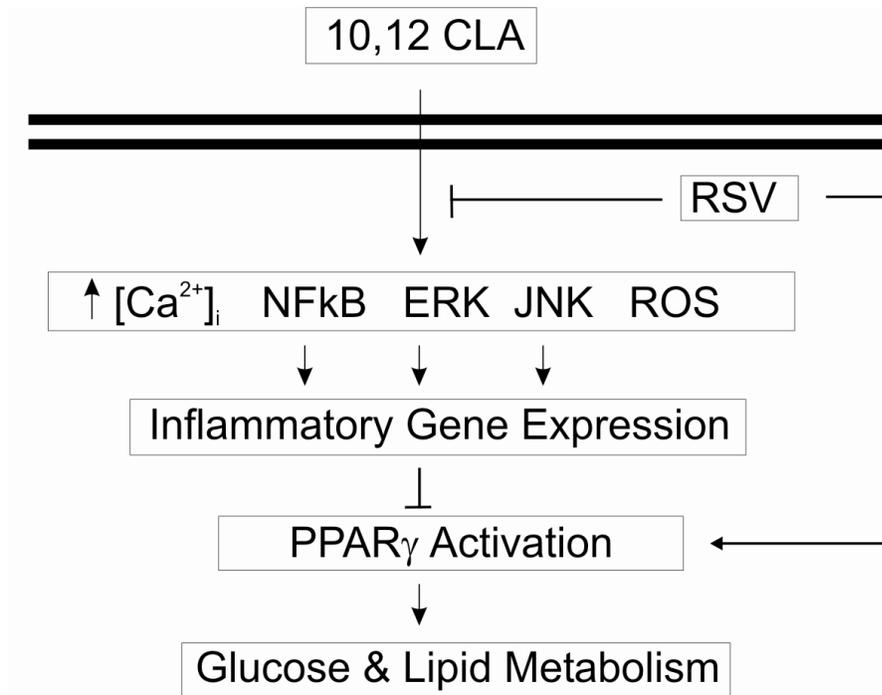
Figure 5.7. Working model. RSV initially blocks 10,12 CLA-mediated increase in the levels of ROS and $[Ca^{+2}]_i$, thereby preventing ROS and calcium signaling. Without these signals to activate ERK, JNK, NFκB, inflammatory cytokines, and PGs, PPARγ activity is not suppressed. This allows for normal insulin signaling, glucose and FA uptake and metabolism, and TG accumulation in adipocytes. RSV may also directly activate PPARγ.

Discussion

Feeding mixed isomers of CLA, or 10,12 CLA alone, has been shown to reduce body fat and the TG content of adipocytes, especially in murine models (House et al. 2005). However, adverse metabolic complications (i.e., inflammation, ISR, insulin resistance) have been reported with CLA supplementation of humans, particularly for the 10,12 isomer (Riserus et al. 2002, Riserus et al. 2002, Tholstrup et al. 2007, LaRosa et al. 2007). In this article, we demonstrate for the first time that RSV, a phytoalexin with antioxidant properties, attenuates markers of inflammation, cellular stress, ROS production, insulin resistance, and delipidation in cultures of newly differentiated human adipocytes treated with 10,12 CLA. Central to this mechanism is our discovery that RSV blocks 10,12 CLA-mediated 1) increase in $[Ca^{+2}]_i$, which is essential for CLA-mediated inflammation and cellular stress (Kennedy et al. 2007), and 2) suppression of PPARγ activity in primary cultures of human adipocytes treated with 10,12 CLA.

Based on these and our previously (un)published data, we propose the following working model (Fig. 5.7) by which RSV prevents 10,12 CLA-mediated inflammation (Figs. 5.1, 5.2), cellular stress (Fig. 5.3), insulin resistance (Fig. 5.4), and ultimately delipidation (Fig. 5.5). We speculate that RSV initially blocks CLA-mediated ROS production or accumulation, and release of calcium from the ER. This prevents cellular stress initiated by ROS and calcium signaling. Without these signals to activate MAPKs (e.g., ERK, JNK) and other inflammatory proteins (e.g., NFκB, COX-2, PLA$_2$) or PGs (e.g., PGF$_{2α}$), 1) inflammatory cytokines (e.g., IL-6, IL-1β) and chemokines (IL-8) are not induced, 2) insulin signaling is not disrupted, and 3) the protein levels and activity

of PPARγ and the TG levels are preserved (Figs. 5.5, 5.6). This allows for normal insulin signaling, glucose and FA uptake and metabolism, and TG accumulation in adipocytes.

Consistent with our data in human adipocytes, 50-150 uM RSV has been shown *in vitro* to reduce inflammation in murine 3T3-L1 adipocytes (Ahn et al. 2007), reduce oxidative stress in human lung epithelial cells (Kode et al. 2008, Robb et al. 2008), reduce ER stress in mouse macrophages (Tabata et al. 2007), decrease TNFα-mediated NFκB activation in hepatocytes (Yu et al. 2008) and coronary arterial endothelial cells (Csiszar et al. 2006), and enhance glucose transport in muscle (Park et al. 2007). Thus, RSV reduces inflammation and enhances glucose and FA utilization *in vivo* and *in vitro*, although the mechanism is unknown.

Further support for our working model comes from studies showing that RSV decreases $[Ca^{+2}]_i$ levels following stimulation with various inflammatory agents or disease states. For example, elevated levels of $[Ca^{+2}]_i$ induced by severe acute pancreatitis was attenuated by RSV (Wang et al. 2008). Similarly, RSV reduced $[Ca^{+2}]_i$ levels in stress-induced oxygen-glucose deprivation/reperfusion in primary neurons of neonatal rats (Gong et al. 2007). Lastly, trans-RSV prevented platelet aggregation thru inhibiting elevated $[Ca^{+2}]_i$ levels (Dobrydneva et al. 1999). Thus, RSV suppression of the levels of ROS and $[Ca^{+2}]_i$ may be an important mechanism by which RSV prevents these deleterious side effects of 10,12 CLA.

Alternatively, RSV may suppress inflammation by activating PPARγ. Support for this hypothesis comes from studies showing that RSV activates PPARγ in CaCo2 cells (Ulrich et al. 2006) and macrophages (Ge et al. 2006). Additionally, our data show the

RSV robustly induces PPARγ activity and prevents CLA-mediated suppression of TZD-induced PPARγ activation (Fig. 5.6). Further support comes from studies showing that compounds that enhance PPARγ activity and insulin sensitivity antagonize NFκB-mediated signaling, and vice-versa (Suzawa et al. 2003, Adams et al. 1997, Ruan et al. 2003, Nie et al. 2005). For example, TZDs, which are high affinity PPARγ ligands, suppress inflammation (Ruan et al. 2003, Nie et al. 2005). In contrast, PPARγ depletion via siRNA enhances the inflammatory responses of TNFα (Liao et al. 2007). Consistent with these data, Rosiglitazone prevents CLA-mediated insulin resistance and hepatic steatosis in rats (Lui et al. 2007, Purushotham et al. 2007), and delipidation of human adipocytes (Kennedy et al. 2008).

One proposed mechanism for the anti-inflammatory actions of PPARγ ligands is via (trans) repression of inflammatory gene transcription (Pascual et al. 2005, Ricotte et al. 2007, Straus et al. 2007, Ghisletti et al. 2007). Activation of PPARγ can repress the transcriptional activation of inflammatory genes by 1) direct interaction with the mediator of transcription (i.e., NFκB, JNK), 2) inhibition of its co-activator recruitment, or 3) inhibition of its co-repressor clearance. For an example of transrepression, ligand-activated PPARγ becomes SUMOylated, which then binds to co-repressor complexes at inflammatory gene promoters, thereby inhibiting dismissal of NCoR/HDAC, which blocks inflammatory gene transcription (Pascual et al. 2005, Ricotte et al. 2007). Notably, this pathway does not interfere with transactivation of PPARγ-responsive genes (Pascual et al. 2005, Ricotte et al. 2007). Studies are underway to determine if this is the mechanism by which RSV prevents inflammation in CLA-treated adipocytes.

RSV has also been shown to enhance insulin sensitivity and glucose uptake in C2C12 myotubes by enhancing AMPK activity (Ruan et al. 2003). AMPK is activated by adiponectin, and by SIRT1 and PGC1α (Lagouge et al. 2006, Sun et al. 2007). These events are directly linked to mitochondrial biogenesis and oxidative metabolism, which are positively regulated by PPARα. Consistent with these data, RSV prevented or attenuated insulin resistance and the suppression of SIRT1 and adiponectin mRNA levels in CLA-treated adipocytes (Fig. 5.4). This effect of RSV may be responsible for increasing glucose and FA uptake for oxidative metabolism, which we have previously shown are suppressed by 10,12 CLA (Brown et al. 2004). However, we do not know if this was due to increased SIRT1 or PGC-1α activation by RSV.

SIRT1 activation appears to be an important means by which RSV enhances glucose and FA utilization, at least in muscle. SIRT1 increases the activation of PGC-1α and FOXO1-C, thereby increasing mitochondrial biogenesis and oxidative metabolism (Qiao et al. 2006). Furthermore, several studies demonstrated that feeding 20-400 mg/kg body weight/ d of RSV prevents insulin resistance and adiposity in mice fed a high fat diet (Baur et al. 2006, Lagouge et al. 2006). These studies suggest that RSV shifts excess calories away from storage in WAT and towards oxidation in muscle and brown adipose tissue (BAT), in part, by activating SIRT1. In contrast to our hypothesis that RSV activates PPARγ, SIRT1 overexpression in murine adipocytes decreased PPARγ activity and treatment of murine adipocytes with 50-100 uM RSV enhanced FA mobilization and release. However, PPARγ activities in WAT of mice or in cultures of adipocytes treated with RSV were not investigated (Picard et al. 2004). Thus, SIRT1 activation by RSV is

clearly linked to enhanced glucose and FA metabolism in muscle, and involves AMPK activation. However, the effects of RSV on SIRT1 activation and adipocyte metabolism are still unclear, and differences in SIRT1 regulation by RSV between mouse and human adipocytes are unknown.

Taken together, these data demonstrate that RSV prevents 10,12 CLA-mediated inflammation, insulin resistance, and delipidation of human adipocytes. Potential anti-inflammatory mechanisms for RSV include preventing CLA-mediated ROS accumulation and release of calcium from the ER, which are associated with cellular stress and inflammation, and antagonism of PPARγ activity. Alternatively, RSV may be directly activating PPARγ, which has the potential to (trans) repress inflammatory gene transcription. Studies are underway to examine these proposed mechanisms.

CHAPTER VI

EPILOGUE

Obesity is an important health issue in the U.S. and world-wide. Identifying safe and effective treatments for obesity is critical for decreasing risk factors associated with obesity such as diabetes and cardiovascular disease. Dietary supplementation with conjugated linoleic acid (CLA) has been shown to reduce body fat mass in a number of animals such as mice, rats, and pigs. CLA's efficacy in humans is still controversial. Aside from altering body weight, CLA supplementation has also been reported to increase markers of inflammation, induce insulin resistance, and in some cases, cause lipodystrophy. Therefore, it is necessary to understand the mechanism of action of CLA in white adipose tissue (WAT), and its potential adverse side effects.

Our research group has previously demonstrated that 10,12 CLA activates mitogen-activated protein kinase / extracellular signal-regulated kinase kinase (MEK/ERK) and nuclear factor kappa B (NFκB) signaling. Activation of these proteins induces interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF) α production and attenuates peroxisome proliferator activated receptor (PPAR)γ target gene expression, leading to reductions in glucose and fatty acid uptake and adipocyte delipidation. Based on these findings, we have investigated 1) 10,12 CLAs impact on PPARγ activity, 2) upstream signals (i.e., intracellular calcium $[Ca^{2+}]_i$, reactive oxygen species [ROS],

prostaglandins [PGs]) responsible for the activation of NFκB and MAPKs by 10,12 CLA, and 3) the role of inflammation and insulin resistance in 10,12 CLA-induced delipidation.

From these data, I propose (Fig. 6.1) that 10,12 CLA activates a G-protein coupled receptor (GPCR) or generates metabolites that activate phospholipase C (PLC), thereby increasing levels of diacylglycerol (DAG) and inositol triphosphate (IP3). These proposed signals induce the release of calcium from intracellular stores such as endoplasmic reticulum (ER). The elevation of $[Ca^{2+}]_i$ leads to activation of calcium-calmodulin dependent kinase II (CAMKII), ER stress, and/or mitochondrial dysfunction resulting in production of ROS. These events result in the activation of NFκB and MAPKs, which induce inflammation and antagonize PPARγ activity, resulting in the suppression of adipogenic and lipogenic genes and insulin resistance and delipidation of cultures of human adipocytes. Based on these findings, I have developed the following new questions 1) To what extent to does 10,12 CLA activate GPCRs?, 2) Does 10,12 CLA activate PLC and increase DAG and IP3 levels?, 3) What impact does 10,12 CLA have on mitochondrial and ER function?, and 4) What role does c-Jun N-terminal kinase (JNK) play in 10,12 CLA-mediated inflammation, insulin resistance, and delipidation?

Q1. To what extent does 10,12 CLA activate GPCRs?

Transient increases of $[Ca^{2+}]_i$ can occur when a ligand binds to a cell surface receptor such as a GPCR. We have demonstrated that 10,12 CLA increases $[Ca^{2+}]_i$ levels within 5 min in cultures of human adipocytes. Also, our research group has demonstrated pertussis toxin, an inhibitor of G proteins, attenuates 10,12 CLA activation of ERK.

Using data acquired from a microarray gene analysis that I conducted with MERCK Pharmaceuticals, I will identify possible GPCR receptors that are induced by 10,12 CLA in cultures of human adipocytes. Once I have identified candidate GPCRs, I will utilize PCR to verify the findings from the microarray analysis. Next, I will perform siRNA experiments to knockdown the gene expression of potential GPCRs, and then measure $[Ca^{2+}]_i$, inflammatory gene expression, and insulin-stimulated glucose uptake in the presence or absence of 10,12 CLA.

Q2. Does 10,12 CLA activate PLC and increase DAG and IP3 levels?

I demonstrated that D609, a PLC inhibitor, attenuated 10,12 CLA increase of $[Ca^{2+}]_i$, ROS, and inflammation, suggesting that PLC activity may be involved in 10,12 CLA-induced inflammation. The major drawbacks of interpreting chemical inhibitor data are that inhibitors are not typically specific for the target of interest. This was a major issue for interpretation of the results in Chapter IV when using KN-62, the CAMKII inhibitor, and TMB-8, the inhibitor of calcium release from the ER. Therefore, use of siRNA is more specific for the target of interest. To investigate the extent to which PLC is involved in 10,12 CLA-induced inflammation, insulin resistance, and delipidation, I will first measure the phosphorylation levels of the PLC using phospho-specific antibodies for the four PLC isoforms (PLCβ, PLCγ, PLCδ and PLCε) in the absence or presence of 10,12 CLA. Next, once I determine which PLC isoform is phosphorylated by 10,12 CLA, I will utilize siRNA to knockdown the target of interest. Once I achieved successful knockdown, $[Ca^{2+}]_i$, markers of inflammation, and insulin-stimulated glucose uptake will be measured in the absence or presence of 10,12 CLA.

PLC generates the lipid-borne second messengers DAG and IP3 from the phospholipids PIP2 in response to activation by hormones, growth factors, and lipids. If 10,12 CLA is activating PLC, then the levels of IP3 and DAG should be elevated. To investigate this hypothesis, the levels of IP3 will be measured using an IP3 fluorescence polarization assay kit from DiscoverX (Fremont, CA). Cultures of human adipocytes cultured in 96 well plates will be treated in the absence or presence of 10,12 CLA. To each well quench reagent will be added. The IP3 tracer and binding protein will then be added simultaneously. Fluorescence polarization will be measured. Bound IP3 tracer will "tumble" more slowly in solution than on-bound IP3, creating a polarized signal (high mP). Also, the levels of DAG and phosphatidic acid will be measured by thin layer chromatography.

Q3. What impact does 10,12 CLA have on mitochondrial and ER function?

There is considerable evidence from our research group and others that 10,12 CLA elicits a stress response in adipocytes. Currently, it remains to be determined the origin of the stress response. LaRosa et al. (2007) demonstrate that 10,12 CLA increases the expression of ER stress-related genes such as XBP-1, eIF2α, and CHOP. However, there was limited protein data in support of these findings. I investigated 10,12 CLA induction of ER stress in cultures of human adipocytes, but was unable to demonstrate activation or induction of key proteins or genes involved in ER stress. Based on these findings, I hypothesized that 10,12 CLA induced mitochondria dysfunction. I initiated studies to investigate the impact of 10,12 CLA on mitochondria function by measuring mitochondria membrane potential and release of cytochrome C to the cytoplasm. The

findings of these experiments were negative, but there were some issues concerning the dye used to measure membrane potential. Particularly, the dye aggregated towards the lipid droplets making it unclear to decipher whether the dye was staining the mitochondria or areas around the lipid. Therefore, another approach I will take is to measure oxygen consumption in adipocytes treated with 10,12 CLA using oxygen sensitive plates.

Q4. What role does JNK play in 10,12 CLA induced inflammation, insulin resistance, and delipidation?

I recently discovered that 10,12 CLA increases the phosphorylation levels of JNK and it's downstream target cJun, a member of the redox-sensitive transcription factor activating protein-1 (AP-1), that induces inflammatory gene transcription and cell survival. Notably, I found that the polyphenolic compound resveratrol blocked 10,12 CLA activation of JNK. Data acquired from a microarray gene analysis demonstrated that 10,12 CLA increased the gene expression of JNK and cJun, and resveratrol attenuated this induction. Based on these preliminary data, I hypothesized that 10,12 CLA activation of JNK is a key player in 10,12 CLA-mediated inflammation, insulin resistance, and delipidation.

To investigate the role of JNK, the isomer-specific effects of 10,12 CLA on the activation of JNK and cJun will be determined in the presence and absence of specific chemical inhibitors of JNK (i.e., SP600125) and siRNA targeted to JNK1 and 2, followed by immunoblotting for JNK and cJun using phospho-specific antibodies. The impact of JNK activation on 10,12 CLA's induction of proinflammatory genes (i.e., IL-8, IL-6, IL-

1β, COX2, and ATF3) and suppression of lipogenic and adipogenic genes and protein expression (i.e., PPARγ, GLUT4, adiponectin, ACC, and LPL) will be measured. The impact of these JNK inhibitors and siRNA targeted to JNK1 and 2 on insulin-stimulated glucose uptake and delipidation will also be investigated.

In conclusion, further investigation of potential upstream signals responsible for calcium release and ROS production needs to continue. These studies will provide mechanistic insights for delineating the role of isomer-specific role of 10,12 CLA for the prevention or treatment of obesity and inflammation, and identifying lipid-borne signals induced by CLA that regulate glucose and lipid metabolism in human adipocytes. Thus, we will gain more knowledge for the development of therapeutic treatments for obesity and metabolic diseases.
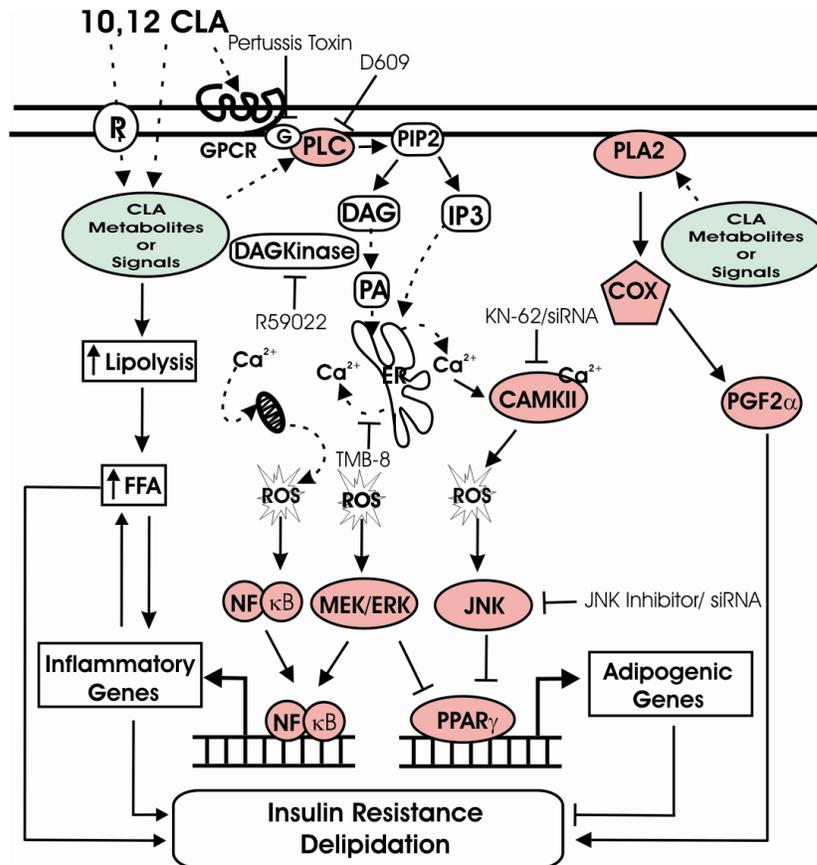
Figure 6.1. Working Model-10,12 CLA's antiobesity mechanism of action in adipocytes. 10,12 CLA activates a GPCR or generates metabolites that activate PLC thereby increasing levels of DAG and IP3. These proposed signals induce the release of calcium from intracellular stores such as ER. The elevation of $[Ca^{2+}]_i$ leads to activation of CAMKII, ER stress, and/or mitochondrial dysfunction resulting in production of ROS. These events result in the activation of NFκB and MAPKs, which induce inflammation and antagonize PPARγ activity, resulting in the suppression of adipogenic and lipogenic genes and insulin resistance and delipidation of cultures of human adipocytes.

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APPENDIX A

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Sarah McCormack

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Also attached is the information for the Graduate School

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