T10c12 Conjugated Linoleic Acid Causes Delipidation in 3T3-L1 Adipocytes and Mice

shan jiang
biojs@hotmail.com

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By

Shan Jiang

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Shan Jiang, Ph.D

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Adviser: Jess L. Miner

T10c12 conjugated linoleic acid (t10c12 CLA) causes the reduction of triglyceride contents in adipocytes. T10c12 CLA's delipidation effect is associated with decreased nutrient uptake, adipogenesis, lipogenesis, and increased energy expenditure, lipolysis, fatty acid oxidation in adipocytes. However, the molecular mechanisms of CLA's delipidation effects are still unknown. AMP-activated protein kinase (AMPK), a central regulator of cellular energy levels, is activated by an increase in the cellular AMP:ATP ratio or various cellular stresses. We demonstrated that t10c12 CLA activated AMPK in 3T3-L1 adipocytes, leading to inhibition of anabolic biosynthesis and increase of energy expenditure. Strong activation of AMPK can induce an inflammatory response, which is required for effective delipidation in t10c12 CLA-treated adipocytes. Sirtuin 1 (SIRT1), a NAD+-dependent histone/protein deacetylase that regulates energy homeostasis, is functionally required for robust TG reduction in response to t10c12 CLA in adipocytes. SIRT1 activated by t10c12 CLA is able to activate AMPK, and functions coordinately with AMPK to enhance TG loss. Activated SIRT1 is also capable of repressing peroxisome proliferator-activated receptor γ (PPARγ) activity to increase delipidation in t10c12 CLA-treated adipocytes. However, the activation of PPARγ by its agonists, essential for adipogenesis and maintenance of adipocytes, strongly reduced AMPK activity and attenuated TG loss in t10c12 CLA-treated adipocytes. T10c12 CLA also activated PPARδ,

a nuclear receptor that regulates fatty acid oxidation, and PPARδ agonists and antagonists increased or decreased AMPK activity, respectively. Activated PPARδ competed more effectively for RXR, an obligate partner for many nuclear receptors, and reduced the amount of PPARγ/RXR protein complex present. Our studies define the Sirt1/AMPK/PPARγ axis as the key regulatory point for determining whether adipocytes increase or decrease TG levels, with activated SIRT1 and AMPK promoting TG loss and PPARγ opposing this loss.

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Conjugated linoleic acid and delipidation

Introduction

Obesity has become a global epidemic disease, with more than 1 billion adults overweight and more than 300 million of them clinically obese. Recently, at least 155 million children worldwide are overweight or obese, causing concerns about the increasing incidence of child obesity [1]. Even in many developing countries, the rising rate of overweight and obesity is a growing concern, with a faster increase than in the developed world [2]. Obesity is associated with many diseases, particularly heart disease, type 2 diabetes, breathing difficulties during sleep, certain types of cancer, and osteoarthritis [1,2]. Currently, obesity and type 2 diabetes are the leading metabolic diseases. There is a great need for the prevention and therapeutic strategies against these health diseases. For the obesity therapeutics, many current strategies are based on inhibiting satiety and doing exercise, but none of the current treatments are effective for controlling body weight or reducing obesity.

It has been clearly demonstrated that conjugated linoleic acid (CLA) is able to significantly reduce body fat, particularly abdominal fat, decrease the level of serum lipids, decrease glucose uptake, and increase lipid oxidation [3]. CLA [18:2(n-6)] refers to a family of polyunsaturated fatty acids (PUFAs) that exist as conjugated stereoisomers of linoleic acids [4]. Early in 1979, Pariza et al. found that tumorigenesis in a mouse model was inhibited by certain components from grilled beef [5]. In 1987, they identified CLA as the active component to provide beneficial effects [6]. CLA has at least 13 isomers, which are found primarily in the meat and dairy products of ruminants [4]. CLA can be synthesized by anaerobic bacteria through biohydrogenation in the ruminant animals [7]. CLA has very broad beneficial effects such as anticarcinogenic, antioxidant, antiatherosclerosis, antiatherogenic, and antidiabetogenic properties [8-10]. The promising application of CLA as a therapeutic strategy to these diseases

has received great attention. Whereas, some differences appear in CLA effects between species [11]. Two main CLA isomers, c9t11 and t10c12, have some different biological effects. Compared to the c9t11 isomer, t10c12 CLA has much stronger delipidation effects both in vitro and in vivo [12-15].

CLA induces delipidation

As early as 1997, the delipidation effect of CLA was reported by Park et al. Consumption of feed including 0.5% mixed CLA for 4-5 weeks (1:1 ratio of c9t11 and t10c12) in ICR (Institute for Cancer Research) male and female mice decreased body fat mass by 60% [16]. In this study, the delipidation mechanism proposed that t10c12 CLA increased lipolysis and fatty acid oxidation, but reduced fatty acid uptake in adipocytes. In another study of atherogenic diet-fed hamsters, diet containing 0.5% t10c12 CLA for 6 weeks caused a significant reduction of fat mass [17]. For Zucker diabetic fatty rats, 1.5% mixed CLA was able to decrease fat mass significantly [10]. For the delipidation effect of CLA, most of the experiments showed that t10c12 isomer, not the c9t11 isomer, plays the primary role in the reduction of fat mass. The data from our lab demonstrated that mice fed 0.5% t10c12 CLA for 2 weeks lost more than 75% of white adipose tissue (WAT) mass in the retroperitoneal (RP) and epididymal (EPi) fat tissues [12]. Interestingly, more fat loss is induced by CLA in RP than EPi. This suggests that the response sensitivity to CLA is dependent on the type of fat tissue. T10c12 CLA was also reported to induce significant delipidation in other animals, including chickens [18], rabbits [19] and porcine [20]. Interestingly, when eliminating CLA from the diet, the accumulation of TG is recovered in the fat tissues. This suggests that the fat loss is reversible when CLA is removed, indicating CLA doesn't destroy adipocytes.

CLA's delipidation effect on animals is associated with exercise. It has been recently reported that high-fat diet-fed BALB/C male mice treated with the mixture of 0.4% CLA (50:50 c9t11+t10c12) and moderate treadmill exercise for 14 weeks showed reduced body fat mass, decreased serum leptin levels, as well as increased oxygen consumption and energy expenditure [21].

In vitro, the delipidation effect was shown in the CLA-treated 3T3-L1 cell line and human primary cells. Park et al. reported that 20-200 μM of t10c12 CLA was able to cause the decrease of TG content in 3T3-L1 adipocytes [22]. Studies in our lab also showed 100 μM of t10c12 CLA caused the robust loss of TG content by 50% in the mature 3T3-L1 adipocytes, as compared to control treatment [23]. McIntosh group showed that 3-30 μM t10c12 CLA was able to inhibit the preadipocyte differentiation and decrease TG content in primary human stromal vascular (SV) cultures [24]. Although the robust delipidation effect of t10c12 CLA was reported, the isomer-specific delipidation mechanism of CLA is still not clearly known.

T10c12 CLA has been demonstrated to induce dramatic fat loss in human SV adipocyte cultures [24]. However, most of the human studies have not shown the significant delipidation effect observed in animal and cell line studies. There are few positive delipidation effects of t10c12 CLA reported in some short- and long-term studies in human trials, showing the beneficial effects of t10c12 CLA in reducing fat mass [25-27]. The reason for the limited delipidation effects of t10c12 CLA in human trials may be because t10c12 CLA dosage used in human studies is much lower than used in animal studies [28]. In these human studies, a dosage of 3.4 g per day has been used , while the dosage used in mice is at least 10 times this amount per kg bodyweight [29]. To increase effective delipidation in humans, our goal is to combine t10c12 CLA with other nutritional supplements to attain the synergistic effects to induce respectable fat

loss at a lower CLA dose than used in mice. This has great potential for inducing adiposity in human trials.

Adipocyte

Adipocytes are the main target of the CLA delipidation effect. The majority of dietary CLA enters into adipocytes. Improving our understanding of adipocyte biology is the key to determining the mechanism of CLA's delipidation effects. Mesenchymal stem cells (MSC) can generate a common early preadipocyte, which can be further differentiated to committed white adipocyte under certain stimuli. The number of preadipocytes in adipose tissue is limited [30]. During adulthood, the number of fat cells remains consistent in lean and obese humans, even after marked weight loss. About 10% of adipocytes renew every year [31].

Peroxisome proliferator activated receptor-γ (PPARγ) and CCAAT/Enhancer Binding Protein α (C/EBPα) are two important transcriptional factors in the differentiation of preadipocytes to adipocytes. As the master regulator of adipogenesis, PPARγ is both necessary and sufficient to enable the preadipocytes to differentiate into adipocytes [32]. PPARγ is a nuclear hormone receptor and has two different isoforms produced by alternative splicing of the PPARγ mRNA. PPARγ1 is ubiquitously expressed, but PPARγ2 is mainly expressed in adipose tissue [33]. Some synthetic PPARγ ligands, such as thiazolidinediones (TZDs), can activate PPARγ. PPARγ forms a heterodimer with retinoid acid receptor (RXR). The heterodimer is able to activate the transcription of adipogenic target genes by binding to peroxisome proliferator response elements (PPREs) in their promoters [34]. Adipocyte differentiation was promoted by overexpression of PPARγ2 in fibroblasts [35]. In contrast, adipocytes can not be formed without PPARγ2 [36]. In a conditional knockout to create a PPAR γ2-deficient mouse model, the amounts of adipose tissue were decreased compared to wild type mice [37].

C/EBPα is a transcription factor that also plays an important role in adipogenesis by interacting with PPARγ. C/EBPα can activate the transcription of adipogenesis genes such as PPARγ and aP2 by binding to their promoters [33]. C/EBPα is sufficient to enhance preadipocyte differentiation. In 3T3-L1 preadipocytes, C/EBPα knockdown prevents adipocyte differentiation [38]. In vivo, with C/EBPα-deficient mice, the number of adipocytes was reduced in the adipose tissue, compared to wild-type mice [36].

Interestingly, PPARγ and C/EBPα promote adipogenesis in a coordinated manner: PPARγ and C/EBPα positively increase the expression each other. Whereas in the absence of C/EBPα, reduced levels of PPARγ still can stimulate adipogenic differentiation but weakly. However, reduced levels of C/EBPα can't induce adipogenesis in the absence of PPARγ [36]. This suggests that PPARγ is essential in the differentiation of adipocytes.

Preadipocyte factor-1 (Pref-1) is a transmembrane protein containing epidermal growth factor-like domains. Pref-1 has very high expression in 3T3-L1 preadipocytes, while it is highly suppressed during adipocyte differentiation, becoming undetectable in mature adipocytes. Pref-1 prevents adipogenesis by inhibiting PPARγ and C/EBPα expression [39]. Suppression of Pref-1 is required for adipocyte differentiation. In 3T3-L1 preadipocytes, decreasing Pref-1 levels by siRNA knockdown promotes adipogenesis [40]. In vivo, in Pref-1 deficient mice, the body weight and fat depots were increased more rapidly, compared to wild-type mice [39]. Conditional transgenic mice overexpressing Pref-1 in adipose tissue, using the aP2 promoter, showed decreased adipose tissue mass, compared to wild-type mice [41, 42]. This indicates that overexpression of a preadipocyte specific gene in differentiating adipocytes can inhibit further differentiation.

There are two types of adipose tissue in mammals. White adipose tissue (WAT) stores energy as triglycerides. When energy shortage such as fasting happens, release of fatty acids from triglyceride in white adipocyte into the plasma by lipolysis is used to supply the energy to the body. Although brown adipose tissue (BAT) shares many features with white adipocytes, BAT plays a physiological function in fatty acid oxidation and thermogenesis, which is opposite to that of white adipocyte. The brown adipocyte contains lots of smaller droplets and numerous mitochondria which contain iron which make the tissue brown. In contrast, the white adipocyte contains a single and large lipid droplet and fewer mitochondria [43].

White adipose tissue has a low intrinsic oxidative capacity. Current research showed that the limited fatty acid oxidation in white adipocytes comes from the low protein level of uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor coactivator-1α and fatty acid re-esterification involved enzymes [44]. The expression of uncoupling protein 1 (UCP1) in numerous mitochondria is essential for fatty acid oxidation and heat generation in brown adipocytes. UCP1 uncouples ATP synthesis and oxygen consumption to enhance energy dissipation as heat. The detailed mechanism is that UCP1 diverts energy from ATP synthesis to thermogenesis in the mitochondria by leaking protons out of the mitochondria [45]. Transgenic C57BL6/J mice expressing UCP1 from the adipocyte lipid-binding protein (aP2) promoter are capable of reducing genetic and diet-induced obesity [46]. However, in human intraperitoneal adipose tissue, it was estimated that 0.5-1% of adipocytes express UCP1 [47, 48]. Interestingly, the microarray data in our studies showed that UCP1 mRNA levels dramatically increased by 26 fold in RP tissues, as compared to the control group, when the mice were fed 0.5% CLA for 2 weeks [12].

The mechanism of t10c12 CLA-induced delipidation

T10c12 CLA causes little change in feed intake

Generally, food intake is a starting point to study t10c12 CLA's delipidation effect. Most studies, including our recent studies [12], in mice, rats and pigs showed that t10c12 CLA has no effect on feed intake. However, a few studies showed that t10c12 CLA caused a little loss of appetite in animals [49]. Further studies indicated a slight reduction in feed intake does not completely account for a significant reduction in fat mass [50].

T10c12 CLA inhibits adipogenesis

Adipogenesis is a differentiation process in which preadipocytes become adipocytes. T10c12 CLA can inhibit differentiation in a dose-dependent manner via the inhibition of the key transcription factors of adipogenesis, including PPARγ, C/EBPα, PPARγ co-activators and fatty acid binding proteins [23]. Our studies based on 3T3-L1 cells and mouse models also showed t10c12 CLA can decrease the protein level of adipogenesis associated genes, and increase the expression of preadipocyte factor-1 (Pref-1) , which is an inhibitor of adipogenesis [12].

T10c12 CLA inhibits lipogenesis

Lipogenesis is a process by which fatty acids are synthesized and esterified to glycerol. Sterol regulatory element-binding proteins (SREBPs) are one class of important transcription factors to regulate the process of lipogenesis. SREBPs function by binding to the sterol regulatory element DNA sequence. Inactive SREBPs attach to the membranes of the nuclear envelope and endoplasmic reticulum. Low sterol levels is enough to trigger the cleavage of SREBPs to a water soluble N-terminal domain, which is able to translocate into the nucleus and enhance the sterol synthesis. The cleavage of SREBPs is then inhibited by increased sterol level through a negative feedback regulation. SREBP-1c is responsible for regulation of fatty acid

synthesis [51]. It was reported that t10c12 CLA's delipidation effect was associated with the inhibition of SREBPs [23]. T10c12 CLA may down-regulate SREBP-1c and LXR-α, then inhibit the transcription of genes involved in fatty acid synthesis and fatty acid elongation, including acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD), glycerol phosphate acyltransferase and acylglycerol phosphate acyltransferase [12]. Other down-regulated pathways involved in sterol biosynthesis are associated with leucine degradation, ubiquinone biosynthesis, and amino acid metabolism. Our microarray data also showed that the mRNA levels of lipid biosynthetic genes decrease, including ATP citrate lyase and acetyl Co-A carboxylase alpha and beta, in t10c12 CLA-treated 3T3-L1 adipocytes and WAT [12]. Other genes involved in the inhibition of lipogenesis include acylation-stimulating protein, caveolin I, and lipin 1, which had decreased mRNA level in t10c12 CLA treatment.

T10c12 CLA increases energy expenditure

T10c12 CLA was reported to increase energy expenditure [52]. As a mitochondrial enzyme, carnitine palmitoyltransferase 1 (CPT1) is a rate-limiting enzyme for fatty acid beta-oxidation. CPT1 helps transport fatty acids across the membrane via carnitine, and enhance fatty acid beta-oxidation in the mitochondria. This enzyme can be inhibited by malonyl CoA, which is produced by acetyl-CoA carboxylase (ACC). ACC is able to convert acetyl-CoA to malonyl-CoA and supply the malonyl-CoA substrate for fatty acid synthesis. Therefore, the reduction of malonyl-CoA increases the activity of CPT-1, increases the transport of fatty acids into mitochondria, and consequently, increases fatty acid oxidation rate [53]. The work from Park et al. showed that t10c12 CLA increased the activity of CPT1 in mitochondria [22]. In their further studies, they concluded that fatty acid oxidation is an important factor to decrease body fat in the treatment of t10c12 CLA. It was also reported that CPT1 mRNA increased in t10c12 CLA-fed mice [12].

Other studies showed most of the energy expenditure was derived from increased metabolic heat output in t10c12 CLA-fed mice [52]. Our recent studies also demonstrated that transcriptional levels of key genes involved in fatty acid oxidation such as UCP1, UCP2 and CPT1 increased in the fat tissues when the mice were fed with the diet contained 0.5% CLA for 2 weeks [12]. The levels of the induced mRNAs of UCP1 and UCP2 genes are very abundant, accounting for 95% and 90% of the most abundant mRNAs in WAT, respectively [12].

T10c12 CLA increases lipolysis

The form of energy stored in white adipose tissue is triglyceride. Lipolysis is the breakdown of triglycerides into free fatty acids, which are then released into the blood. Lipolysis is a main means to export energy with free fatty acids and glycerols from adipocytes [54]. Perilipin proteins coat oil droplets in adipocytes, acting as a protective coating from breakdown by lipases into glycerol and free fatty acids [55]. Perilipin A is most abundant in mature adipocytes. Adipocyte differentiation-related protein (ADRP) is a major constituent of the globule surface of oil droplets in adipocytes [56]. Compared to perilipin, ADRP has high mRNA level in the early stage of adipocyte differentiation, but low at the large lipid droplet stage in mature adipocytes. This suggests ADRP inhibits the formation of oil droplets and tends to decrease TG accumulation in adipocytes. Chung et al. reported that t10c12 CLA increased lipolysis in human SV adipocyte cultures [57]. The mechanism was t10c12 CLA increased ADRP protein expression, which eliminates the perilipin A proteins. The decreased perilipin A protein coating enhanced fat acid release via lipolysis. Further studies indicated that phosphorylation of perilipin (active form) was regulated by the cAMP-PKA axis in t10c12 CLA treatment. The recent microarray data from our lab supported the increased lipolysis in CLA treated mice [12]. The highly expressed genes involved in lipolysis include monoglyceride lipase,

hormone sensitive lipase and lipase A. Interestingly, the products glycerol and FFA from lipolysis can be re-utilized to synthesize TG, and ATP consumption is required [58]. In this process, called a futile cycle, the increased lipolysis enhances energy expenditure. In this case, t10c12 CLA might utilize a novel mechanism to increase energy expenditure, which is beneficial for obesity and diabetes therapeutics.

T10c12 CLA inhibits nutrient uptake

Glucose and fatty acids are two major sources for triglycerol synthesis in adipocytes. Brown et al. reported t10c12 CLA inhibited the uptake of glucose into primary human adipocytes by decreasing glucose transporter 4 (GLUT4) gene expression [59]. In this study, t10c12 CLA also inhibited the uptake of oleic acids. In the study of 3T3-L1 adipocytes and mouse models, including our recent studies, t10c12 CLA significantly reduced the activity of lipoprotein lipase (LPL), which is an enzyme involved in the uptake of fatty acids by adipocytes [12]. The inhibition of glucose and fatty acid uptake resulted in the reduction of synthesis of triglycerides. In our studies, another gene involved in the inhibition of glucose and fatty acid uptake is the facilitated glucose/fructose transporter member SLC2A5, which had decreased mRNA levels in the t10c12 CLA treatment.

T10c12 CLA induces inflammation response

Inflammation response is induced in the t10c12 CLA-treated adipocyte cultures and WAT [12]. The studies from the McIntosh group showed that t10c12 CLA imparted its effects to activate ERK1/2 and NFκB [60]. NFκB p65/p50 was activated by phosphorylation of ERK when the NFκB subunit complex translocated to the nucleus of SV cells. Activated NFκB enhanced the expression of some cytokines like TNF-α, IL-6 and IL-8. Subsequently, these secreted cytokines bind to their receptors on the adipocyte surface, leading to enhanced inflammation response in a

positive feedback. As a consequence, the inflammation response results in the suppression of PPARγ, C/EBPα, aP2, sterol-CoA desaturase (SCD), LPL, FAS, Glut4 and PLIN. The inflammation response causes a reduction in glucose and fatty acid uptake, and inhibition of Adipogenesis and lipogenesis, but increase of lipolysis, consequently contributing to delipidation in adipocytes. Histologically, our recent studies in mouse model showed the density of macrophages was increased in t10c12 CLA-treated RP fat tissue, about 2 times as many as in the control [12]. The microarray data showed that the first several days of dietary CLA induced an inflammatory response, including IL-1, 6, 10, 15 and 17, their receptors and CXC or CC ligand family members [12]. Interestingly, this initial inflammatory response was transient and the expression levels of many of these genes returned to normal levels after the first week of CLA treatment. Most of these genes express less at later times than in the initial response. Some pathways involved in the CLA-induced inflammation response include Jak/Stat, ERK/MAPK, PI3K/AKT signaling.

T10c12 CLA induces apoptosis

Apoptosis is a form of programmed cell death (PCD) in multicellular organisms. T10c12 CLA was reported to reduce adipose tissue mass by initiating apoptosis [12]. This study showed that CLA induced apoptosis through p53-dependent and/or independent pathways, depending on the cell type [61]. In an in vitro study in the estrogen-unresponsive MDA-MB-231 breast cell line, CLA triggered apoptosis through the reduction of the anti-apoptotic protein Bcl-x and up-regulation of the pro-apoptotic protein Bak [62, 63]. Further studies showed that t10c12 CLA induced caspase-dependent apoptosis, due to decreased bcl-2 and increased p21 (WAF/Cip1) expression [64]. This suggests that t10c12 CLA mediates its effects partially via the modulation of apoptosis and cell cycle control [65]. Previous studies in our lab using a polygenic obese line

of mice (M16) by Miner et al. also showed that apoptosis in MI-CLA fed mouse retroperitoneal fat pads was 4 fold higher than the control mice [66]. Our recent studies in the mouse model showed the number of adipocytes in fat tissues was reduced by 15% [12]. Interestingly, adipocyte cell size became smaller, with about a 90% reduction in cell volume. Our microarray analysis in CLA-treated 3T3-L1 adipocytes and WAT demonstrated the induction of numerous genes involved in apoptotic signaling, including a number of death receptors and their ligands, TNF ligands, and TNF responsive genes, MdM-2, APAF1, BAX, PMAIP1, MCL1 and DNA repair involved such as PCNA and GADD45B, and cell cycle arrest-associated gene CDKNA1 (pRB), proinflammatory initiator caspases 1 and 4 [12].

T10c12 CLA induces integrated stress response

The inflammation response and apoptosis contribute to t10c12 CLA-induced delipidation. In our recent studies, integrated stress response (ISR) appears early and is able to trigger an inflammation response and apoptosis [67]. Integrated stress response (ISR) is triggered by phosphorylation of eukaryotic initiation factor 2α (eIF2 α) under certain stress stimuli such as endoplasmic reticulum (ER) stress in response to an accumulation of unfolded or misfolded proteins in the lumen of ER. We found ISR to be the earliest transcriptional response to t10c12 CLA treatment in 3T3-L1 adipocytes or in WAT in mice [12]. The ISR might be a key regulatory point in the delipidation response to CLA. By examining gene expression between 4 and 24 hrs after t10c12 CLA treatment for both mice and 3T3-L1 adipocyte cell line, our study suggested that the release of proinflammatory cytokines by ISR played an important role in the induction of the later inflammatory response. The release of proinflammatory cytokines may explain how ISR causes lipid loss in mature adipocytes. We further studied the potential t10c12 CLA effect-mediated pathways associated with the increase of phosphorylation of eIF2, which

reduces the rate of protein translation initiation. Phosphorylation of eIF2 induces ATF4, activating ISR/UPR genes including ATF3, CHOP and TRB3, which are involved in cell stress survival and apoptosis, and NFκB, which initiates an inflammatory response. ISR can induce the dephosphorylation of GSK3β by excluding AKT2 from nucleus, and subsequently causes the inhibition of SREBP1c [68].

Conclusion

T10c12 CLA is able to cause dramatic delipidation in cell culture and animal models. The mechanism is associated with inhibited nutrient uptake, Adipogenesis and lipogenesis, but increased energy expenditure, lipolysis, fatty acid oxidation, inflammation, apoptosis and ISR. Despite numerous studies to describe the responses to t10c12 CLA, its molecular mechanism are still unknown. My thesis attempted to discover additional mechanisms by which delipidation is induced by t10c12 CLA at the molecular level. We were particularly interested in the early events in an effort to eventually discover the mechanism initially responding to the presence of CLA. My results explain much of the mechanisms leading to the delipidation response to CLA. The initial "receptors" are waiting for further investigation .

Chapter II. Conjugated linoleic acid activates AMP-activated protein kinase and reduces

adiposity more effectively when used with metformin in mice

Introduction

Conjugated linoleic acid (CLA) isomers of linoleic acid (LA) are found in dairy products and cause reduced adiposity in mice [69]. The *trans*-10, *cis*-12 CLA (*t*10*c*12 CLA) isomer of CLA causes rapid fat loss when fed to mice or in human adipocytes cultured in its presence [16, 60, 69]. *T*10*c*12 CLA increases fatty acid oxidation [70], apoptosis [71], lipid export [57] and an inflammatory response [72] in differentiating or mature adipocytes. It also reduces glucose and fatty acid uptake [73] as well as reducing lipogenesis [71, 73, 74]. These cumulative physiological changes account for the fairly rapid and dramatic loss of lipids in *t*10*c*12 CLA-treated adipocytes [24, 75].

The molecular responses accompanying these physiological changes are remarkably diverse [24] and include the integrated stress response [ISR; [67]] or the unfolded protein response [UPR; [76]], a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated inflammatory response [60], and mitogen-activated protein kinase (MAPK) cascades [57]. The ISR induces activating transcription factor 3 (ATF3) and activating transcription factor 4 (ATF4) proteins as well as possibly activating NF-κB and an inflammatory response [60, 67]. The occurrence of the UPR, which includes the ISR and inflammation, is an emerging theme in chronic metabolic diseases such as type 2 diabetes [77, 78]. The ability of *t*10*c*12 CLA to selectively activate only the ISR component of the UPR [67] may account for its ability to cause selective reduction of the white adipose tissue (WAT) mass [79]. The transcripts and/or protein activities of key adipocyte transcription factors such as CCAAT enhancer binding protein α (C/EBPα), peroxisome proliferator-activated receptor γ *(*PPARγ), sterol regulated element binding protein 1c (SREBP1c), carbohydrate-response-element-binding protein (ChREBP), and

important lipogenic enzymes including acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are reduced when adipocytes are exposed to $t10c12$ CLA [12, 24, 50, 67].

AMP-activated protein kinase (AMPK) is a central regulator of cellular energy levels. Increases in the cellular AMP/ATP ratio or various cellular stresses result in the activation of AMPK [80, 81]. AMPK is a heterotrimeric protein and the activity of the AMPKα catalytic subunit is controlled by the AMPKβ and AMPKγ regulatory subunits as well as phosphorylation at a conserved threonine (Thr-172). AMPK substrates include a number of biosynthetic enzymes such as ACC, FAS, *glycerol*-3-phosphate acyltransferase (GPAT), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). Phosphorylation of these proteins reduces their activity and leads to decreased energy consumption in their respective biosynthetic pathways as well as increased fatty acid oxidation to increase energy production [80, 82]. The activity of AMPK can be modulated by several known chemical inhibitors and activators. Compound C is a potent inhibitor of AMPK while drugs such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), metformin, and thiazolidinediones activate AMPK [80].

In the present study, we analyzed the participation of AMPK in the $t10c12$ CLA-mediated lowering of triglycerides (TG) in 3T3-L1 adipocytes. Our objectives were to determine whether $t10c12$ CLA activated AMPK, AMPK activators or an inhibitor affected the biochemical and transcriptional responses, an inflammatory response was required, and whether metformin enhanced the reduction in adiposity in mice given a low dose of $t10c12$ CLA.

Methods

Reagents. Insulin, isobutyl-1-methylxanthine, dexamethasone, bovine serum albumin (BSA, > 99% fat free), AICAR and metformin were from Sigma (St. Louis, MO). Bay11-7082 and AMPK inhibitor compound C were purchased from Calbiochem (San Diego, CA). Antibodies to

ATF3, ATF4, NF-κB p65, Lamin B, PPARγ, SREBP-1c, and C/EBPα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while those to p-AMPK, AMPK, p-ACC, and ACC were from Cell Signaling (Beverly, MA). The antibodies to p-ACC and ACC recognize both ACC1 and ACC2.

Animals. Forty-two metabolism-low (ML) and forty-two metabolism-control [MC; [83]] 12-wk old male mice were used. Mice were housed individually at 22 °C with 12 h light and 12 h dark. All mice were fed AIN-93G diet ([84]; Dyets, Inc., Bethlehem, *PA*) for 5 d before beginning the treatments. Mice were blocked by initial body weight (ML 33.8 ± 2.2 g; MC 35.7 ± 1.9 g; within genotype) and assigned to one of the seven treatment diets. Specific diets contained either 70 g/kg soy oil without *t*10*c*12 CLA or 67.5 g/kg soy oil with 2.5 g/kg *t*10*c*12 CLA. Mice were allowed to eat *ad libitum.* Metformin, when present, was dissolved in distilled drinking water to provide 2 or 20 mmol/L, and these solutions were changed every 3 days. After 14 d of these treatments, mice were killed in the fed state between 09:00 and 13:00 h by carbon dioxide asphyxia. Retroperitoneal and epididymal fat pads were harvested, weighed, flash frozen in liquid nitrogen, and stored at −80 °C. All procedures were approved by the University of Nebraska Institutional Animal Care and Use Committee.

3T3-L1 cell culture and differentiation. 3T3-L1 fibroblasts [85] were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% bovine calf serum (Fisher, Pittsburgh, PA) and differentiated as described [86]. In brief, two days after growing to confluence, the cells were induced to differentiate by DMEM containing 10% fetal bovine serum (FBS; Fisher, Pittsburgh, PA), 0.17 μmol/L insulin, 0.5 mmol/L isobutyl-1-methylxanthine, and 1 μmol/L dexamethasone for 3 days. The medium was then changed to 10% FBS/DMEM with 0.17 μmol/L insulin for another 2 days, at which time the cells were usually differentiated to at

least 90% adipocytes, followed by basal 10% FBS/DMEM medium before initiating fatty acid treatments. All media contained 100,000 U/L penicillin and 172 µmol/L streptomycin (Invitrogen, Carlsbad, CA). When present, Bay11-7082 was used at 2 µmol/L and compound C was used at 10 µmol/L, and were added 1 h before adding fatty acids.

Fatty acid treatments. Fatty acids (>99%, Nu-chek Prep, Elysian, MN), either linoleic acid or *trans*-10, *cis*-12 CLA, were complexed to fatty acid free BSA (>99%) in a 1:1 ratio (2 mmol/L BSA: 2 mmol/L fatty acid), pH adjusted to 7.4, and added to the cultures containing 5 d post-differentiated 3T3-L1 adipocytes.

Western blots. Nuclear and cytosolic extracts were isolated based on the manufacturer's protocol (Active Motif, Carlsbad, CA). Equal amounts of proteins were separated by SDS-PAGE, transferred to Immun-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA) and probed with the indicated antibodies. Enhanced chemiluminescence (Pierce, Rockford, IL) was used for detection. Band intensities were determined from digital images from exposures in the linear range using software (Quantity One, Biorad, Hercules, CA). All western blot analyses were repeated at least three times.

Quantification of TG content. Cells were collected in phosphate buffer saline, aggregates dispersed by pipetting several times, and aliquots were used to obtain cell counts using a hemacytometer (Hausser Scientific, Horsham, PA), and used to calculate the original density of the cells. Preparation of extracts and TG measurements were performed according to the manufacturer using TG reagent (T2449; Sigma, St. Louis, MO) and free glycerol reagent (F6428; Sigma, St. Louis, MO). TG data are expressed as nmol of TG per 10^6 cells.

Measurement of MCP-1 mRNA. Total RNA was extracted by TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA (2 μg) was used for cDNA synthesis. Real-time PCR was performed by a Bio-Rad iCycler using iQ SYBR Green Supermix reagent (Bio-Rad, Hercules, CA). The primer sequences for MCP-1 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were taken from [87]. MCP-1 mRNA expression was normalized to GAPDH, which showed no significant variation in microarrays between linoleic acid and $t10c12$ CLA treatments. Experiments were repeated three times, and each sample was analyzed using one RNA control and two replicates of each cDNA pool, and the relative amounts of MCP-1 and GAPDH were calculated using the comparative C_T method [according to the manufacturer's software (Bio-Rad, Hercules, CA) and [88]]. Cycle numbers were used to calculate gene expression levels in the linear amplification range, and qRT-PCR efficiency was close to 100%.

Microarray data analysis. RNA isolation and labeling of the 3T3-L1 adipocytes was done according to [12]. Results of the four treatments (linoleic acid, $t10c12$ CLA, linoleic acid + 2 mmol/L metformin, $t10c12$ CLA + 2 mmol/L metformin) of each three biological replicates were collected from Affymetrix® GeneChip Mouse Genome 430 2.0 arrays via the Affymetrix GeneChip Operating Software (GCOS; Affymetrix, Santa Clara, CA). The raw data for microarray results were normalized using the methods described by Wu et al [89]. To identify differentially expressed genes between the control (linoleic acid) and other treatments, the linear models for microarrays [Limma; [90]] package in R/Bioconductor were used, with Benjamini-Hochberg [91] adjusted *P*-values. Those genes with an absolute log2 ratio of 2 or more (four-fold up- or down-regulated) and an adjusted *P*-value ≤ 0.1 were deemed to be significant and taken for further analysis. Calculation of correlation coefficients used a Spearman correlation (two-

tailed test, $\alpha = 0.05$). The gene expression data were deposited at the NCBI Gene Expression Omnibus with series number GSE14888.

Statistical Analysis. Two-way ANOVA was used to analyze the data as follows. For Figures 1A, and 2A-E, the main effects of CLA (LA or CLA) and Compound C (- or +), and their interaction (CLA x Compound C) were analyzed. For Figures 1B, and 1C, the main effects of CLA (LA or CLA) and time (0.5, 1, 8, and 12 h), and their interaction (CLA x time) were determined. For Figures 3A-C, the main effects of CLA (LA or CLA) and Bay11-7082 (Bay11, - or +), and their interaction (CLA x Bay11) were determined. For Figures 4A-C, the main effects of CLA (LA or CLA) and AICAR (- or +) or Met (- or +), and their interaction (CLA x AICAR or Met) were determined. Data were analyzed as a 2 x 2 x 3 factorial ANOVA in the ML and MC mouse experiment. The model included the main effects of mouse line, CLA, and metformin, all two-way interactions, and the three-way interaction, and used $\alpha = 0.1$. Initial body weight was included as a covariate. Pairwise comparisons were calculated using Tukey's test (criterion for significance, $\alpha \leq 0.05$, unless otherwise noted). All analyses were performed using SAS software (SAS, Cary, NC).

Results

3T3-L1 adipocytes treated with compound C, or $t10c12$ CLA and compound C, had TG levels similar to samples treated with the control linoleic acid (LA). Adipocytes treated with $t10c12$ CLA alone produced a 50% reduction in TG levels (Fig. 1A). This result suggested active AMPK was involved in the response to $t10c12$ CLA. This suggestion was confirmed as $t10c12$ CLA-treated adipocytes had increased levels of both phosphorylated AMPK and phosphorylated ACC as early as 30 min after exposure and this enhanced phosphorylation level increased during the 12 h duration of the experiment (Fig. 1B and 1C). The total amounts of AMPK and ACC

remained constant during the experiment (Fig. 1B and 1C), and the amounts of the isoforms of ACC were expected to be similar to their relative mRNA abundance in 3T3-L1 adipocytes, which was 4:1 for ACC1:ACC2 in a microarray analysis (data not shown).

Compound C attenuated the ability of $t10c12$ CLA to increase the phosphorylation of AMPK and ACC (Fig. 2A), to increase in the ISR-induced transcription factors ATF4 and ATF3, after 2 or 8 h of treatment, respectively (Fig. 2B and 2C), and to increase the amount of NF-κB in the nucleus (Fig. 2C). Compound C also attenuated the ability of $t10c12$ CLA to reduce the amounts of the key adipogenic transcription factors C/EBPα, SREBP1c, and PPARγ (Fig. 2D) and to increase the transcript levels of monocyte chemotactic protein-1 (MCP-1; Fig. 2E).

NF-κB inhibitor Bay 11-7082 attenuated the $t10c12$ CLA-mediated reduction in 3T3-L1 adipocyte TG levels (Fig. 3A), and inhibited the increased nuclear localization of the p65 subunit of NF-κB (Fig. 3B). Bay 11-7082 also reduced the amount of MCP-1 mRNA produced in the presence of $t10c12$ CLA (Fig. 3C).

3T3-L1 adipocytes treated with $t10c12$ CLA, metformin, or AICAR showed TG reductions of 14 to 20% when compared to the control LA treatments (Table 1). Adipocytes treated with a combination of either metformin or AICAR and t10c12 CLA had reductions in TG levels of almost 50% (Table 1).

3T3-L1 adipocytes treated with metformin, AICAR, or $t10c12$ CLA, alone or in combination, had increased phosphorylation of AMPK and ACC after 2 or 8 h (Fig. 4 and Table 1). The combination of $t10c12$ CLA with metformin produced significantly higher levels of phosphorylated AMPK at each time. The combination of metformin or AICAR and $t10c12$ CLA reduced the levels of MCP-1 mRNA relative to $t10c12$ CLA alone (Table 1). Neither metformin nor AICAR alone produced a significant induction of MCP-1 mRNA.

The transcriptional responses to $t10c12$ CLA, metformin, or the combination treatment were further analyzed using microarrays. The hypothesis was that if similar pathways were being activated by the two treatments the microarray results should show a high degree of similarity. The gene expression changes for 3T3-L1 adipocytes treated with either metformin or $t10c12$ CLA or the combination of the two produced very similar responses (Table 2 and Supplemental Tables 1-6). The correlation coefficients for genes with significant changes in transcript levels were 0.75 or better for pairwise analysis of these treatments (Table 2). We also compared the expression changes for these treatments using specific lists of genes in the ISR (Supplemental Table 4) and lipogenesis (Supplemental Table 5) pathways that were determined in prior experiments with $t10c12$ CLA or the UPR-inducing chemical tunicamycin [67]. The correlation coefficients for these specific gene lists for the above treatments were 0.91 or better, and indicated all three treatments had a strong ISR (Table 2 and Supplemental Table 4). One apparent difference was that the inflammatory response was weaker in the metformin treatment. Metformin treatment produced lower levels of mRNA induction for the CD14 antigen (*CD14*), cardiotrophin-like cytokine factor 1 (*Clcf1*), cyclo-oxygenase 2 (*Cox2*), colony stimulating factor 1 (*Csf1*), MCP-1, oncostatin M receptor (*Osmr*), plasminogen activator inhibitor type 1 (*PAI* or *Serpine1*) and suppressor of cytokine signaling 3 (*Socs3*) genes that respond more strongly with t10c12 CLA treatment (Supplemental Table 6).

Metformin's ability to enhance the effectiveness of a low dose of $t10c12$ CLA was then evaluated in ML and MC mice. The three-way interaction of mouse line x $t10c12$ CLA x metformin was significant for epididymal (EPI) WAT ($P = 0.08$) and approached significance for retroperitoneal (RP; $P = 0.22$). Within the ML mice fed $t10c12$ CLA, those without metformin had only a 3% reduction in fat pad weights and had significantly greater weights for the pair of

RP or EPI fat pads than mice that also received the high dose of metformin (Supplemental Fig.1 and Table 3). The MC mice fed t10c12 CLA had a 58% reduction in WAT mass, making an interaction with metformin difficult to evaluate (Table 3).

Discussion

*T*10*c*12 CLA causes isomer specific reductions in lipids in adipocytes and here we demonstrated that *t*10*c*12 CLA activated AMPK, although it is not clear if this was a direct activation of AMPK or an indirect activation via upstream regulatory proteins [80, 82]. The *cis*-9, *trans*-11 isomer of CLA (*c*9*t*11 CLA) generally does not cause lipid loss [24], but has been recently reported to stimulate AMPK activity in myotubes [92]. In those studies *c*9*t*11 CLA alleviated the palmitate-mediated suppression of AMPK activity [92]. However, those investigators observed the highest levels of AMPK activity in the untreated control samples, and they did not present data indicating c9t11 CLA independently activated AMPK, leaving this issue unresolved. We used LA (*cis*-9, *cis*-12 linoleic acid) as a control fatty acid because it is an isomer that differs from *t*10*c*12 CLA only in the 9-cis position of the double bond. In our experiments, the isomer specific ability of *t*10*c*12 CLA to strongly activate AMPK in adipocytes correlated with its isomer-specific lipid lowering properties in adipocytes.

AMPK's central role in reducing anabolic biosynthesis and increasing catabolic processes helps explain the dramatic fat loss and variety of physiological and molecular responses observed in WAT in mice fed *t*10*c*12 CLA [12, 24, 50, 67]. Key metabolic or regulatory proteins known to be inhibited by AMPK phosphorylation include ACC, SREBP1c, C/EBPα, FAS, HMGR, and glycogen synthase [82, 93]. We found compound C attenuated the reduction in the levels of the SREBP1c, C/EBPα and PPARγ proteins and the increase in the phosphorylation of ACC typically caused by *t*10*c*12 CLA. Activated AMPK is also expected to phosphorylate

transcription co-activator p300 which should reduce its interaction with transcription factors PPARγ and retinoid X receptor, inhibiting their ability to induce lipogenesis [94]. The DNA binding activity of the transcription factor ChREBP, which regulates genes involved in glycolysis and lipogenesis, is also known to be inhibited when phosphorylated by AMPK [95]. The presence of activated AMPK in these cells and its known ability to cause reductions in the activity or amounts of these key biosynthetic and regulatory proteins helps explain the reduction in lipogenesis [71, 73, 74] and the reduced transcript levels of many lipogenic genes in $t10c12$ CLA-treated adipocytes.

An ISR is initiated by $t10c12$ CLA in WAT in mice and 3T3-L1 adipocytes [67]. Increased AMPK activity was sufficient to initiate an ISR when the AMPK activator AICAR was added to F442a adipocytes [96]. Our microarray analysis indicated an ISR occurred in 3T3-L1 adipocytes treated with AMPK activator metformin, whose chemical structure is unrelated to AICAR's. We also found that compound C inhibited the ability of $t10c12$ CLA to induce an ISR. Together, these results strongly suggest that activation of AMPK by $t10c12$ CLA is responsible for the ISR observed. Activation of the ISR has been observed to cause reductions in WAT of mice fed a leucine deficient diet [79]. This latter observation supports a functional role for an ISR in lipid loss in adipocytes.

An inflammatory response occurs during the response to $t10c12$ CLA *in vitro* and *in vivo* [12, 60, 72]. Inhibition of NF-κB reduces the inflammatory response in $t10c12$ CLA-treated human adipocytes and attenuates the reduction in glucose transporter GLUT-4 and PPARγ protein levels [60]. We found that inhibition of NF-κB, and its associated inflammation response, attenuated TG reduction in $t10c12$ CLA-treated 3T3-L1 adipocytes. Metformin activated AMPK, induced an ISR that did not induce MCP-1, and produced a weaker TG loss in 3T3-L1

adipocytes or in WAT in mice. AICAR seems to repress inflammatory responses as AICAR reduced the production of MCP-1 from stimulated adipocytes [97]. An ISR might be sufficient to cause an inflammatory response through activation of NF-κB [98], but this inflammatory response was partially suppressed when metformin or AICAR was used in combination with $t10c12$ CLA, possibly by AMPK independent mechanisms [99]. Taken together, these results indicate that although some level of an inflammatory response appears to be needed for robust TG loss, higher levels of inflammation are not required.

Many physiological responses are known to participate in mediating the lipid loss in $t10c12$ CLA treated adipocytes [12, 24, 50, 67] and activated AMPK may play a role in many of these processes [80, 82]. In particular, activated AMPK might facilitate the increased rates of fatty acid oxidation that occur in $t10c12$ CLA-treated 3T3-L1 adipocytes [70]. AMPK phosphorylation of ACC is expected to reduce the amount of malonyl-CoA that ACC produces. In addition to its role in fatty acid biosynthesis, malonyl-CoA is a key regulatory molecule that inhibits the activity of carnitine palmitoyltransferase-1 (CPT-1) [100]. A more active CPT-1 will increase the rate of fatty acid transport into mitochondria and produce an increased fatty acid oxidation rate [93]. Previous microarray studies of WAT of mice fed $t10c12$ CLA for 1 to 14 or 17 days showed increased transcript levels of both CPT-1 and uncoupling protein 1 (UCP-1) after several days [12, 50]. The combination of reduced amounts of malonyl-CoA, increased amounts of CPT-1 fatty acid transporter activity, and higher levels of UCP-1 would support an increased rate of fatty acid oxidation *in vivo,* which is consistent with the increased rates of metabolism that have been observed in some experiments with $t10c12$ CLA-fed mice [52]. The much higher levels of UCP-1 mRNA observed in $t10c12$ CLA fed mice [12, 50] might provide a longer term mechanism of reducing adiposity as transgenic mice overexpressing UCP-1 in WAT

were found to have elevated AMPK activity levels and to be resistant to diet-induced obesity [101].

The question remains as to whether activation of AMPK by $t10c12$ CLA is necessary to mediate the majority of the lipid reduction effects observed in treated adipocytes. Either AICAR or metformin activated AMPK in adipocytes, but produced only limited reductions in lipid levels relative to the reductions observed with $t10c12$ CLA treatments. This difference might be explained by our finding that $t10c12$ CLA more strongly activated AMPK over longer times, thereby increasing its effects. The ability of either AMPK activator to allow a lower concentration of $t10c12$ CLA to cause a more robust lipid loss in adipocytes further supports the involvement of AMPK activity with the amount of lipid lost. The complete inhibition of $t10c12$ CLA-mediated TG reduction we observed on addition of compound C indicates AMPK is probably a key component of this response. The reduced phosphorylation of AMPK and ACC that occurred when treated with compound C, provided confirmation that compound C is inhibiting AMPK in our experiments. However, compound C could potentially inhibit other protein kinases that might also affect the response. This possibility of another pathway playing a major role in the response is not supported by our finding of very similar transcriptional responses to metformin, $t10c12$ CLA, or the combination of the two. Altogether, these results are consistent with a hypothesis that activated AMPK is a necessary component of the mechanisms utilized by $t10c12$ CLA to mediate lipid loss in adipocytes.

In tests of $t10c12$ CLA and metformin in mice, metformin's enhancement of the effects of $t10c12$ CLA in ML mice was consistent with our results in 3T3-L1 adipocytes while the results from the MC mice were inconclusive. This is probably because the 58% loss of WAT mass caused by $t10c12$ CLA alone made further reductions in lipid amounts in the WAT more

difficult to achieve in MC mice. Additionally, the MC and ML mice are not inbred lines and were bred to have substantial genetic variability within each line [102], possibly contributing to the variation in the responses observed, which reduced the statistical significance of the results. Our interpretation of these results is that metformin is probably more effective when combined with $t10c12$ CLA at a marginal low dose than with a higher dose of $t10c12$ CLA that is sufficient to cause robust lipid loss.

The AMPK activators, metformin and members of the thiazolidinediones class (e.g., rosiglitazone and pioglitazone), are widely used in the treatment of insulin resistance and type 2 diabetes [80, 103, 104]. These drugs activate AMPK indirectly, and partially reverse the decreased AMPK activity and increased malonyl-CoA levels associated with metabolic syndrome [105-107]. AMPK also affects the organism's energy perception through its regulation by adipokines such as leptin and adiponectin [107]. Our results suggest that $t10c12$ CLA should be added to this list of chemicals used in humans that elicit mechanisms that activate AMPK. These results may have relevance to human trials by suggesting that $t10c12$ CLA's limited effectiveness for reducing adiposity in humans [108] might be increased in combination with metformin or other dietary or medicinal chemicals that activate AMPK. However, AMPK activators that are also potent PPARγ agonists, such as the thiazolidinediones, are not likely to be as useful as they antagonize the effects of $t10c12$ CLA [109].

Tables and figures

Table 1. Levels of TG, relative levels of AMPK and ACC phosphorylation, or MCP-1 mRNA in 3T3-L1 adipocytes treated with AMPK activators alone or in combination with $t10c12$ CLA[1]

Drug	Drug0		Drug2		Significance		
	-CLA	+CLA	-CLA	+CLA	Drug	CLA	Drug x CLA
TG (nmol/L)[2]							
AICAR	309±11[a]	266±3[b]	256±8[c]	167±2[d]	<0.001	<0.001	0.01
Metformin	309±11[a]	266±3[b]	247±9[c]	158±13[d]	<0.001	<0.001	0.03
p-AMPK/AMPK (fold)[3]							
AICAR 2h	0.9±0.2[c]	2.3±0.1[b]	1.0±0.3[c]	3.3±0.2[a]	0.004	<0.001	0.01
AICAR 8h	1.3±0.3[b]	3.8±0.3[a]	1.7±0.7[b]	3.7±0.2[a]	0.340	<0.001	0.13
Metformin 2h	0.9±0.2[d]	2.3±0.1[b]	2.0±0.1[c]	6.1±0.5[a]	<0.001	<0.001	<0.01
Metformin 8h	1.3±0.3[d]	3.8±0.3[b]	2.0±0.1[c]	9.1±0.8[a]	<0.001	<0.001	<0.01
p-ACC/ACC (fold)[3]							
AICAR 2h	0.9±0.4[c]	3.1±0.2[a]	1.7±0.3[b]	3.4±0.2[a]	0.040	<0.001	0.33
AICAR 8h	1.2±0.3[d]	4.2±0.1[b]	2.0±0.3[c]	4.8±0.1[a]	0.020	<0.001	0.42
Metformin 2h	0.9±0.4[d]	3.1±0.2[b]	1.6±0.3[c]	3.8±0.2[a]	0.020	<0.001	0.85
Metformin 8h	1.2±0.3[d]	4.2±0.1[b]	2.4±0.2[c]	8.8±0.2[a]	<0.001	<0.001	<0.01
MCP-1 mRNA (fold)[4]							
AICAR	1.0±0.5[c]	23.7±1.0[a]	0.7±0.6[c]	9.7±2.3[b]	0.001	<0.001	0.02
Metformin	1.0±0.5[c]	23.7±1.0[a]	0.2±0.6[c]	16.9±0.7[b]	0.001	<0.001	<0.01

[1]Differentiated adipocytes were treated with LA (-CLA) or $t10c12$ CLA (+CLA), 2 mmol/L AICAR, or 2 mmol/L metformin alone, or in combination. Data are presented as the mean ±

SEM, n=3 independent experiments. Means in a row not sharing a common letter differ, $P \leq$ 0.05.

[2]TG levels were measured 24 h after treatment with 50 µmol/L of either fatty acid.

[3]Immunoblots were analyzed as described in Figure 4.

[4]Cells were treated with 100 µmol/L of either fatty acid for 12 h and the amounts of MCP-1 and GAPDH mRNA determined.

Table 2. Spearman correlation coefficients for gene expression changes in 3T3-L1 adipocytes treated with $t10c12$ CLA, metformin or the combination[1]

	Treatment compared	
Gene list compared[2]	Metformin	$T10c12$ CLA + Metformin
All significant genes[3]		
$t10c12$ CLA	0.75 ($n = 246$)	0.86 ($n = 410$)
$t10c12$ CLA + Metformin	0.92 ($n = 408$)	
ISR[4] genes ($n = 63$)		
$t10c12$ CLA	0.91	0.96
$t10c12$ CLA + Metformin	0.97	
Lipogenesis[4] ($n = 69$)		
$t10c12$ CLA	0.95	0.97
$t10c12$ CLA + Metformin	0.98	

[1] Data for each treatment were derived from 3 microarrays and analyzed for the Spearman correlation coefficient for the comparisons indicated, and had P-values $\leq 1 \times 10^{-10}$.

[2] The number of genes being compared in each gene list is represented by n.

[3] The 'all significant genes' list is derived from the union of genes in either treatment that show a four fold change in expression and a Benjamini-Hochberg-adjusted P-value ≤ 0.1 in at least one of the treatments being compared.

[4]The ISR and lipogenesis gene lists were derived from a list of genes previously found to be responsive to t10c12 CLA or tunicamycin in 3T3-L1 adipocytes [67].

Table 3. The effect of combining metformin and *t*10*c*12 CLA on lipid loss in ML and MC mice[1]

BW change (g/2 wk) or Tissue (g)[2]	Met0[3]		Met2		Met20		Significance		
	-CLA	+CLA	-CLA	+CLA	-CLA	+CLA	Met	CLA	Met x CLA
ML mice									
BW	2.46±0.48a	1.89±0.95ab	2.89±1.00a	0.68±0.40b	2.54±0.51a	0.88±0.48b	0.07	0.01	0.50
EPI	0.90±0.09ab	0.89±0.17ab	1.06±0.13a	0.71±0.10bc	1.08±0.12a	0.64±0.07c	<0.01	<0.01	<0.01
RP	0.28±0.05a	0.27±0.08ab	0.37±0.10a	0.19±0.04bc	0.33±0.06a	0.15±0.02c	0.76	0.02	0.34
MC mice									
BW	1.51±0.53a	1.42±0.46ab	1.96±0.90a	0.27±0.48b	2.82±0.96a	0.47±0.96b	0.77	0.03	0.31
EPI	0.64±0.11a	0.31±0.07b	0.59±0.07a	0.36±0.11ab	0.48±0.09a	0.29±0.04b	0.48	<0.01	0.72
RP	0.28±0.05a	0.10±0.01b	0.23±0.04a	0.10±0.04b	0.23±0.06a	0.06±0.01b	0.48	<0.01	0.77

[1]Data are presented as mean ± SEM, n = 6. Means in a row not sharing a common superscript differ, $P< 0.1$.

[2]The change in body weight (BW) or the final weight of the epididymal (EPI) or retroperitoneal (RP) was measured after 14 d of treatments.

[3]Mice were fed diets supplemented with soy oil (-CLA), or *t*10*c*12 CLA (+CLA), and given drinking water lacking (Met0) or containing metformin at 2 (Met2) or 20 mmol/L (Met20) for 14 d.
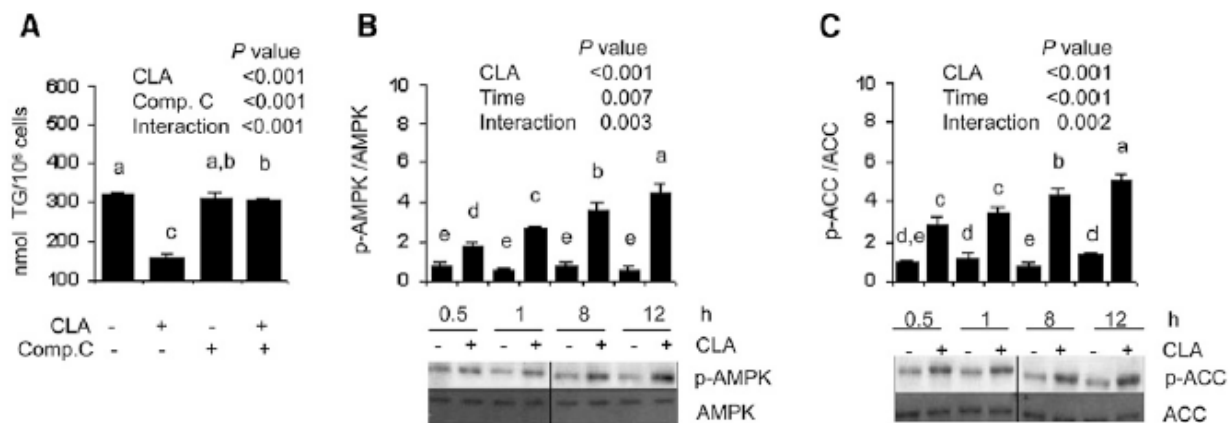
Figure 1. TG (A) and phosphorylated AMPK (B) and ACC (C) levels in 3T3-L1 adipocytes treated with *t*10*c*12 CLA, in combination with compound C (A), or alone (B, C). Differentiated 3T3-L1 adipocytes were incubated with 100 μmol/L LA or *t*10*c*12 CLA, with or without compound C (Comp.C) for 24 h or the indicated times. Representative western blots indicate the proteins detected with antibodies to p-AMPK (p-Thr 172), total AMPK, p-ACC (p-Ser 79), or total ACC. Each bar represents the mean + SEM, n=3 independent experiments. Means not sharing a common letter differ, $P \le 0.05$.
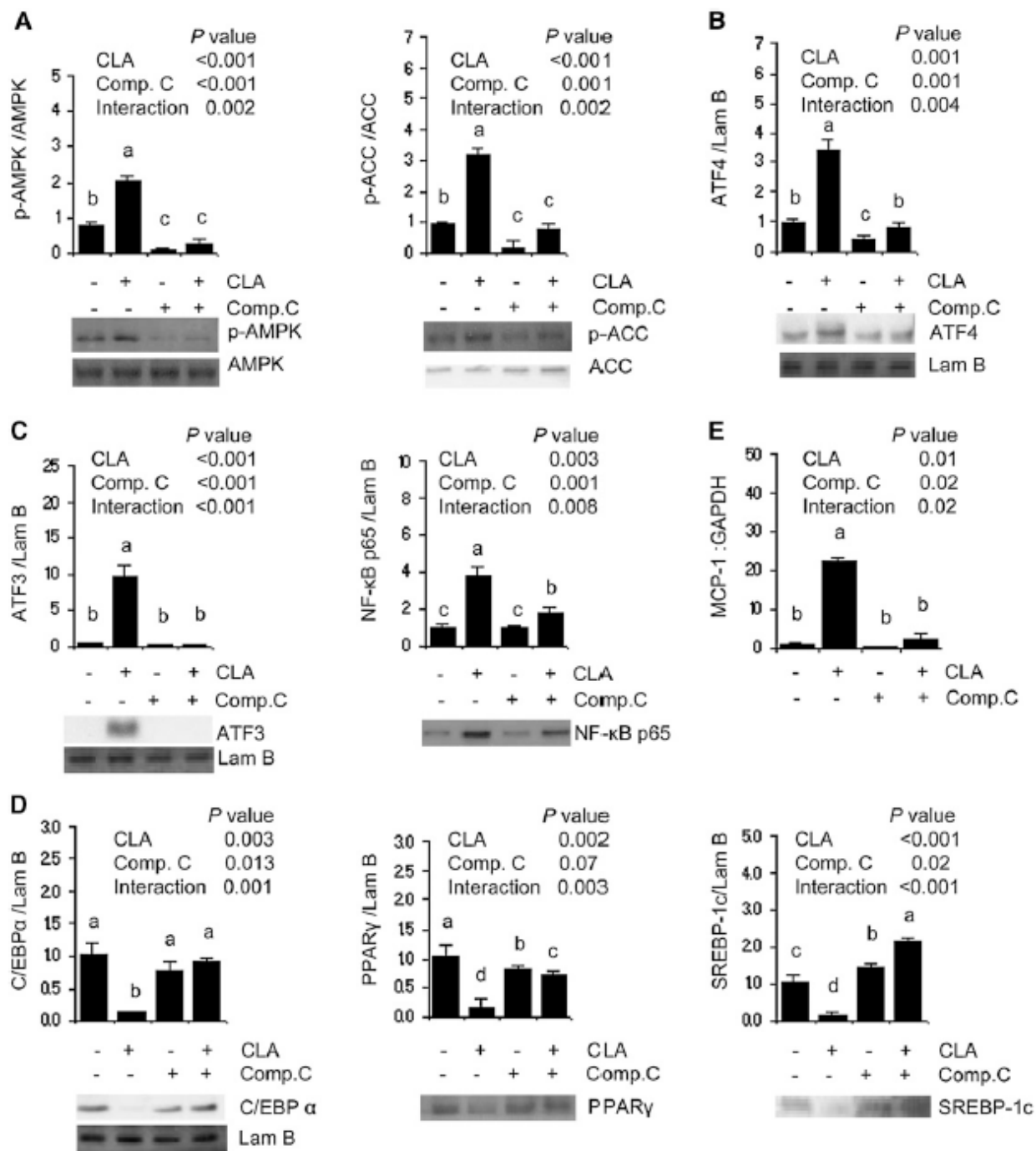
Figure 2. Compound C attenuates t10c12 CLA effects on phosphorylated AMPK and ACC (A); ATF4 (B); ATF3 and NF-κB (C); C/EBPα, SREBP1c, and PPARγ (D); or MCP-1 mRNA levels (E) in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with 100 μmol/L LA (-) or *t*10*c*12 CLA (+), with or without compound C (Comp.C) as indicated and the cytoplasmic or

nuclear fractions harvested at the following times for analysis of the indicated proteins: A) 1 h

cytoplasmic: p-AMPK, AMPK, p-ACC, ACC; B) 2 h nuclear: ATF4; C) 8 h nuclear: ATF3, NF-

κB p65; D) 12 h nuclear: C/EBPα, SREBP1c, PPARγ.   Representative western blots are shown

for the cytoplasmic or nuclear extracts analyzed with the antibodies to the proteins indicated.

Lamin B (Lam B) was used as a control for the amount of nuclear protein loaded for each blot in

B, C and D, and is only shown once in each panel. E) The indicated treatments were for 12 h.

MCP-1 levels and GAPDH were quantitated by real time PCR of cDNA. Each bar represents the

mean + SEM, n=3 independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.
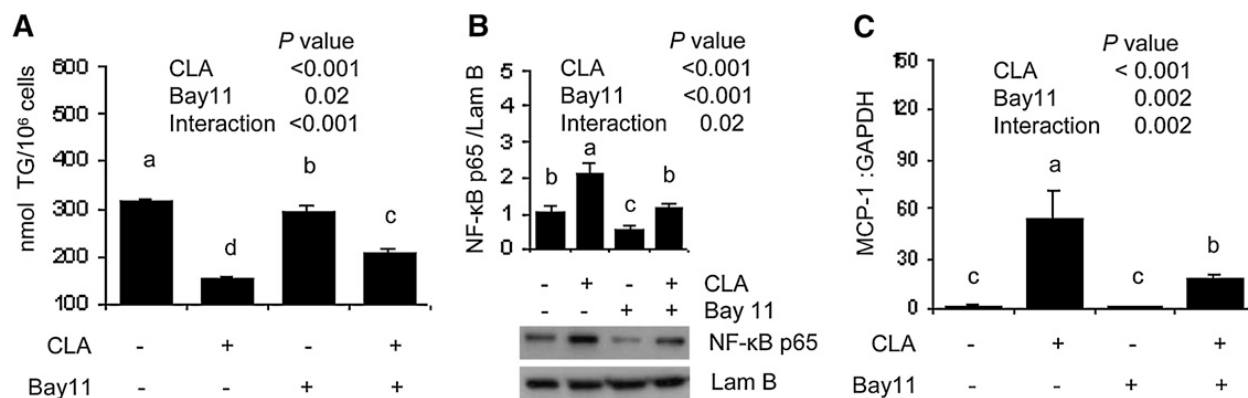
Figure 3. Bay 11-7082 inhibits *t*10*c*12 CLA's ability to reduce TG levels (A), translocate NF-kB to the nucleus (B), or induce MCP-1 mRNA levels in 3T3-L1 adipocytes (C). Differentiated 3T3-L1 adipocytes were treated with 100 μmol/L LA (-) or *t*10*c*12 CLA (+) for 24 (A) or 12 h (B, C), with or without 2 μmol/L Bay11-7082 (Bay11). Representative western blots are shown for nuclear extracts analyzed with antibodies specific for the p65 subunit of NF-kB or lamin B (Lam B). The mRNA levels of MCP-1 and GAPDH were quantitated by real time PCR of cDNA. Each bar represents the mean + SEM, n=3 independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.
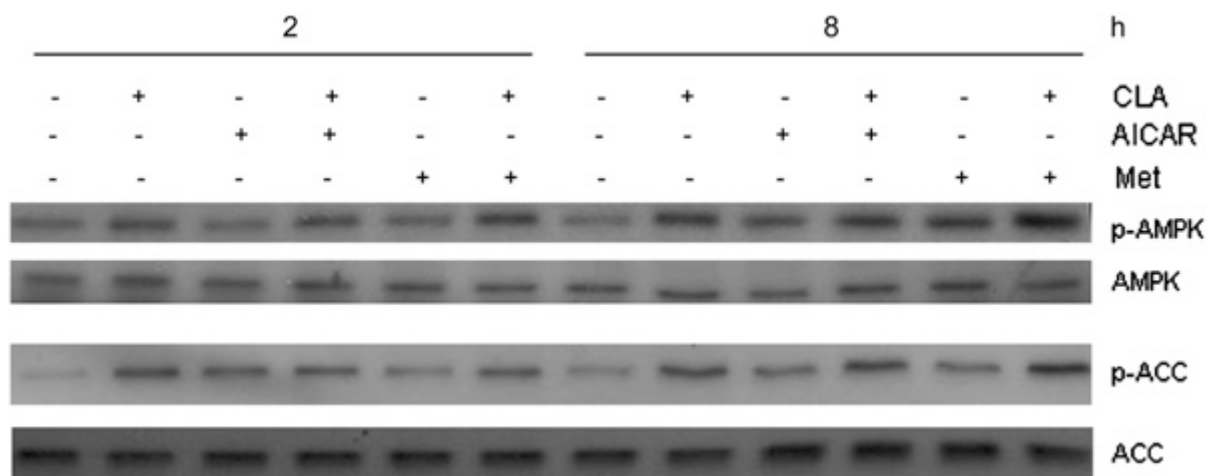
Figure 4. AMPK activators affect the ability of t10c12 CLA to increase AMPK and ACC phosphorylation in 3T3-L1 adipocytes. Differentiated adipocytes were treated with LA (-) or *t*10*c*12 CLA (+ or CLA), 2 mmol/L AICAR, or 2 mmol/L metformin (Met) alone, or in combination. Representative immunoblots are shown of proteins extracts from cells treated with 100 µmol/L of either fatty acid for 2 or 8 h, and analyzed with antibodies to p-AMPK (p-Thr 172), total AMPK, p-ACC (p-Ser 79), or total ACC. Immunoblots analyses were repeated three times for each antibody. The data from the quantitative analysis of the immunoblots is presented in Table 1.
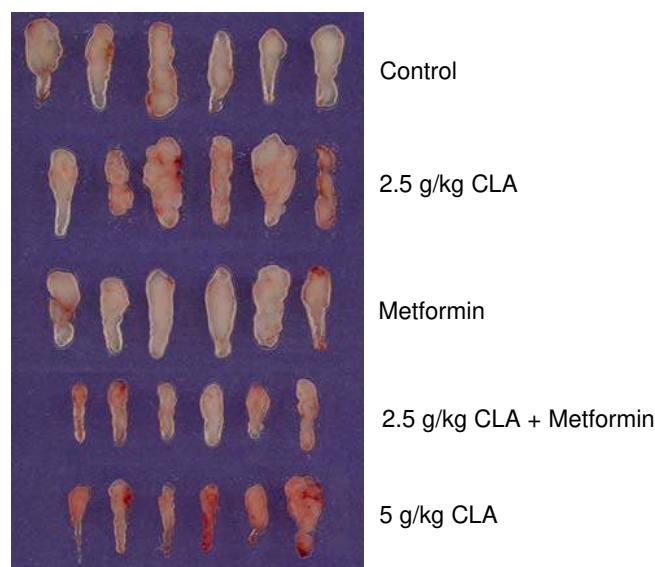
Figure 5. Metformin increases the lipid loss response to *t*10*c*12 CLA in retroperitoneal fat pads of ML mice. ML were fed diets supplemented with 2.5 g/kg soy oil (Control), or *t*10*c*12 CLA (CLA) at either 2.5 or 5 g/kg, and given drinking water lacking or containing metformin (20 mmol/L) for 14 d. One retroperitoneal fat pad is shown for each of the 6 mice for each of the treatments indicated.

Chapter III. Activated AMPK and prostaglandins are involved in the response to conjugated linoleic acid and are sufficient to cause lipid reductions in adipocytes

INTRODUCTION

Dietary conjugated linoleic acid (CLA) causes dramatic reductions in adiposity in mice and in human adipocyte cultures [16, 24, 60, 69]. An active isomer for reductions in white adipose tissue (WAT) is the *trans*-10, *cis*-12 isomer (*t*10*c*12 CLA; [24]). The molecular responses to *t*10*c*12 CLA are remarkably diverse and include the integrated stress response (ISR; [67, 110]) or the unfolded protein response (UPR; [76]), a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-mediated inflammatory response [60], mitogen-activated protein kinase (MAPK) cascades [57], and AMP-activated protein kinase (AMPK) [111, 112].

AMPK is a central regulator of cellular energy levels that is activated by increases in the cellular AMP/ATP ratio, cellular processes [80, 81], and *t*10*c*12 CLA in adipocytes [111] or mixed isomers of CLA in mice [112]. AMPK is a heterotrimeric protein and activated AMPK requires phosphorylation at AMPKα threonine 172 [80, 81]. Activated AMPK phosphorylates acetyl-CoA carboxylase (ACC) to inhibit fatty acid biosynthesis, and the amount of phosphorylated ACC provides a measurement of AMPK activity *in vivo* [113]. Drugs such as 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), metformin, and phenformin directly or indirectly activate AMPK [80, 93], while compound C is a potent inhibitor of AMPK. Either AICAR or metformin is sufficient to initiate an ISR in adipocytes [96, 111], supporting a role for activated AMPK in this response. An ISR can activate NF-κB [98], and compound C blocks the ISR, activation of NF-κB, and NF-κB-dependent inflammatory response [60] that are induced in *t*10*c*12 CLA treated adipocytes [111], indicating these responses are AMPK dependent. Whether

activation of AMPK is sufficient to explain the TG loss response caused by $t10c12$ CLA remains unresolved.

The inflammatory response has been proposed as a critical component of the triglyceride (TG) reduction that occurs in adipocytes exposed to $t10c12$ CLA [12, 24, 50, 60, 67, 72]. NF-κB is required for the inflammatory response, and inhibition of NF-κB attenuates IL6 induction, the repression of GLUT4 and Peroxisome Proliferator-Activated Receptor γ (PPARγ) protein activity, and the reduced glucose transport that otherwise occur in $t10c12$ CLA treated human adipocytes [60]. Inhibition of NF-κB also attenuates the reduction in TG levels in $t10c12$ CLA treated 3T3-L1 mouse adipocytes [111]. A key difference in the weak TG reduction that occurs in the presence of AMPK activator metformin, and the more robust lipid loss mediated by $t10c12$ CLA is the stronger inflammatory response that occurs with $t10c12$ CLA [67, 111]. The proinflammatory cytokine tumor necrosis factor α (TNF-α) is sufficient for lipid loss in adipocytes [114], and is moderately induced by $t10c12$ CLA in human adipocytes [60], but not in 3T3-L1 adipocytes [67]. PPARγ is a master regulator of adipogenesis and a key regulatory point for controlling inflammation in adipocytes [115]. PPARγ and NF-κB are mutually antagonistic, as PPARγ inhibits NF-κB transcriptional activity through transrepression [115], and NF-κB activity indirectly reduces PPARγ protein levels in the $t10c12$ CLA response [60].

$T10c12$ CLA increases prostaglandin (PG) biosynthesis in human adipocytes [110] and in mouse WAT [116]. PG biosynthesis is controlled in part by the rate-limiting release of arachidonic acid (AA) from the cell membrane by phospholipase A2 (PLA2). AA is then converted into PGH2, the precursor to the prostanoids, including the

prostaglandins, by either cyclo-oxygenase (COX)-1 or by the inflammation-inducible COX2 [117]. PG production is increased in $t10c12$ CLA-treated adipocytes through increased activation of PLA2 [110], as measured by its increased phosphorylation, and increased levels of COX2 mRNA [67, 110]. At least one member of the PG family, PGF2α, negatively affects adipocyte differentiation [118], but the functional role of increased PG biosynthesis in $t10c12$ CLA treated adipocytes has not been established.

The interplay between AMPK activation and the inflammatory response, particularly its PG component, in causing the reduction of TG levels that occurs in $t10c12$ CLA treated adipocytes, is not currently well defined. The goal of this study was to test the following hypotheses: 1) A moderate activation of AMPK and an inflammatory response are sufficient to reduce triglycerides, and 2) Strong activation of AMPK is also sufficient. Our experimental approach was to establish the possible involvement of specific pathways through chemical inhibitors, confirm that these pathways are activated by $t10c12$ CLA, and then attempt to activate these pathways independently of $t10c12$ CLA to functionally assay their ability to reduce TG levels in adipocytes. Our rationale was that independent activation of the critical pathways should produce responses similar to those caused by $t10c12$ CLA, and thereby provide additional support for the functional roles these pathways play. A potent activator of AMPK was analyzed for its similarity to $t10c12$ CLA in its ability to lower TG levels, in its whole genome transcriptional response, and in its cell-type dependent induction of the ISR. The results of these studies support our hypotheses and provide new insights into the mechanisms utilized by $t10c12$ CLA to reduce TG levels in adipocytes.

METHODS AND MATERIALS

Reagents. Arachidonic acid, bovine serum albumin (BSA, > 99% fat free), CP-24879, dexamethasone, Ibuprofen, insulin, isobutyl-1-methylxanthine, metformin, Naproxen, PGE2, phenformin and TNF-α were from Sigma (St. Louis, MO). AMPK inhibitor compound C was purchased from Calbiochem (San Diego, CA). Antibodies to p-AMPK (α1 and α2), AMPK (α1 and α2), p-ACC (ACC1 and ACC2), ACC (ACC1 and ACC2), p-PLA2, and PLA2 were from Cell Signaling (Beverly, MA). PGF2α, PGH2, SP600125, and U0126 were from Fisher (Pittsburgh, PA).

3T3-L1 cell culture and differentiation. 3T3-L1 fibroblasts [85] were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 25mM glucose and 10% bovine calf serum (Fisher, Pittsburgh, PA) and differentiated as described [86]. In brief, two days after growing to confluence, the cells were induced to differentiate in DMEM containing 25mM glucose and 10% fetal bovine serum (FBS; Fisher, Pittsburgh, PA), 0.17 μmol/L insulin, 0.5 mmol/L isobutyl-1-methylxanthine, and 1 μmol/L dexamethasone for 3 days. The medium was then changed to 10% FBS/DMEM containing 25mM glucose and with 0.17 μmol/L insulin for another 2 days, at which time the cells were usually differentiated to at least 90% adipocyte, followed by basal 10% FBS/DMEM medium containing 25mM glucose before initiating fatty acid treatments. All media contained 100,000 U/L penicillin and 172 μmol/L streptomycin (Invitrogen, Carlsbad, CA).

Fatty acid treatments. Fatty acids (>99%, Nu-check Prep, Elysian, MN), either linoleic acid or trans-10, cis-12 CLA, were dissolved in 0.1 M KOH, diluted into fatty acid free (>99%) bovine serum albumin (BSA) in phosphate buffered saline at a 1:1 ratio (2

mmol/L BSA: 2 mmol/L fatty acid), pH adjusted to 7.4, and added to the cultures containing 5.5 to 6 d post-differentiated 3T3-L1 adipocytes.

Western blots. Nuclear and cytosolic extracts were isolated based on the manufacturer's protocol (Active Motif, Carlsbad, CA). Equal amounts of proteins were separated by SDS-PAGE, transferred to Immun-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA) and probed with the indicated antibodies. Enhanced chemiluminescence (Pierce, Rockford, IL) was used for detection. Band intensities were determined from digital images from exposures in the linear range using imaging software (Quantity One, Biorad, Hercules, CA). All western blot analyses were repeated at least three times.

Quantification of TG content. Cell isolation and TG measurements were performed according to the manufacturer using TG reagent (T2449; Sigma, St. Louis, MO) and free glycerol reagent (F6428; Sigma, St. Louis, MO). Protein concentrations were measured using Bradford dye (Biorad, Hercules, California) to obtain results as μg of TG per mg of protein.

Measurement of PGF2α. Cell culture concentrations of PGF2α were determined using culture media samples obtained immediately prior to cell harvest for TG analysis. PGF2α levels were measured by enzyme immunoassay (EIA) using the Correlate-EIA PGF2α Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions. A standard curve was plotted using dilutions of a known concentration of the PGF2α standard, and sample concentrations were determined based on this standard curve. Data were derived from three independent experiments, with two replicates in each experiment.

Real time PCR analysis of cDNA from mRNA. Total RNA was extracted by TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA (2 μg) was used for cDNA synthesis. Real-time PCR was performed by a Bio-Rad iCycler using iQ SYBR Green Supermix reagent (Bio-Rad, Hercules, CA). The primer sequences for MCP-1 and GAPDH were taken from [87], and all other primer sequences of genes analyzed by real time PCR are shown in Supplementary Table 5. The cDNA levels were normalized to GAPDH, which showed no significant variation in microarrays between LA and $t10c12$ CLA treatments. Experiments were repeated three times, and each sample was analyzed using one RNA control and two replicates of each cDNA pool, and the relative amounts of each target cDNA and GAPDH were calculated using the comparative C_T method [according to the manufacturer's software (Bio-Rad, Hercules, CA) and [88]]. Cycle numbers were used to calculate gene expression levels in the linear amplification range, and qRT-PCR efficiency was close to 100%.

Microarray data analysis. RNA isolation and labeling of the 3T3-L1 adipocytes was performed as previously reported [67]. Results of the treatments of each of the three biological replicates were collected from Affymetrix® GeneChip Mouse Genome 430 2.0 arrays via the Affymetrix GeneChip Operating Software (GCOS; Affymetrix, Santa Clara, CA). The raw data for microarray results were normalized using the methods described by Wu et al [89]. To identify differentially expressed genes between the control (linoleic acid) and other treatments, the linear models for microarrays [Limma; [90]] package in R/Bioconductor was used, with Benjamini-Hochberg [119] adjusted P-values. Those genes with an absolute log2 ratio of 2 or more (four-fold up- or down-regulated) and a Benjamini-Hochberg adjusted P-value $\leq .05$ were deemed to be significant and analyzed

further. The gene expression data has been deposited at the NCBI Gene Expression Omnibus with series number GSEXXXXX (access activated upon publication).

Statistical Analysis.

Experiments represented in Figures 1 through 6 were subjected to analysis of variance using fixed effect statistical models. F-statistics, least-squares means, and standard errors were calculated using software of SAS (Cary, NC). The main effects or interactions were considered significant if $P \leq .05$, unless otherwise noted. For Fig. 1A, the main effects of TNF-α (0, 0.1, 0.3 or 0.6 nmol/L) and Met (- or +), and their interaction (TNF-α x Met) were analyzed. For Fig. 1B, the main effects of TNF-α (- or +) and compound C (- or +), and their interaction (TNF-α x Comp.C) were determined. The data presented in each panel of Fig. 2 were analyzed using a statistical model that included a single main effect. For Fig. 3, the main effects of CLA (- or +) and Ibuprofen (- or +), Naproxen (- or +) or CP-24879 (- or +), and their interaction (CLA x Ibuprofen, Naproxen or CP-24879) were separately determined for both the TG and PGF2α experiments. For Fig. 4, the main effects of Met (- or +) and PGE2 (- or +), PGF2α (- or +), PGH2 (- or +), or AA (- or +) and their interaction (Met x PGE2, PGF2α, PGH2 or AA) were separately determined. For Fig. 5A, the main effects of CLA (- or +) and time (2, 4, 8, and 12 h), and their interaction (CLA x time) were determined. For Fig. 5B and 5C, the main effects of CLA (- or +) and SP600125 (- or +) or U0126 (- or +), and their interaction (CLA x SP or U0126) were determined. For Fig. 6, the large range of the response values of ATF3 required both a log10 transformation and separating the data into a first group (panel A) and second group (panel B). The main effects of CLA (- or +), phenformin (- or +), and day (-2, 0, 1-5 d), and their interaction (CLA or Phen x d) were determined. Individual

pairwise comparisons of least-squares means were calculated by Least Significant Difference Test. Data are presented as least-squares means + SEM. Means not sharing a common superscript differ significantly ($P \leq .05$), unless otherwise noted.

RESULTS

Our hypothesis, based on prior use of chemical inhibitors, was that moderate activation of AMPK and an inflammatory response are sufficient to reduce TG levels in adipocytes. This was tested independently of $t10c12$ CLA as follows. Metformin was used to activate AMPK, as it was found previously to moderately activate AMPK without initiating a strong inflammatory response [111]. TNF-α, an inflammatory cytokine capable of causing reductions in TG levels in adipocytes [114], was used at several concentrations as the inflammatory stimulus. As a control, the biological activity of the TNF-α sample was separately verified in adipocytes by its induction of MCP-1 and CXCL1 mRNAs (Supplemental Fig.1), which were induced to levels similar to those caused by treatments with $t10c12$ CLA (data not shown). Either metformin or 0.6 nmol/L TNF-α caused reductions in TG levels of 14 ± 2% or 19 ± 4%, respectively (Fig. 1A). The reduction in TG levels was 40 ± 4% when both metformin and 0.6 nmol/L TNF-α were used in combination (Fig. 1A).

This result appeared to support our hypothesis, and to further strengthen this analysis, we checked whether the response to TNF-α was dependent on activated AMPK. Surprisingly, when the AMPK inhibitor compound C was tested for its ability to inhibit the TG loss caused by TNF-α alone, the majority of the TNF-α mediated reduction in TG was attenuated (Fig. 1B). This latter result indicated the above experiments had more relevance for our second hypothesis that higher levels of activated AMPK were sufficient

to cause robust TG loss. This hypothesis was separately tested by using phenformin, a more potent AMPK activator that is structurally related to metformin. Phenformin was used at increasing concentrations, which resulted in progressively lower TG levels (Fig. 2A). 100 µmol/L phenformin was able to reduce TG levels by $45 \pm 5\%$, nearly the same reduction as achieved by 100 µmol/L $t10c12$ CLA ($50 \pm 2\%$; Fig. 2A).

To gain further insight into the association of the amount of activated AMPK with reduced TG levels and inflammation, the ability of TNF-α, phenformin, metformin, or $t10c12$ CLA to activate AMPK, reduce TG levels, and induce COX2 mRNA was measured. Each of these chemicals increased phosphorylation of AMPK, as well as of ACC, a known *in vivo* substrate of AMPK (Fig. 2B). Phenformin and $t10c12$ CLA produced more phosphorylated AMPK and ACC than TNF-α or metformin at the concentrations used for each compound (Fig. 2B). The effects of these compounds on concurrent changes in TG levels and inflammation, using COX2 mRNA as an indicator of inflammation, was also measured (Fig. 2C). The effects of these treatments suggested the amounts of activated AMPK and COX2 mRNA both affected TG levels. $T10c12$ CLA or phenformin caused higher activation of AMPK and more TG loss than metformin or TNF-α. Within the $t10c12$ CLA and phenformin treatments, $t10c12$ CLA induced higher levels of COX2 mRNA and lost more TG despite its lower amount of activated AMPK. Within the metformin and TNF-α treatments, TNF-α induced higher levels of COX2 mRNA and lost more TG than metformin despite its lower amount of activated AMPK. Taken together, this data showed a trend of more highly activated AMPK causing more TG loss. For treatments with somewhat similar AMPK activation levels, higher COX2 mRNA levels seemed to be associated with more TG loss.

This latter observation supported a hypothesis that PGs might be a critical component of the inflammatory response to $t10c12$ CLA. The functional role of PGs was then investigated by using chemicals that inhibit PG production. Treatment of 3T3-L1 adipocytes with Naproxen, Ibuprofen, or CP-24879 (an inhibitor of AA biosynthesis) inhibited the ability of $t10c12$ CLA to cause TG reductions (Fig 3A). The effectiveness of these chemicals in reducing PG levels, using PGF2α levels as an indicator of PG production, was then determined (Fig 3B). The modest reductions of PGF2α levels by Naproxen or Ibuprofen treatments were not significant, while CP-24879 caused a significant reduction in PGF2α in $t10c12$ CLA treated adipocytes (Fig 3B). The effectiveness of these inhibitors at reducing PGF2α levels was in agreement with their effectiveness at attenuating the TG loss caused by $t10c12$ CLA (Fig 3A).

The functional importance of PG in the TG reductions in $t10c12$ CLA treated adipocytes generated an alternative to TNFα for providing an inflammatory stimulus to test our first hypothesis (a moderate activation of AMPK and an inflammatory response are sufficient to reduce TG levels in adipocytes) independently of $t10c12$ CLA. This hypothesis was tested by adding a combination of metformin and a PG or AA, a precursor to PG biosynthesis, to 3T3-L1 adipocytes. Metformin alone produced a 15 ± 4% reduction in TG levels (Fig. 4), while $t10c12$ CLA reduced TG levels by 47 ± 5% (data not shown). PGE2 alone had no effect, while the combination of PGE2 and metformin increased the amount of TG (Fig. 4). PGF2α alone reduced TG levels by 6 ± 2%, while PGF2α and metformin caused a 32 ± 7% TG loss (Fig. 4). PGH2 alone had no effect on TG levels, while the combination of PGH2 and metformin reduced TG levels by 41 ± 2% (Fig. 4). Similarly, AA alone had no effect, while AA and metformin reduced

TG levels by 45 ± 7% (Fig. 4). Metformin combined with PGH2 or AA was nearly as effective as $t10c12$ CLA in causing reductions of TG levels in these experiments, providing support for our first hypothesis, where the inflammatory component is supplied by exogenous PGH2 or AA.

The mechanism by which PG production is regulated in $t10c12$ CLA treated adipocytes was then investigated. PLA2 releases AA by hydrolysis from the glycerol backbone of membrane lipids. PLA2 activity is activated by phosphorylation, and its phosphorylation has been reported to be increased by $t10c12$ CLA in human adipocytes, with the possible involvement of ERK and JNK [110]. The involvement of the ERK and JNK signaling pathways in the activation of PLA2 by phosphorylation was therefore examined. Increasing amounts of phosphorylated PLA2 were observed during longer times of exposure to $t10c12$ CLA (Fig. 5A). The phosphorylation of PLA2 was found to be reduced in the presence of JNK inhibitor SP600125 or MEK inhibitor U0126 (Fig. 5B), both of which also inhibited $t10c12$ CLA's ability to reduce TG levels (Fig. 5C).

The abilities of $t10c12$ CLA and phenformin to strongly activate AMPK and to cause robust reductions in TG levels suggested phenformin might initiate cellular responses very similar to those caused by $t10c12$ CLA. Our hypothesis was that if similar signaling pathways were being utilized by the two chemicals, the microarray results should show a high degree of similarity when analyzed for their correlation coefficients. Microarray analysis generated a list of genes with changes in transcript levels of at least 4-fold in either $t10c12$ CLA- or phenformin-treated adipocytes (Supplementary Table 1). Previous lists of genes that responded to $t10c12$ CLA in the ISR, lipid metabolism, or inflammatory pathways [67] were also used to compare the changes in the two treatments

(Supplementary Tables 2-4). These lists were separately analyzed for the Spearman correlation coefficients and each comparison indicated a strong similarity between the transcriptional responses of 3T3-L1 adipocytes treated with phenformin or $t10c12$ CLA (Table 1). The changes in expression of selected functionally relevant genes were checked by real time PCR and were in good agreement with the microarray results (Table 2).

Our earlier report indicated that differentiated adipocytes responded more robustly than their non-differentiated fibroblast precursors to $t10c12$ CLA, as measured by ATF3 mRNA, an indicator of the ISR [67]. The ISR is an early component of the response to $t10c12$ CLA and can occur in both cell types [67], unlike the TG accumulation that is specific for maturing or mature adipocytes. This differential response in differentiated adipocytes provided an additional method to compare the similarities in the responses caused by phenformin or $t10c12$ CLA. Additionally, phenformin is a highly polar, water-soluble small molecule, while $t10c12$ CLA is a hydrophobic fatty acid, providing an additional test as to whether the hydrophobic or hydrophilic nature of the chemical affected the response of cells at different differentiated states. The competence of a developmental series consisting of 3T3-L1 fibroblasts, differentiating adipocytes, and mature adipocytes to respond to phenformin or $t10c12$ CLA was measured by the induction of ATF3 mRNA as an indicator of the ISR. The abilities of phenformin or $t10c12$ CLA to induce ATF3 were similar and increased dramatically at about 3 d after differentiation, with the highest levels induced at 4 and 5 d after differentiation when young and maturing adipocytes were present (Fig. 6). The developmental competence to

respond to both chemicals appeared to be the same, strengthening the premise that both chemicals activated the same developmentally-regulated pathways.

DISCUSSION

We examined the contributions the AMPK and inflammatory pathways, particularly PGs, make towards reducing TG levels in $t10c12$ CLA treated adipocytes. A key part of our approach was to activate these pathways independently of $t10c12$ CLA to assess their contributions away from the complex response initiated by $t10c12$ CLA [12, 24, 67]. However, the interpretation of these "chemical mimicry" studies also relies on the fact that these pathways are activated by, and their inhibition attenuates the response to, $t10c12$ CLA. These chemical mimicry studies benefited from metformin's ability to moderately activate AMPK without producing a strong inflammatory response in adipocytes [111], and phenformin's ability to strongly activate AMPK and an inflammatory response as demonstrated here. TNF-α, a pro-inflammatory cytokine, was initially used to increase the inflammatory response in adipocytes treated with metformin. TNF-α negatively regulates AMPK activity in muscle cells [120], and the extent to which TNF-α relied on AMPK activation to mediate TG reductions in adipocytes was previously unclear [114, 121]. Our data indicated TNF-α's major contribution to reducing TGs appeared to be through activated AMPK as TNF-α treated adipocytes had increased AMPK activity, and the majority of TNF-α's ability to reduce TG levels was inhibited by compound C. TNF-α's activation of AMPK confounded the interpretation of its contribution to the inflammatory response, but proved useful as one of several treatments we used to establish a correlation of AMPK activity levels with TG reductions.

In addition to the level of activated AMPK, the amount of TG reduction also appeared to be influenced by the level of induction of COX2 mRNA, which supported a role for PGs in facilitating TG reductions. PG levels increase in $t10c12$ CLA treated human adipocytes [110], mice [116], and 3T3-L1 adipocytes as demonstrated here. Much of the mechanism of the increased production of PGs can be explained by the activation of PLA2 [110] to increase AA production, the substrate for COX2, and the induction of COX2 mRNA levels [67, 110]. Inhibitors of MEK or JNK interfered with $t10c12$ CLA's ability to reduce TG levels, and reduced phosphorylation of PLA2, indicating the MEK and JNK pathways are involved in regulating PLA2. This is in agreement with earlier findings of the involvement of the MEK and JNK pathways in the $t10c12$ CLA response in human adipocytes [57, 110]. Other inhibitors that interfered with either AA or PG biosynthesis also inhibited the $t10c12$ CLA-mediated reduction in TGs in adipocytes. These results provided support for a functional role for PGs in lowering TG levels in $t10c12$ CLA treated adipocytes.

The functional role of the PGs was verified independently of $t10c12$ CLA by testing them in the presence of metformin. We found metformin combined with either PGH2 or AA was nearly as effective as $t10c12$ CLA at reducing TG levels. Importantly, PGH2 and AA did not significantly reduce TG levels when used alone, which suggested activated AMPK was necessary for them to enhance the TG reduction response. If we make the assumption that AMPK and PGs have similar biological activity in the $t10c12$ CLA-treated and alternatively-treated adipocytes, then these two components account for most of the TG reduction that occurs. These results support our hypothesis that a moderate activation of AMPK and an inflammatory response are sufficient to reduce

triglycerides, where most of the requirement for an inflammatory component can be provided by increased levels of PGs.

The similar effectiveness of PGH2, the precursor to the prostanoid family, and AA, the precursor to the entire eicosanoid pathway, suggests prostanoids play the major role in the response, without ruling out minor contributions from the leukotriene component of the eicosanoid pathway. The combination of PGF2α and metformin was not quite as effective. This could be due to the participation of other eicosanoids or prostanoids derived from AA or PGH2, respectively, or possibly a more rapid metabolic degradation of exogenous PGF2α [122]. PGE2 in combination with metformin resulted in increased TG levels. PGE2 can be a precursor to PGF2α, but this conversion might not be efficient with exogenously added PGE2. Consistent with these effects, PGF2α indirectly suppresses PPARγ activity [118], while PGE2 can also be a precursor to 15-keto-PGE$_2$, an agonist of PPARγ [123], which would be expected to oppose the effects of *t*10*c*12 CLA [109].

Phenformin was sufficient to cause a robust reduction in TG levels comparable to the reduction caused by *t*10*c*12 CLA, supporting our second hypothesis that strong activation of AMPK is sufficient for TG loss. This hypothesis is consistent with the first hypothesis that moderate AMPK activation and an inflammatory response are sufficient for robust TG loss, as stronger activation of AMPK is accompanied by a stronger inflammatory response as described here. We previously reported the transcriptional responses to metformin or *t*10*c*12 CLA were also very similar, but metformin caused a weaker inflammatory response than *t*10*c*12 CLA [111]. The inability of metformin to cause the robust reductions in TG levels that result from *t*10*c*12 CLA treatments, left

open the question of whether $t10c12$ CLA activated other critical pathways or whether its stronger activation of AMPK was sufficient to explain the response to $t10c12$ CLA. The results with phenformin, a more potent activator of AMPK, indicate highly activated AMPK is sufficient to cause a larger inflammatory response than metformin, although still less than that caused by $t10c12$ CLA, that in combination with its ability to strongly activate AMPK, can result in a robust TG reduction.

A detailed microarray analysis of the transcriptional response of the individual genes and pathways in 3T3-L1 cells and mice WAT treated with $t10c12$ CLA was done previously [12, 50, 67]. These analyses identified increases in uncoupling protein (UCP)-1 and carnitine palmitoyltransferase-1 (CPT1) transcripts, suggesting increased fatty acid oxidation could be occurring as has been observed in CLA-fed mice mice [52]. Fatty acid oxidation would be expected to be further enhanced by AMPK's phosphorylation of ACC, reducing the malonyl CoA pool, which typically inhibits CPT1 transport of fatty acids into mitochrondria [124]. Microarray analyses also identified a large increase in many inflammatory cytokines, and moderate reductions in key adipogenic transcription factors including PPARγ, CCAAT/Enhancer Binding Protein α (C/EBPα), and Sterol regulatory element binding protein (SREBP)-1c, suggesting reduced rates of lipid and cholesterol biosynthesis would result. Activated AMPK should further reduce biosynthesis by reducing the activity of key enzymes involved in the biosynthesis of fatty acids, cholesterol, glycogen, and proteins [124]. Our comparison of the transcriptome response of adipocytes treated with $t10c12$ CLA or phenformin found the changes in overall gene expression were very similar to those changes observed previously,

particularly induction of the ISR, reduction in mRNAs of genes encoding regulatory and enzymes in the lipid biosynthesis, and induction of inflammatory pathways.

Within this overall strong similarity of the transcriptional responses, the most apparent differences were $t10c12$ CLA's stronger induction of the immediate early genes and of some of the inflammatory response genes. The immediate early genes are a group of genes that are predominantly transcription factors and other signaling molecules that are induced by a large variety of cellular stresses [125]. Their higher induction in $t10c12$ CLA treated adipocytes indicates that $t10c12$ CLA either causes a more general cell stress than phenformin or alternatively, specifically activates this pathway by an unknown mechanism. In the inflammatory response, the genes induced to higher levels in response to $t10c12$ CLA included COX2, suppressor of cytokine signaling 3 (SOCS3), cardiotrophin-like cytokine factor 1 (CLCF1) in the IL6 cytokine family, tumor necrosis factor receptor superfamily member 12a (TNFRSF12a), and oncostatin M receptor (OSMR). The highly induced COX2 plays an important functional role in reducing TG levels through producing PGs as demonstrated here. SOCS3 mediates local insulin resistance through inhibition of insulin receptor substrate (IRS)-1 and IRS2 [126], and can be induced by cardiotrophin [127], and therefore, possibly by CLCF1. TNFRSF12a and OSMR are receptors for members of the TNFα or IL6 family of cytokines. Although the levels of TNF-α and IL6 mRNAs are low in 3T3-L1 adipocytes, other family members of these cytokines may signal through these receptors. Despite the overall strong similarity of the transcriptional responses to phenformin and $t10c12$ CLA, they were not identical, and this could be due to differences between phenformin's ability to

indirectly activate AMPK by inhibition of complex 1 of the mitochondrial respiratory chain [128, 129], and the still unknown pathways used by $t10c12$ CLA to activate AMPK.

The role of PGs *in vivo* may be less important as COX2 mRNA levels increased only 2 to 3 fold in 24 h in WAT of mice fed $t10c12$ CLA [12, 67]. COX2 mRNA levels then decreased to about control levels on subsequent days [12]. This is considerably less than the 8-fold or 12- to100-fold observed in human [110] and mouse adipocytes [67], respectively. PGE2 levels were previously found to be increased about 3.4 fold in WAT of mice fed $t10c12$ CLA for two weeks. In that study, inhibition of PG production with aspirin prevented this increase in PG levels without reducing the effect of $t10c12$ CLA on WAT [116]. This suggests the role of PG in WAT might be less important *in vivo* than in adipocyte cultures. The initial and subsequent inflammatory response to $t10c12$ CLA treatment is much more robust and diverse in WAT than in adipocytes in culture [67], in part due to the multiple types of cells in WAT, as well as the increased infiltration of macrophages [72]. The relative contributions of these diverse inflammatory pathways to reducing WAT *in vivo* are unclear and require additional investigation.

Despite the effectiveness of $t10c12$ CLA, phenformin, and even metformin to a lesser extent, in reducing TG levels in mouse adipocytes, and $t10c12$ CLA's effectiveness in mice [24] and primary human adipocytes [60], these chemicals have limited abilities to reduce adiposity in humans. The limited effectiveness of $t10c12$ CLA in humans remains unexplained [108], despite $t10c12$ CLA's preferential accumulation in adipose tissue [130]. Phenformin is no longer used to treat type 2 diabetes due to a higher frequency of lactic acidosis than occurs with metformin [131, 132], and it did not reduce weight in non-diabetic patients [133]. Metformin is important in the treatment of type 2 diabetes,

but it has a limited ability to either reduce weight or inhibit weight gain in patients without type 2 diabetes [134]. Our finding of a correlation of the amount of activated AMPK with the amount of TG reduction in 3T3-L1 adipocytes, supports a hypothesis that higher levels of activated AMPK are needed in adipocytes for effective reduction of adiposity in humans. It is unclear how to best accomplish this, although direct AMPK activators are promising [135], and our demonstration of the differential sensitivity of adipocytes to AMPK activators increases the potential for cell selectivity in this approach. We recently demonstrated metformin increases the ability of a moderate dose of $t10c12$ CLA to reduce WAT in mice [111], suggesting combinations of chemicals that activate AMPK are another possible approach. Adding a compound that leads to higher prostanoid levels in adipocytes might also be possible, but the complex biological responses to members of the prostanoid pathway [122, 136] indicate considerable research will be required to determine if this is possible in mice, and whether it can be accomplished in a therapeutically acceptable manner.

Tables and figures

Table 1. Spearman correlation coefficients for gene expression changes in treatments containing t10c12 CLA or phenformin

	Correlation coefficient
All significant genes ($n = 541$)	0.83
ISR genes ($n = 63$)	0.87
Lipid metabolism ($n = 69$)	0.95
Inflammation (n = 49)	0.85

Each treatment contained three replicates. The number of genes being compared is represented by *n*. For the 'all significant genes' the gene list is derived from the union of genes in either treatment that show a four fold change in expression and a Benjamini-Hochberg-adjusted *P*-value ≤.05 in at least one of the treatments being compared. The ISR, lipogenesis, and inflammation gene lists were derived from a list of genes previously found to be responsive to t10c12 CLA or tunicamycin in 3T3-L1 adipocytes. The Spearman correlation was used with associated *P*-values ≈ 0.

Table 2. Quantitative real time PCR verification of selected genes

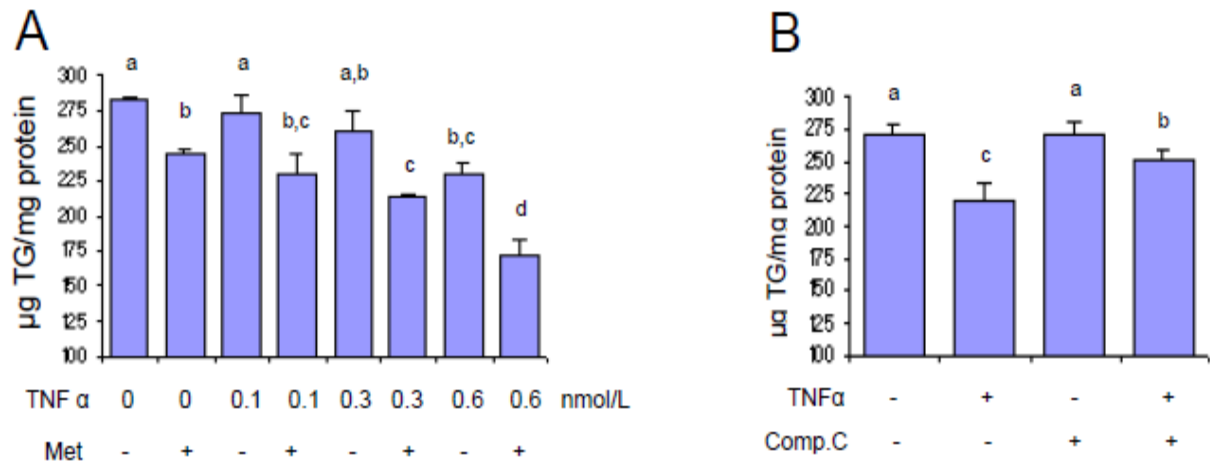| Gene symbol	CLA vs LA		Phenformin vs LA	
	RT-PCR fold change	Microarray fold change	RT-PCR fold change	Microarray fold change
ATF3	15.1±1.9	21.9 ± 3.1	4.8±0.6	9.6 ±1.8
MCP1	20.0±2.0	22.0±1.3	6.2±0.6	6.5± 0.3
CXCL1	3.5±0.5	6.0 ± 0.9	1.6±0.2	4.0 ± 0.7
CEBPα	0.9±0.2	0.9 ± 0.2	1.0±0.2	1.1 ± 0.3
PPARg	0.6±0.2	0.4 ± 0.1	0.9±0.2	0.7 ± 0.2
GLUT4	0.4±0.2	0.7 ± 0.2	1.6±0.6	2.1 ± 0.5
Adiponectin	1.1±0.8	1.1 ± 0.3	1.2±0.9	1.1 ± 0.3
IL6	7.0±0.0	5.2 ± 2.5	2.9±0.0	5.1 ± 2.0

Figure 1. TNFα increases the ability of metformin to reduce TG levels in adipocytes.

A) 3T3-L1 adipocytes were untreated or treated with either 2 mmol/L metformin (Met), the indicated amounts of TNF-α, or combinations of these compounds, and TG levels were measured after 24 h. The effects of metformin and TNF-α were statistically significant, but their interaction was not. B) 3T3-L1 adipocytes were either untreated or treated with 0.6 nmol/L TNF-α, 10 μmol/L compound C (Comp.C), or the combination, and TG levels were measured after 24 h. Their interaction was significant. Each bar represents the mean + SEM (n=3) of three independent experiments.
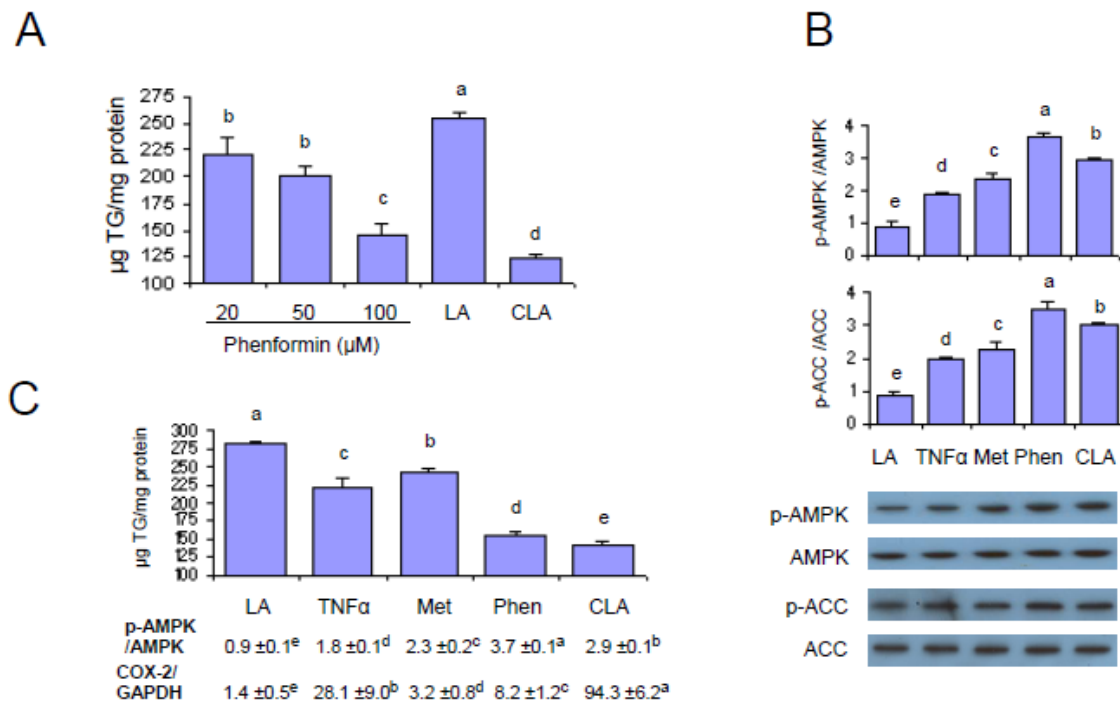
Figure 2. Phenformin causes robust delipidation and highly activates AMPK.

A) 3T3-L1 adipocytes were treated with the indicated concentrations of phenformin, and TG levels were measured after 24 h. For comparison, the TG levels for adipocytes treated with either 100 µmol/L LA or $t10c12$ CLA are also shown. B) 3T3-L1 adipocytes were treated with 100 µmol/L LA, 0.6 nmol/L TNF-α, 2 mmol/L metformin (Met), 100 µmol/L phenformin (Phen), or 100 µmol/L $t10c12$ CLA for 12 h. Cellular extracts were immunoblotted and the amounts of AMPK (α1 and α2), p-AMPK (α1 and α2), ACC (ACC1 and ACC2), and p-ACC (ACC1 and ACC2) were determined with specific antibodies. The immunoblots shown were representative of three independent experiments. The ratios of the phosphorylated form (p-AMPK or p-ACC) to the total amount of each respective protein (AMPK or ACC) were determined and are shown in the bar charts above the immunoblots. C) The same treatments were used to determine their effects on TG levels after 24 h. Both the p-AMPK/AMPK ratio from B and the fold

induction of Cox2 mRNA at 12 h, as measured by quantitative PCR, are shown below the graph. The mean + SEM (n=3) of three independent experiments is shown for each type of measurement.
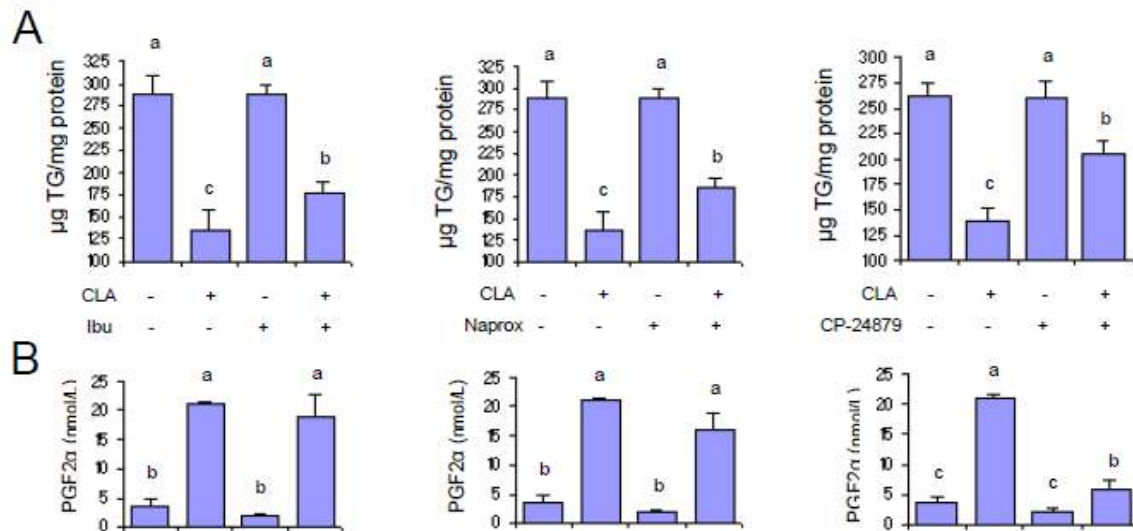
Figure 3. Inhibitors of prostaglandin biosynthesis inhibit the reduction in TG levels and increase in PGF2α levels caused by *t*10*c*12 CLA.

A) 3T3-L1 adipocytes were treated with 100 µmol/L LA (-) or 100 µmol/L *t*10*c*12 CLA (+), with or without either 50 µmol/L Ibuprofen (Ibu), 200 µmol/L Naproxen (Naprox), or 50 µmol/L CP-24879, and TG levels were measured after 24 h. The interactions of *t*10*c*12 CLA with Naproxen, or CP-24879 were significant, while the interaction with Ibuprofen approached significance ($P \leq .08$). B) Media from treatments of A were analyzed for PGF2α concentrations. The interaction of *t*10*c*12 CLA with Ibuprofen or Naproxen was not significant, but was significant for CP-24879. Each bar represents the mean + SEM (n=6) of three independent experiments.
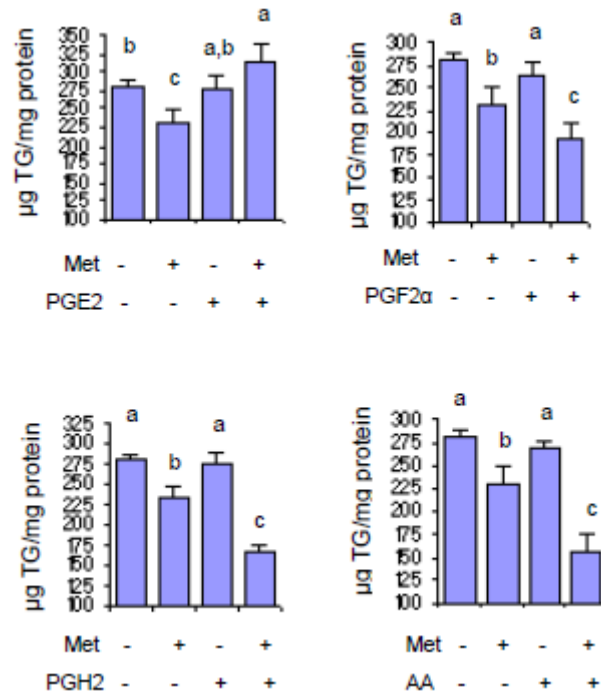
Figure 4. Prostaglandins affect metformin's ability to reduce TG levels. 3T3-L1 adipocytes were treated with or without 2 mmol/L metformin (Met), and with or without a prostaglandin (25 µmol/L PGE2, 25 µmol/L PGF2α, or 1 µmol/L PGH2) or arachidonic acid (150 µmol/L AA). TG levels were measured after 24 h. The interaction of metformin with PGE2, PGH2, or AA was significant, but was not significant for PGF2α. Each bar represents the mean + SEM (n=3) of three independent experiments.
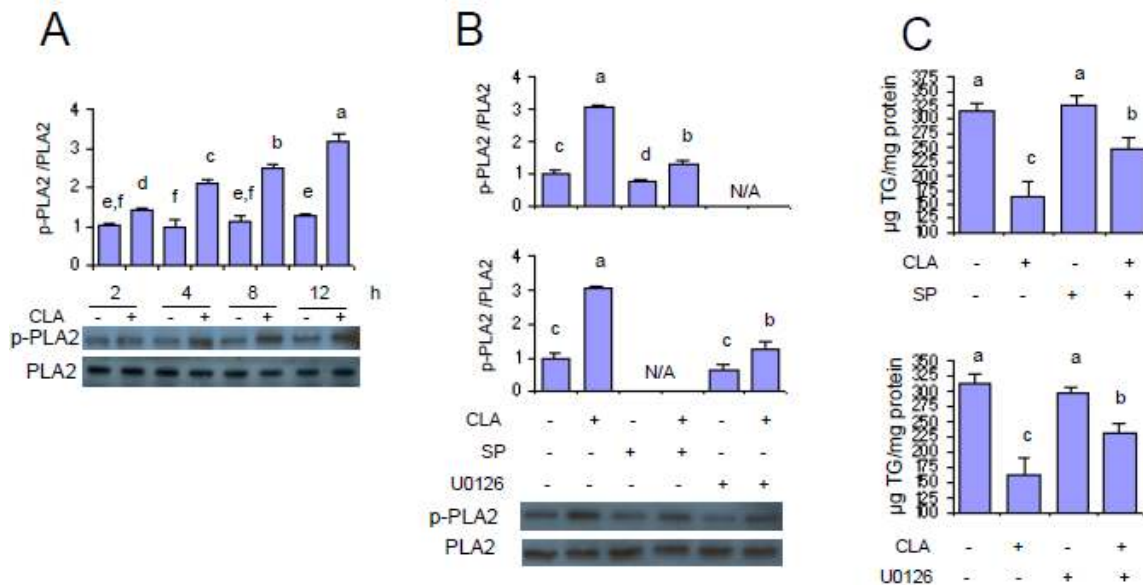
Figure 5. *T*10*c*12 CLA induces PLA2 phosphorylation through the JNK and ERK pathways.

A) PLA2 phosphorylation was measured at various times during exposure to *t*10*c*12 CLA in 3T3-L1 adipoctyes. The amount of phosphorylated PLA2 (p-PLA2) and total PLA2 was determined by immunoblots with specific antibodies. The ratio of p-PLA2 to the total PLA2 was determined and is shown in the bar chart above the immunoblots. The effects of *t*10*c*12 CLA and time were significant, but their interaction was not significant. B) 3T3-L1 adipocytes were either not treated or pretreated for 1 h with 20 μmol/L of JNK inhibitor SP-600125 (SP) or 20 μmol/L of MEK inhibitor U0126. Either 100 μmol/L LA or 100 μmol/L *t*10*c*12 CLA was then added for 12 h. Cellular extracts from these treatments were analyzed by immunoblot as in A. The ratio of p-PLA2 to the total PLA2 was determined and is shown in the bar charts above the immunoblots. Both SP-600125 and U0126 had a significant inhibitory interaction with *t*10*c*12 CLA. C) Adipocytes were treated as in B and TG levels were measured after 24 h. Both SP-600125 and U0126 had a significant inhibitory interaction with *t*10*c*12 CLA. The

immunoblots shown in A and B are representative of three independent experiments, and

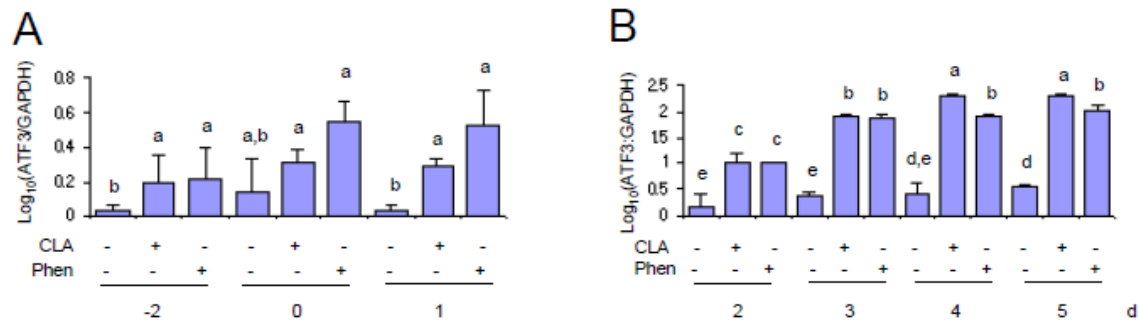each bar represents the mean + SEM (n=3) of three independent experiments.

Figure 6. The ability of phenformin or t10c12 CLA to induce the ISR is dependent on the stage of differentiation. 3T3-L1 cells at various developmental stages, either fibroblasts (two days before confluence; -2), differentiating adipocytes (from confluence (0) to five days post-confluence (days 1-5)), were treated with 100 μmol/L LA (-), 100 μmol/L phenformin (Phen), or 100 μmol/L $t10c12$ CLA (CLA). RNA was isolated from the cells after 12 h of treatment and analyzed for ATF3 and GAPDH mRNA levels by reverse transcription and quantitative PCR. The relative amounts of ATF3 mRNA are shown as bar graphs. Note the change in the scale of the log10 y-axis starting at day 2. The effects of the treatments and interactions were not significant for A. For B, the main effects of $t10c12$ CLA, phenformin, and day of differentiation and their interaction (CLA or Phen x d) were significant. Each bar represents the mean + SEM (n=4) of four independent experiments.

Chapter IV. Cross regulation of Sirtuin 1, AMPK, and PPARγ in conjugated linoleic acid treated adipocytes

Introduction

Conjugated linoleic acid (CLA) reduces adiposity in human and mouse adipocytes [16, 24, 60, 69], and the *trans*-10, *cis*-12 CLA (*t*10*c*12 CLA) isomer is capable of causing this response [137]. The rates of fatty acid oxidation and lipolysis increased in *t*10*c*12 CLA-treated 3T3-L1 adipocytes [70], while *t*10*c*12 CLA reduced fatty acid oxidation [138], but increased lipolysis in human adipocytes [57]. Molecular responses to *t*10*c*12 CLA include AMP-activated protein kinase (AMPK) [111], integrated stress response (ISR; [67, 110]) or unfolded protein response (UPR; [76]), mitogen-activated protein kinase (MAPK) cascades [57], a nuclear factor kappa-B (NF-κB)-mediated inflammatory response [12, 24, 50, 60, 67, 72] that includes increased prostaglandin biosynthesis in human adipocytes [110] and in mouse white adipose tissue [116], and reduced amounts of peroxisome proliferator-activated receptor γ (PPARγ) protein [60]. Despite this progress, the understanding of the pathways involved in the initial perception of *t*10*c*12 CLA, and the complex regulation of the subsequent responses, is incomplete.

AMPK is a central regulator of cellular energy levels that is activated by increases in the cellular AMP/ATP ratio, various cellular stresses [80, 81], or treatment of adipocytes [111] or mice [139] with *t*10*c*12 or mixed isomers of CLA. AMPK activation requires phosphorylation at Thr172 [135], and two of the proteins inhibited by phosphorylation by activated AMPK are acetyl CoA carboxylase (ACC), and fatty acid synthase, two key enzymes in fatty acid biosynthesis. Through this and other mechanisms [80, 81], activated AMPK decreases lipogenesis, increases fatty acid oxidation, and increases lipolysis in adipocytes *in vitro* and *in vivo* after an initial delay [140]. Phenformin and metformin are structurally related chemicals that can be used to activate

AMPK [80, 141]. Metformin increases the TG loss, while compound C, a potent inhibitor of AMPK, strongly attenuates the TG loss typically caused by $t10c12$ CLA in adipocytes [111].

PPARγ is a ligand-activated nuclear receptor that regulates lipogenesis and is a key regulatory point for controlling inflammation in adipocytes [115]. PPARγ forms a complex with nuclear receptor corepressors 1 or 2 (NCoR1 or 2) in the absence of its bound ligand. PPARγ transactivation activity is also reduced by phosphorylation at Ser112 of PPARγ2 by extracellular signal-regulated kinase (ERK), c-Jun N-terminal Kinase (JNK), or p38 MAPKs, and a phosphorylation-dependent sumoylation at K107 [142]. In addition, a non-genomic role for PPARγ is emerging, as a number of PPARγ-dependent processes are too rapid to involve transcriptional responses [143, 144]. The critical role of PPARγ in the response to $t10c12$ CLA is demonstrated by the attenuated responses that occur on addition of PPARγ agonists [109, 110].

SIRT1 is a NAD$^+$-dependent histone/protein deacetylase that is involved in regulating cell energy metabolism, cell stress, and cell fate [145]. SIRT1 directly binds to NCoR1 and directly or indirectly to PPARγ to repress PPARγ transactivation activity, inhibit adipogenesis, and increase fat loss in adipocytes [146]. SIRT1 deacetylates liver kinase B1 (LKB1), facilitating the ability of LKB1 to phosphorylate AMPK, defining a SIRT1/LKB1/AMPK signaling pathway that provides one of the connections between SIRT1 and AMPK for regulating energy metabolism [147, 148]. SIRT1 also has an anti-inflammatory effect due to its removal of acetyl groups from NF-κB, thereby inhibiting NF-κB transactivation activity [149]. SIRT1 activity can be influenced by exogenous chemicals. Sirtinol and nicotinamide inhibit SIRT1 and 2, and possibly other sirtuin

proteins [150]. Etomoxir inhibits fatty acid transport into mitochondria which prevents a rise in NAD$^+$ levels that would facilitate SIRT1 activity [151], while SRT1720 preferentially activates SIRT1 [152].

In this study, we analyzed the functional role of SIRT1 in the TG reduction that occurs in *t*10*c*12 CLA-treated 3T3-L1 adipocytes. Our objectives were to test whether SIRT1 was functionally required for robust triglyceride reduction, and whether SIRT1, AMPK, and PPARγ cross regulated each other in the response. These experiments were performed by using activators, inhibitors, or siRNA that affected these pathways and analyzing their effects on TG levels, fatty acid metabolism, and post-translational modifications or activity levels of SIRT1, AMPK, and PPARγ proteins.

Experimental Procedures

Reagents

Compound C was purchased from Calbiochem (San Diego, CA). Bovine serum albumin (BSA, > 99% fat free), dexamethasone, etomoxir, GW9662, insulin, isobutyl-1-methylxanthine, metformin, nicotinamide, phenformin, and sirtinol were purchased from Sigma (St. Louis, MO). Ciglitazone, pioglitazone, rosiglitazone, SP600125, troglitazone, and U0126 were from Fisher (Pittsburgh, PA). SRT1720 was from Cayman Chemical (Ann Arbor, MI). T10c12 CLA (90%, #UC-61-A) was from Nu-Chek Prep, Inc (Elysian, MN). Antibodies to acetyl-NF-κB p65 (acetyl K310), p-PPARγ (phospho S112) and negative control siRNA were from Abcam (Austin, TX). Protein A agarose beads, antibodies to β-actin, NCOR1, NF-κB p65, PPARγ, SIRT1, or anti-goat or anti-rabbit secondary antibodies coupled to horseradish peroxidase, and Sirt1 siRNA were purchased

from Santa Cruz Biotechnology (Santa Cruz, CA), while antibodies to p-AMPK, AMPK, p-ACC, and ACC were from Cell Signaling (Beverly, MA).

3T3-L1 cell culture, differentiation, and chemicals

3T3-L1 fibroblasts [85] were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% bovine calf serum (Fisher, Pittsburgh, PA) and differentiated as described [111]. When present, chemicals were dissolved in DMSO, with the exception that 2 mmol/L metformin and 0.1 mmol/L phenformin were dissolved in water, and were added directly to the media at $\leq 0.2\%$ of the final volume in the media at the following concentrations: 5 µmol/L ciglitazone, 10 µmol/L compound C, 10 µmol/L etomoxir, 10 µmol/L GW9662, 10 µmol/L nicotinamide, 5 µmol/L pioglitazone, 5 µmol/L rosiglitazone, 10 µmol/L sirtinol, 10 µmol/L SP600125, 8 µmol/L SRT1720, 5 µmol/L troglitazone, or 10 µmol/L U0126, and were added 1 h before adding fatty acids. Fatty acids, either linoleic acid or trans-10, cis-12 CLA, were dissolved in 0.1 M KOH, diluted into fatty acid free (>99%) bovine serum albumin (BSA) in phosphate buffered saline at a 1:1 ratio (2 mmol/L BSA: 2 mmol/L fatty acid), pH adjusted to 7.4, and added to the cultures containing 5.5 to 6 d post-differentiated 3T3-L1 adipocytes [111].

Fatty acid biosynthesis, oxidation, and lipolysis assays

Fatty acid biosynthesis was measured in differentiated adipocytes after 24 h by removing the treatment media and incubating the adipocytes in Hanks' Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA) containing 37 KBq [^{14}C]-acetate [specific activity 2.1 GBg/mmol, (PerkinElmer Radioisotopes, Waltham, MA)] for 30 min (incorporation was linear for 60 min). Cells were washed in PBS three times, pelleted,

and then resuspended in 100 µl PBS and 0.1% SDS. Lipids were extracted in 1 ml of 2:1 chloroform:methanol [153] and measured by scintillation counting. Cells briefly exposed to 37 KBq [^{14}C]-acetate, followed by immediate washing and extracted as above, were used to determine background levels, which were subtracted from sample values. Fatty acid oxidation was measured in differentiated adipocytes in 3.5 cm culture plates 12 h after starting treatments by adding 37 KBq [^{14}C]-oleic acid [specific activity 1.7-2.2 GBg/mmol, (PerkinElmer Radioisotopes, Waltham, MA)] to the treatment media for 2 h and collecting [^{14}C]-CO_2 for 1 hr in collection jars as reported [138]. For lipolysis assays, the TG pool of differentiated adipocytes was labeled by adding 37 KBq [^{14}C]-acetate [specific activity 1.5-2.2 GBg/mmol, (PerkinElmer Radioisotopes, Waltham, MA)] to the media for 4 h, after which time the plates were washed four times with PBS, and specific experimental media treatments were initiated. Media (0.1 ml) was collected after 24 h, lipids extracted in 1 ml of 2:1 chloroform:methanol [153] and measured by scintillation counting. The use of labeled [^{14}C]-acetate and the 2:1 chloroform:methanol extraction step considerably reduced non-specific background to 50 DPM, as determined by using the above protocol on cells that had been briefly exposed to 37 KBq [^{14}C]-acetate in media.

siRNA transfection

3T3-L1 adipocytes, 5 d post differentiation, were transfected by siQUEST transfection reagent (Mirus, Madison, WI), using final concentrations of 2 µl of siQUEST reagent per ml of media and siRNA at 40 nmol/L, 24 hours before adding fatty acids .

Immunoblot analysis

Nuclear and cytosolic extracts were isolated using a nuclear extract kit (Active Motif, Carlsbad, CA). Equal amounts of proteins were separated by SDS-PAGE, transferred to Immun-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA), probed with the indicated primary antibodies, and detected with secondary antibodies. Enhanced chemiluminescence (Pierce, Rockford, IL) was used for detection. Band intensities were determined from digital images from exposures in the linear range using software (Quantity One, Biorad, Hercules, CA). All western blot analyses were repeated at least three times.

Immunoprecipitation

Immunoprecipitations were performed according to the procedure described [146]. In brief, the collected 3T3-L1 adipocytes were sonicated, lysates were centrifuged, and aliquots of the supernatants were immunoprecipitated overnight with specific antibody or control nonspecific IgG serum. Protein A agarose beads were used to bind the specific or non-specific antibody complexes, the protein A beads containing bound proteins were washed five times, and the bound proteins were eluted in SDS sample buffer for immunoblot analysis.

Quantification of TG content

Cell isolation and TG measurements were performed according to the manufacturer' instructions using TG reagent (T2449; Sigma, St. Louis, MO) and free glycerol reagent (F6428; Sigma, St. Louis, MO). TG data are expressed as nmol of TG per mg of protein.

Measurement of MCP-1 and COX2 mRNA

Total RNA was extracted by TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA (2 μg) was used for cDNA synthesis. Real-time PCR was performed by a Bio-Rad iCycler using iQ SYBR Green Supermix reagent (Bio-Rad, Hercules, CA) using PCR primers for MCP-1 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [111], and COX2 [111]. MCP-1 and COX2 mRNA levels were normalized to GAPDH, which showed no significant variation in microarray analyses between linoleic acid and t10c12 CLA treatments. Experiments were repeated three times, and each sample was analyzed using one RNA control and two replicates of each cDNA pool, and the relative amounts of MCP-1, COX2 and GAPDH were calculated using the comparative C_T method [88], according to the manufacturer's software (Bio-Rad, Hercules, CA). Cycle numbers were used to calculate gene expression levels in the log linear amplification range.

Statistical Analysis

One or two-way ANOVA was used to analyze the data. Pairwise comparisons were calculated using Tukey's test and were considered significant for $p \leq 0.05$. All analyses were performed using SAS software (SAS, Cary, NC).

Results

We first determined whether increased SIRT1 activity would lower TG levels in t10c12 CLA treated adipocytes. To specifically activate SIRT1, we used SRT1720, as this chemical is known to preferentially activate SIRT1 at 8 μM [152]. SRT1720, in combination with 50 μM t10c12 CLA, significantly lowered TG to 55% of the linoleic acid (LA) control levels, while 50 μM t10c12 CLA alone only lowered TG to 70% (Fig. 1A; In our experiments, we used 50 μM t10c12 CLA if assaying chemicals that increased

TG loss, but otherwise used 100 μM $t10c12$ CLA in order to obtain large changes in TG levels or in the molecular responses of interest). In contrast, addition of 100 μM $t10c12$ CLA in combination with a SIRT inhibitor, either sirtinol or nicotinamide, significantly increased TG levels to 67% and 68% of the LA control, respectively, relative to the 50% TG level caused by 100 μM $t10c12$ CLA treatment (Fig.1. B). Etomoxir, which inhibits SIRT activity indirectly by reducing NAD^+ levels via inhibition of mitochondrial fatty acid transport and oxidation [151], was used in combination with $t10c12$ CLA, and significantly increased TG levels to 72% of the LA control, relative to the 44% TG level in the $t10c12$ CLA treatment (Fig. 1C). These data indicated SIRT1 activity might be participating in the TG loss response caused by $t10c12$ CLA treatment.

SiRNA was used to reduce SIRT1 expression to confirm the functional involvement of SIRT1 in the response to $t10c12$ CLA. As a baseline for this comparison, we determined that treatment with $t10c12$ CLA did not change SIRT1 or β-actin protein levels (Fig. 1D). SiRNA treatment reduced SIRT1 protein levels by 53% but not affect β-actin levels, while a control siRNA did not change SIRT1 or β-actin amounts (Fig. 1E). In the presence of LA, the siRNA SIRT1 treatment had TG levels that were not significantly different than those in the control siRNA treatment (Fig. 1F). In contrast, in the presence of $t10c12$ CLA, the siRNA SIRT1 treatment had TG levels that were 72% of the LA control, while the control siRNA treatment had TG levels of 47% of the LA control (Fig. 1F). Taken together with the above inhibitor studies, these results suggested that inhibition of SIRT1 significantly interfered with the TG loss caused by $t10c12$ CLA.

The involvement of SIRT1 in $t10c12$ CLA-mediated changes in the rates of fatty acid biosynthesis, oxidation, and lipolysis as well as in the induction of two key

inflammatory mRNAs was then measured to gain insights into how SIRT1 affected these specific pathways. Adipocytes treated with $t10c12$ CLA had a 77% reduction in their rate of lipogenesis (Fig. 2A). The combination of $t10c12$ CLA and sirtinol significantly changed this to a 47% reduction in the rate of lipogenesis (Fig. 2A). This result indicated SIRT1 activity was involved in inhibiting the rate of fatty acid biosynthesis. Adipocytes treated with $t10c12$ CLA exported 61% more radioactively-labeled lipids than LA treated cells (Fig. 2B), but this $t10c12$ CLA-mediated lipolysis was not significantly affected by sirtinol (Fig. 2B). The rate of fatty acid oxidation was increased by 272% in $t10c12$ CLA-treated adipocytes, but sirtinol did not significantly affect $t10c12$ CLA's ability to increase this rate (Fig. 2C). The inflammatory MCP1 and COX2 mRNAs were highly induced by $t10c12$ CLA treatment (Fig. 2D-E). The SIRT1 activator SRT1720, in combination with $t10c12$ CLA, modestly attenuated the induction of MCP1 to 79% of $t10c12$ CLA levels, but did not significantly affect COX2 mRNA levels (Fig. 2D-E). In contrast, the SIRT1 inhibitors, sirtinol or nicotinamide, when used in combination with $t10c12$ CLA, attenuated the induction of the mRNA of MCP1, to 65% or 58% of the $t10c12$ CLA control levels, respectively (Fig. 2D). Similarly, sirtinol or nicotinamide, when used in combination with $t10c12$ CLA, attenuated the induction of the mRNA of COX2, to 54% or 65% of the $t10c12$ CLA control levels, respectively (Fig. 2E). Taken together, these results indicated SIRT1 was involved with the inhibition of the rate of fatty acid biosynthesis and the increased induction of the inflammatory MCP1 and COX2 mRNAs, but did not significantly affect lipolysis or fatty acid oxidation.

We next determined whether SIRT1 affected AMPK regulation during the response to $t10c12$ CLA. AMPK activity was measured by the amount of phosphorylation at

AMPK Thr172 (p-AMPK) and by the amount of phosphorylated ACC (p-ACC), one of AMPK's key substrates *in vivo*. SIRT1 activator SRT1720 or SIRT inhibitors, with or without *t*10*c*12 CLA, had no significant effect on the amount of p-AMPK or p-ACC produced after 2 h of treatment (Fig. 3A). However, when used in combination with *t*10*c*12 CLA for 12 h, SRT1720 significantly increased p-AMPK or p-ACC levels by 37% or 43%, respectively, relative to the amounts present when treated by *t*10*c*12 CLA (Fig. 3B). Conversely, when used in combination with *t*10*c*12 CLA for 12 h, sirtinol or nicotinamide significantly attenuated p-AMPK levels to 66% or 83%, respectively, and p-ACC levels to 76% or 75%, respectively, relative to the amounts of these proteins in adipocytes treated with *t*10*c*12 CLA (Fig. 3B). Similarly, when used in combination with *t*10*c*12 CLA, siRNA targeting of SIRT1 significantly reduced p-AMPK to 54% and p-ACC to 53% of their respective levels in the control treatment (Fig. 3C). Collectively, these results indicated SIRT1 increased AMPK activity levels at 12 h, but not within the first 2 h, after exposure to *t*10*c*12 CLA.

The involvement of PPARγ in the response to *t*10*c*12 CLA was then tested through addition of a PPARγ antagonist or agonist. The PPARγ antagonist GW9662 significantly reduced TG levels to 41%, relative to the LA control level, when used in combination with 50 μM *t*10*c*12 CLA, while 50 μM *t*10*c*12 CLA alone reduced TG levels to 70% (Fig. 4A). Prior to examining the effects of a PPARγ agonist on the response to *t*10*c*12 CLA, we first determined the most potent PPARγ agonist amongst a set of four thiazolidinedione agonists, as measured by their ability to increase the amount of TG produced in differentiating adipocytes. Troglitazone was the most potent of the four agonists tested (Fig. 4B). Troglitazone significantly attenuated *t*10*c*12 CLA 's ability to

reduce TG levels, as the combined treatment was not significantly different from the LA control (Fig. 4C). These results indicated that in adipocytes treated with $t10c12$ CLA, a PPARγ antagonist facilitates TG loss, while a PPARγ agonist interferes with TG loss.

The cross regulation between PPARγ and AMPK was next determined. When used in combination with $t10c12$ CLA for 12 h, PPARγ antagonist GW9662 increased AMPK and ACC phosphorylation levels by 33% and 34%, respectively (Fig. 4D). Conversely, when used in combination with $t10c12$ CLA for 12 h, troglitazone reduced AMPK and ACC phosphorylation levels by 45% and 39%, respectively (Fig. 4D). However, when used in combination with $t10c12$ CLA for 2 h there was no significant effect of these chemicals on AMPK or ACC phosphorylation levels (Fig. 4D). Therefore, although AMPK activation occurred at 2 h, cross regulation by PPARγ was not apparent at 2 h. Phosphorylation of PPARγ increased by 140% at 12 h, but not by 2 h, after $t10c12$ CLA treatment (Fig. 4E-F). When compound C was used in combination with $t10c12$ CLA, the amount of phosphorylated PPARγ was reduced by 41%, relative to the levels when treated by $t10c12$ CLA. This suggests AMPK activity was directly or indirectly involved in the phosphorylation of PPARγ (Fig. 4F). These results indicated AMPK and PPARγ cross regulated each other in the response to $t10c12$ CLA.

We next determined whether this cross regulation between AMPK and PPARγ occurred in the absence of $t10c12$ CLA. This was done by using two other chemicals to activate AMPK, alone or in combination with a PPARγ agonist or antagonist. Phenformin, a strong AMPK activator, reduced TG levels to 60% of those present in the untreated control adipocytes (Fig. 5A). When phenformin was used in combination with troglitazone, the amount of TG significantly increased to 86% of the amount in the

untreated control (Fig. 5A). Metformin, a weaker AMPK activator, only reduced TG levels to 87% of the amount present in the untreated control (Fig. 5B). The TG level was significantly reduced to 66% of the amount present in the untreated control when metformin was used in combination with the PPARγ antagonist GW9662 (Fig. 5B). These results demonstrated that the antagonistic cross regulation between AMPK and PPARγ that was observed in the response to $t10c12$ CLA also occurred in phenformin or metformin treated adipocytes in the absence of $t10c12$ CLA.

The regulation of SIRT1 activity by AMPK and PPARγ was then assessed. The deacetylation of the p65 subunit of NF-κB was used as a measure of SIRT1 activity in adipocytes [149]. The acetylation level of the p65 subunit of NF-κB was not significantly changed at 2 h (Fig. 6A), but was significantly reduced to 38% of control levels in adipocytes treated with $t10c12$ CLA for 12 h, suggesting SIRT1 deacetylation activity was increased. When $t10c12$ CLA was used in combination with sirtinol or nicotinamide, the amount of the acetylated p65 subunit of NF-κB increased to 84% or 60% of LA control levels, respectively (Fig. 6 B). These results supported the premise that SIRT1 activity was involved in the deacetylation of the p65 subunit of NF-κB. The amount of the acetylated p65 subunit of NF-κB increased in a treatment using $t10c12$ CLA in combination with compound C to 80% of the LA control, relative to the 48% acetylation level produced by $t10c12$ CLA (Fig. 6C). This result indicated AMPK was directly or indirectly involved in regulating SIRT1 activity in the response to $t10c12$ CLA. Troglitazone was used to determine whether PPARγ affected SIRT1 activity levels. When used in combination with $t10c12$ CLA for 12 h, troglitazone-treated adipocytes significantly increased the amount of acetylated p65 subunit of NF-κB to 85% of LA

control levels (Fig. 6D). Conversely, adipocytes treated with $t10c12$ CLA and GW9662 significantly reduced the amounts of acetylated p65 subunit of NF-κB to 40% of LA control levels. Collectively, these results indicated that AMPK and PPARγ positively and negatively regulated SIRT1 activity, respectively.

A possible mechanism for cross regulation of SIRT1 and PPARγ is through a direct protein interaction between SIRT1 and PPARγ [146]. Using a co-immunoprecipitation method, we observed that $t10c12$ CLA treatment resulted in a 138% increase in the amount of a SIRT1/PPARγ protein complex relative to the amount of this complex in the LA control, despite a 46% reduction in PPARγ protein levels in $t10c12$ CLA treated cells (Fig. 7). Again using a co-immunoprecipitation method, we observed that the amount of a protein complex containing SIRT1 and NCoR1 increased by 131% in the presence of $t10c12$ CLA (Fig. 7). These results demonstrated that there was increased binding of SIRT1 to PPARγ and NCoR1 in $t10c12$ CLA treated adipocytes.

Discussion

Here we demonstrated that SIRT1 activity was involved in the TG loss response that occurred in $t10c12$ CLA-treated adipocytes. Inhibition of SIRT1 activity or abundance with sirtinol, nicotinamide, etomoxir [151], or siRNA attenuated the TG loss, while SIRT1 activation by SRT1720 increased the TG loss caused by $t10c12$ CLA. The 100% difference in TG levels after 24 h of treatment with LA, relative to TG levels in $t10c12$ CLA-treated adipocytes, was caused by a combination of reduced fatty acid biosynthesis, increased lipolysis, and increased fatty acid oxidation in the $t10c12$ CLA-treated adipocytes. The reduction in the rate of fatty acid synthesis was consistent with AMPK's increased phosphorylation of ACC, thereby inhibiting its biosynthetic activity.

Fatty acid synthesis was also likely reduced by AMPK's ability to phosphorylate and inhibit fatty acid synthase [80, 81]. Our finding that $t10c12$ CLA-treated adipocytes have increased lipolysis and fatty acid oxidation is consistent with a report that activated AMPK increases lipolysis after an initial delay [140], and that 3T3-L1 adipocytes treated with $t10c12$ CLA have increased rates of lipolysis and fatty acid oxidation [70]. Our studies indicated that SIRT1 activity participated in inhibiting fatty acid biosynthesis, but did not significantly affect lipolysis or fatty acid oxidation in the response to $t10c12$ CLA. In light of the cross regulation in the SIRT1/AMPK/ PPARγ axis, a possible explanation is that these pathways were coordinately regulated, but each pathway might have a different threshold for responding to this regulation. For example, an increase in lipolysis or fatty acid oxidation might require less activation of AMPK, and therefore have less dependence on SIRT1 activity, than the inhibition of fatty acid biosynthesis.

Our molecular results indicated the response to $t10c12$ CLA included cross regulation between SIRT1, AMPK and PPARγ, as each protein affected the behavior of the others. Inhibitors or an activator of SIRT1 decreased or increased AMPK activity in $t10c12$ CLA-treated adipocytes, respectively. This is consistent with a SIRT1/LKB1/AMPK axis by which SIRT1 can affect AMPK activity [147, 148]. Treatment with $t10c12$ CLA caused more SIRT1 to bind to PPARγ and NCoRI, which reduces PPARγ activity [146], and which is consistent with the reduced transcription of lipogenic genes observed in $t10c12$ CLA treated adipocytes [12, 50, 67]. Inhibition of AMPK reduced SIRT1 activity as measured by the deacetylation of NF-κB, possibly by a mechanism in which AMPK-mediated changes in fatty acid oxidation affect the NAD^+/NADH ratio that affects SIRT1 activity [151]. AMPK was directly or indirectly

responsible for the increased phosphorylated at Ser112 of PPARγ in $t10c12$ CLA treated adipocytes [109], as this effect was attenuated by compound C. Phosphorylation of PPARγ at Ser112 facilitates its SUMOylation at K107, and thereby decreasing its transactivation activity [142]. Agonists or antagonists of PPARγ caused reductions or increases in the activity of AMPK and SIRT1, respectively, demonstrating the repressive effect PPARγ has on the activities of these proteins. Importantly, the total amounts of AMPK and SIRT1 proteins did not change in the response to $t10c12$ CLA, suggesting that PPARγ did not alter the amounts of these proteins through a transcriptional mechanism. As such, our results support an emerging role for PPARγ in regulating non-genomic processes [143, 144].

An inflammatory response has been demonstrated to be an important component of the TG reduction in $t10c12$ CLA treated adipocytes [60, 111]. Deacetylation of NF-κB by SIRT1 is expected to reduce NF-κB's transcriptional induction of pro-inflammatory genes [154]. In possible support of this, resveratrol, a SIRT1 activator, inhibits the inflammatory and cellular stress response caused by $t10c12$ CLA treatment [110]. However, resveratrol is also an agonist of PPARγ, which opposes the molecular responses to $t10c12$ CLA [109], making it more difficult to interpret the effects of resveratrol. We found that the functional consequence of inhibiting SIRT1 activity was an attenuation of the inflammatory response in $t10c12$ CLA-treated adipocytes. This result is consistent with SIRT1's ability to activate AMPK, possibly via SIRT1's ability to increase the activity of LKB1, an upstream kinase that phosphorylates AMPK [147, 148]. AMPK is critical for the $t10c12$ CLA-mediated inflammatory response in

adipocytes as demonstrated by the reduction in this response when AMPK is inhibited by compound C [111].

Our experiments using AMPK activators other than $t10c12$ CLA to manipulate these pathways gave results consistent with those obtained with $t10c12$ CLA. Phenformin, a potent AMPK activator, caused a TG loss similar to that caused by $t10c12$ CLA treatment. Troglitazone, the most potent PPARγ agonist in our 3T3-L1 adipocyte system, attenuated the TG loss caused by phenformin. Troglitazone reduced the amount of phosphorylated AMPK and ACC caused by $t10c12$ CLA treatment, providing a likely explanation for how it inhibits the TG loss caused by phenformin. A PPARγ antagonist increased the amount of TG loss when used with metformin, a moderate AMPK activator. This latter finding supports a hypothesis that both AMPK activation [111] and reduced PPARγ activity [60] are important for reducing TG levels in the response to $t10c12$ CLA. We interpret these results to mean that the ability of these proteins to cross regulate each other allows this endpoint to be achieved by either strong activation of AMPK (by $t10c12$ CLA or phenformin) or by a combination of a moderate AMPK activator (metformin) and a PPARγ antagonist.

Both AMPK and SIRT1 play major roles in regulating cellular energy homeostasis and in response to caloric restriction [155, 156]. The involvement of AMPK and SIRT1 in the molecular response to $t10c12$ CLA is consistent with an overall similarity to the response to cellular energy restriction. This is supported by the strong similarity of the whole genome transcriptional response of adipocytes treated with $t10c12$ CLA to the response to metformin [111], which affects the cellular AMP/ATP ratio [128, 129], and by the ability of phenformin to cause TG losses similar to those caused by $t10c12$ CLA.

Our results support a non-genomic role for PPARγ [143, 144] in its cross regulation of SIRT1 and AMPK activities. This cross regulation occurred in a later stage of the response after the initial activation of AMPK, consistent with the suggestion that AMPK activation is a critical early event [111]. The signaling pathways used by t10c12 CLA to activate AMPK remain unknown.
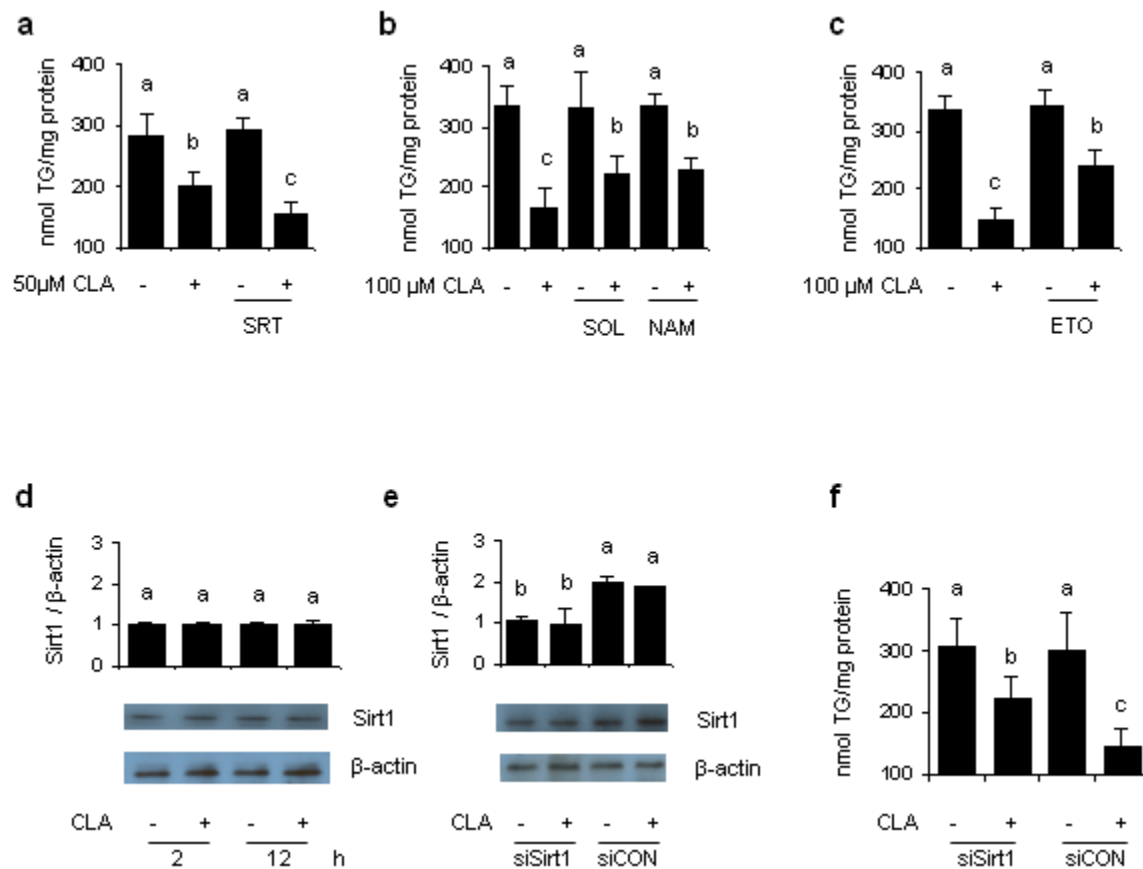
Acknowledgements

Figures

Figure 1. SIRT activity affects TG levels in t10c12 CLA treated 3T3-L1 adipocytes. **a-c** Triglyceride (TG) levels were measured in differentiated adipocytes after incubation with LA (-) or $t10c12$ CLA (+), with or without SRT1720 (SRT), sirtinol (SOL), nicotinamide (NAM), or etomoxir (ETO) for 24 h. **d** Western blot analysis of the amount of SIRT1 and β-actin proteins at 2 or 12 h, or in **e,** after treatment with siRNA against SIRT1 or with control siRNA after 12 h. **f** Triglyceride (TG) amounts 24 h after treatments as in **e.** Each bar in panels **a-f** represents the mean + SEM (n=3), and is representative of three independent experiments (**a-c, and f**) or is the mean of three independent experiments (**d-e**). Means not sharing a common letter differ, $P \leq 0.05$.
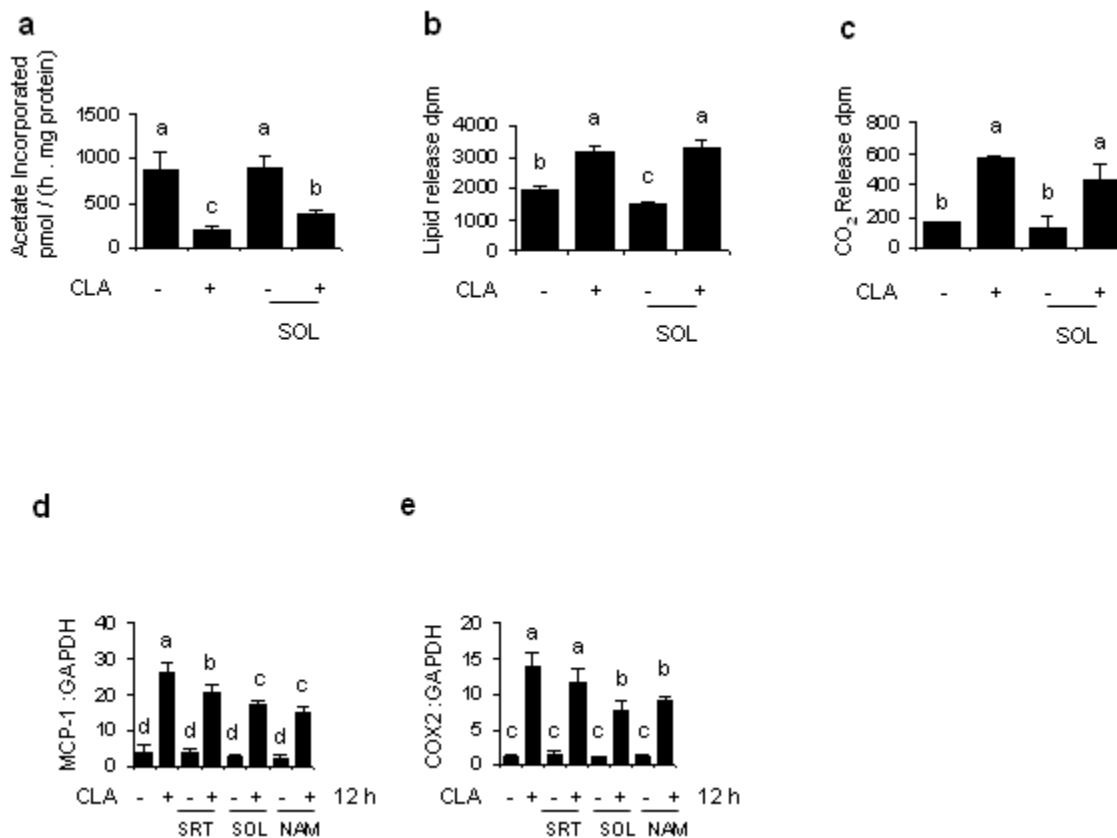
Figure 2. SIRT1 affects fatty acid metabolism and inflammatory mRNA levels. **a-c** The effects of 100 μmol/L of *t*10*c*12 CLA, with or without sirtinol (SOL), on fatty acid biosynthesis, lipolysis, or fatty acid oxidation were measured by incorporation of [1-^{14}C]-acetate (**a**) or release of labeled lipids (**b**) or CO_2 (**c**) derived from [1-^{14}C]-acetate or [1-^{14}C]-oleate, respectively (disintegrations per minute: dpm). **d-e** RNA levels were analyzed for MCP-1 and COX2 relative to GAPDH by reverse transcription and quantitative PCR. The relative amounts of MCP-1 and COX2 mRNA are shown as bar graphs. Each bar in panels **a-e** represents the mean + SEM (n=3 for **a-b** or n=2 for **c-e**), and is representative of three independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.
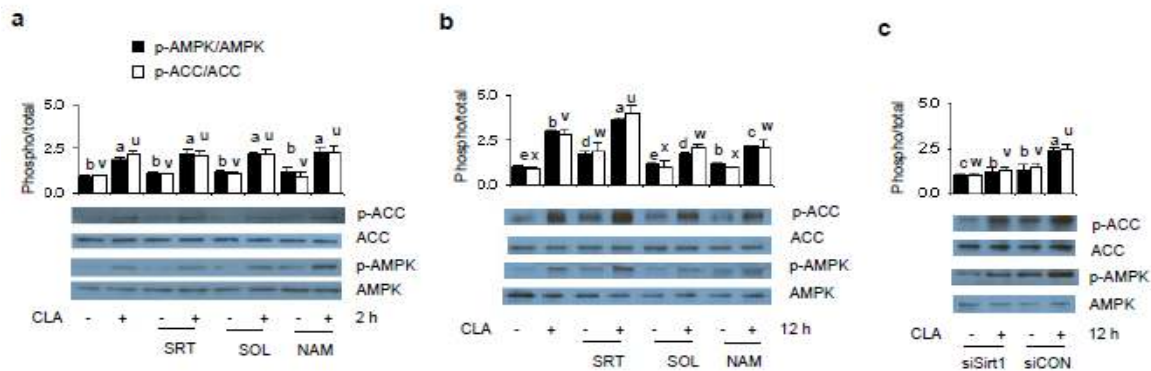
Figure 3. SIRT1 activity affects ACC and AMPK phosphorylation levels after $t10c12$ CLA treatment. Differentiated 3T3-L1 adipocytes were incubated with 100 µmol/L LA (-) or $t10c12$ CLA (+): **a** with or without SRT1720 (SRT), sirtinol (SOL), or nicotinamide (NAM) for 2 or **b** 12 h; or **c** with siRNA to SIRT1 (siSIRT1) or control siRNA (siCON). Representative western blots indicate the proteins detected with antibodies to p-ACC (p-Ser 79), or total ACC, p-AMPK (p-Thr 172), or total AMPK. Each bar represents the mean + SEM of the ratio of the phosphorylated to total form of each protein (phospho/total), n=3 independent experiments. Means for p-AMPK/AMPK (a-e) or p-ACC/ACC (u-x) not sharing a common letter differ, $P \leq 0.05$.
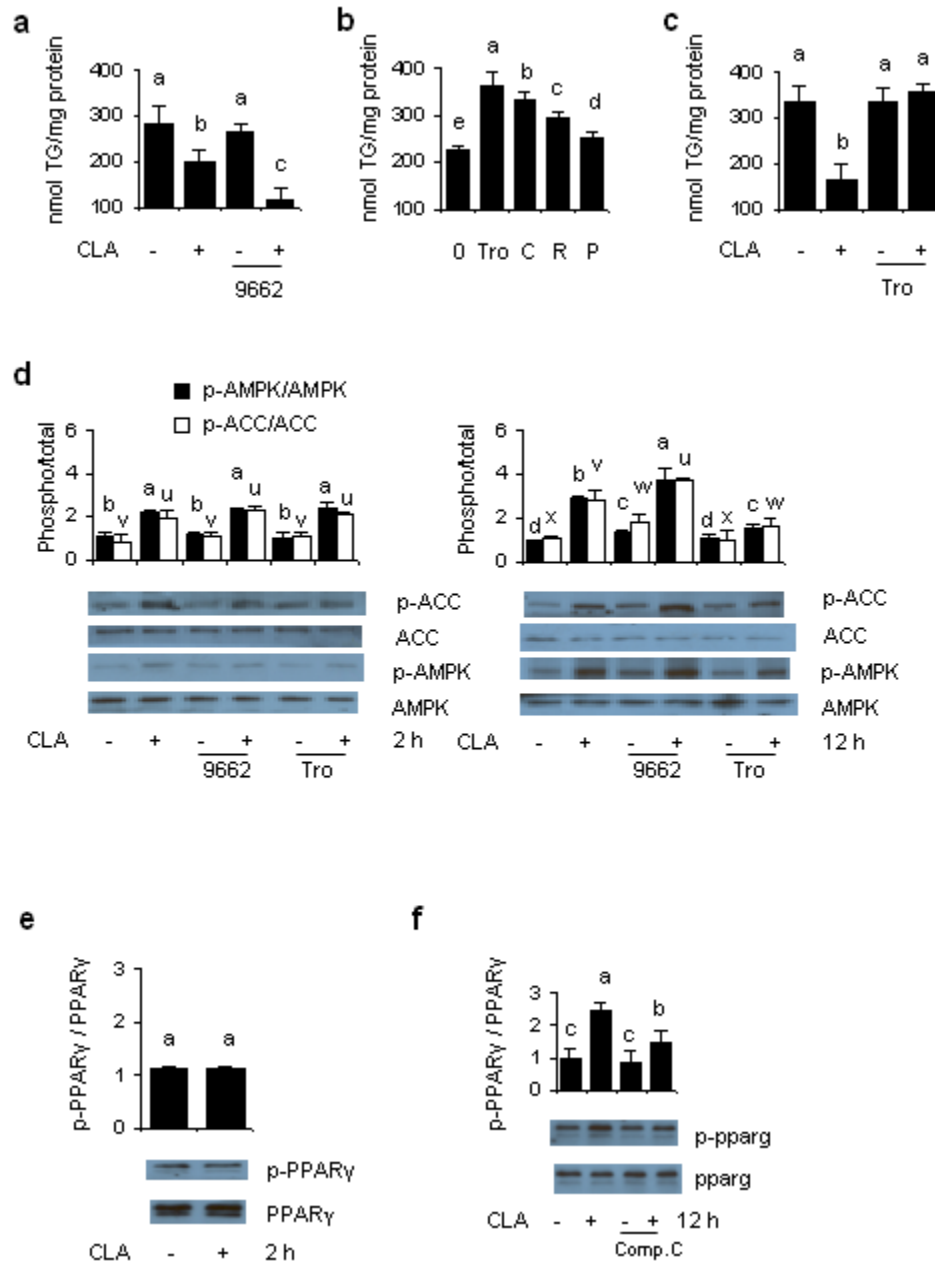
Figure 4. PPARγ antagonists and agonists affect TG levels and modulate p-AMPK activity levels in *t*10*c*12 CLA treated 3T3-L1 adipocytes. **a** Differentiated adipocytes were incubated with 50 µmol/L LA (-) or *t*10*c*12 CLA (+) with or without GW9662 (9662) for 24 h and TG levels were measured. **b** Differentiating adipocytes were treated with either control media (0), troglitazone (Tro), ciglitazone (C), rosiglitazone (R), or

pioglitazone (P) to determine which PPARγ agonist was most effective for increasing TG levels. **c** Differentiated 3T3-L1 adipocytes were incubated with 100 μmol/L LA or *t*10*c*12 CLA, with or without troglitazone (Tro), and TG levels were measured after 24 h. **d** Adipocytes were treated as in **a** or **c** for 2 or 12 h using 100 μmol/L LA (-) or *t*10*c*12 CLA (+), and representative western blots indicate the proteins detected with antibodies to p-ACC (p-Ser 79), total ACC. p-AMPK (p-Thr 172), or total AMPK. The ratio of the phosphorylated to total form of each protein (phospho/total) is shown in the bar graphs. **e-f** The amount of phosphorylated or total PPARγ was measured after 2 or 12 h of treatment with 100 μmol/L LA (-) or *t*10*c*12 CLA (+), and with or without compound C (Comp.C) at 12 h. The ratio of the amount of phosphorylated to total PPARγ is shown in the bar graphs. Each bar in panels **a-f** represents the mean + SEM (n=3), and is representative of three independent experiments (**a-c**) or is the mean of three independent experiments (**d-f**). Means within each data type (a-e or u-x) not sharing a common letter differ, $P \le 0.05$.
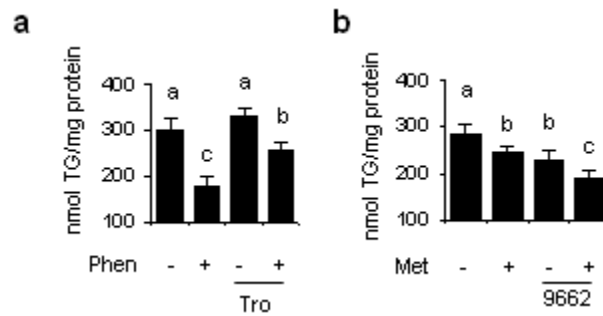
Figure 5. PPARγ agonists or antagonists affect the TG loss caused by AMPK activators.

a Differentiated 3T3-L1 adipocytes were incubated with or without 0.1 mmol/L phenformin (Phen), with or without troglitazone (Tro), and TG levels were measured after 24 h. **b** TG levels were measured in differentiated 3T3-L1 adipocytes in media lacking or containing 2 mmol/L metformin (Met), with or without GW9662 (9662) for 24 h. Each bar represents the mean + SEM (n=3), and is representative of three independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.

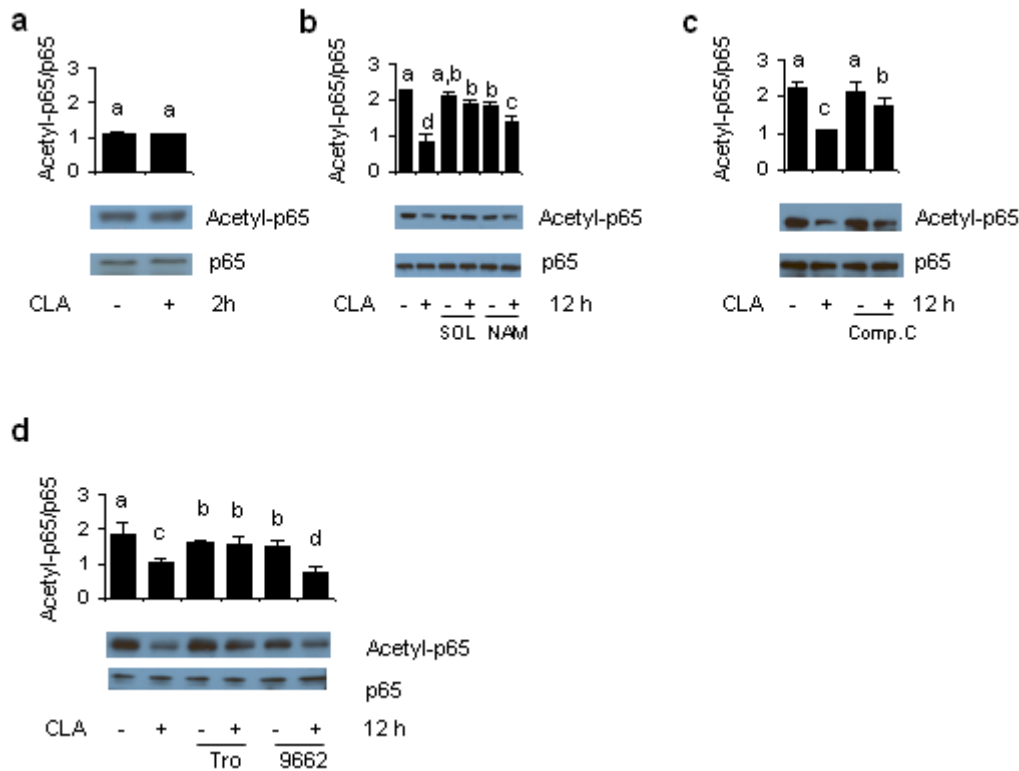**Figure 6.** SIRT1, AMPK and PPARγ affect the *t*10*c*12 CLA-dependent decrease in the amount of acetylated NF-κB. The amount of acetylation on the p65 subunit of NF-κB, or the total amount of p65, was enriched by immunoprecipitation of p65, and detected by immunoblot analysis of the immunoprecipitated proteins with antibodies to detect either acetylated p65 or total p65. The ratio of the acetylated to total p65 is shown (Acetyl-p65/p65). **a-c** Differentiated 3T3-L1 adipocytes were incubated with 100 µmol/L LA (-) or *t*10*c*12 CLA (+) **a** for 2 h, or **b** for 12 h, with or without sirtinol (SOL) or nicotinamide (NAM), or **c** with or without compound C (Comp.C). **d** The effects of troglitazone (Tro) or GW9662 (9662), in combination with LA (-) or *t*10*c*12 CLA (+), on the acetylation of the p65 subunit of NF-κB was measured. Each bar in panels **a-d** represents the mean + SEM (n=3) of three independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.
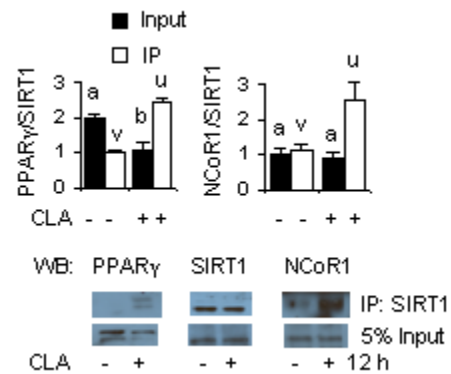
Figure 7. Treatment with $t10c12$ CLA increases the interaction of SIRT1 with PPARγ or NCoR1. Differentiated 3T3-L1 adipocytes were incubated with 100 μmol/L LA (-) or $t10c12$ CLA (+) for 12 h, and a portion of the extracts were immunoprecipitated with antibody to SIRT1. Representative western blots indicate the proteins detected from the crude extracts (5% input) or when the immunoprecipitated (IP) proteins were probed with antibodies to SIRT1, PPARγ, or NCoR1. Each bar represents the mean + SEM (n=3) of three independent experiments. Means within each data type not sharing a common letter differ, $P \leq 0.05$.

Chapter V. PPARδ and AMPK antagonize PPARγ to cause triglyceride loss in conjugated linoleic acid treated adipocytes

Introduction

Conjugated linoleic acid (CLA) reduces adiposity in mouse and human adipocytes, and *trans*-10, *cis*-12 CLA (*t*10*c*12 CLA) is an active isomer capable of causing this response [16, 24, 60, 69, 137]. Treatment of 3T3-L1 adipocytes with *t*10*c*12 CLA increases the rates of lipolysis and fatty acid oxidation [70], and reduces the rate of fatty acid biosynthesis [157]. The molecular responses to *t*10*c*12 CLA are highly dependent on the activity status of AMP-activated protein kinase (AMPK) [158], peroxisome proliferator-activated receptor (PPAR)γ protein [60], and an inflammatory response [60, 158].

AMPK is a central regulator of cellular energy levels that is activated by increases in the cellular AMP/ATP ratio, various cellular stresses [80, 81], AICAR (aminoimidazole carboxamide ribonucleotide), or treatment of adipocytes [158] or mice [139] with *t*10*c*12 CLA or mixed isomers of CLA. Activated AMPK is required for the TG reduction that occurs in *t*10*c*12 CLA treated adipocytes [158], and AMPK activation requires phosphorylation at Thr172 [135]. Two of the proteins phosphorylated and thereby inhibited by activated AMPK are acetyl CoA carboxylase and fatty acid synthase, two rate-limiting lipid biosynthetic enzymes. Through this and other mechanisms [80, 81], activated AMPK decreases lipogenesis, increases fatty acid oxidation, and increases lipolysis in adipocytes *in vitro* [157] and *in vivo* after an initial delay [140].

PPARγ and PPARδ are nuclear receptor transcription factors that can be activated by fatty acids and other ligands in adipocytes. PPARγ regulates lipogenesis and is a key regulatory point for controlling inflammation in adipocytes [115]. PPARδ is a ubiquitously expressed and regulates fatty acid oxidation, and mitochondrial abundance,

primarily in muscles, but a role in WAT is emerging [159-163]. PPARγ plays a critical role in the response to $t10c12$ CLA, as the response is attenuated on addition of PPARγ agonists [109, 110, 157], and enhanced TG loss occurs on addition of a PPARγ antagonist [157, 164]. PPARγ has increased phosphorylation [109] and reduced protein levels in $t10c12$ CLA treated adipocytes [60, 158]. Reduced levels of PPARδ expression caused increased TG accumulation in 3T3-L1 adipocytes, indicating a role for PPARδ in opposing lipid accumulation [165]. Retinoid X receptor (RXR) is a heterodimeric partner for many nuclear receptors, including PPARγ and PPARδ. Competition between nuclear receptors for limiting amounts of RXR is observed in some systems [166]. PPARδ also physically interacts with AMPK, and constitutively activated PPARδ results in activated AMPK in muscle cells, indicating possible cross regulation occurs between these proteins [163]. These aspects of PPARδ biology suggested it could be involved in the TG loss response in $t10c12$ CLA treated adipocytes.

Here we used chemical activators, inhibitors, or siRNA to affect PPARδ, and measured the effects of these treatments on TG levels, fatty acid biosynthesis, lipolysis, and oxidation, as well as on protein interactions in 3T3-L1 adipocytes, and on the ability of $t10c12$ CLA and GW0742 to reduce body and adipose tissue mass in mice. Our objectives were to test whether PPARδ was functionally involved in the TG reduction response to $t10c12$ CLA, and whether this response affected the protein interactions of PPARγ and PPARδ with RXR, and of PPARδ with AMPK.

Methods

Reagents

Bovine serum albumin (BSA, > 99% fat free), dexamethasone, GW0742, GSK0660, insulin, and isobutyl-1-methylxanthine, were purchased from Sigma (St. Louis, MO). T10c12 CLA (90%, #UC-61-A) was from Nu-Chek Prep, Inc (Elysian, MN). Negative control siRNA was from Abcam (Austin, TX). Protein A agarose beads, antibodies to β-actin, PPARδ, and PPARγ, anti-rabbit and anti-goat secondary antibodies coupled to horseradish peroxidase, and siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while antibodies to p-AMPK, AMPK were from Cell Signaling (Beverly, MA).

Animals

C57Bl/6J male mice were housed individually at 22 °C with 12 h light and 12 h dark. All mice were fed AIN-93G diet ([84]; Dyets, Inc., Bethlehem, PA) for 5 d before beginning the treatments. Mice were blocked by initial body weight and assigned to one of the four treatment diets. Specific diets contained either 70 g/kg soy oil without t10c12 CLA or 68 g/kg soy oil with 2 g/kg t10c12 CLA. GW0742, when present, was incorporated in the diet at 7 mg/kg. Diet was changed twice each week, and mice were allowed to eat *ad libitum.* After 14 d of these treatments, body weight was measured and mice were killed in the fed state between 09:00 and 13:00 h by carbon dioxide asphyxia. Retroperitoneal and epididymal fat pads were harvested and weighed. All procedures were approved by the University of Nebraska Institutional Animal Care and Use Committee.

3T3-L1 cell culture, differentiation, and chemicals

3T3-L1 fibroblasts [85] were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% bovine calf serum (Fisher, Pittsburgh, PA) and differentiated as described [158]. When present, chemicals were dissolved in DMSO, and were added directly to the media at ≤ 0.2% of the final volume in the media at the following concentrations: 9 µmol/L GW0742, or 5 µmol/L GSK0660, and were added 1 h before adding fatty acids. Fatty acids, either linoleic acid or $t10c12$ CLA, were dissolved in 0.1 M KOH, diluted into fatty acid free (>99%) bovine serum albumin (BSA) in phosphate buffered saline at a 1:1 ratio (2 mmol/L BSA: 2 mmol/L fatty acid), pH adjusted to 7.4, and added to the cultures containing 5.5 to 6 d post-differentiated 3T3-L1 adipocytes [158].

Fatty acid biosynthesis, oxidation, and lipolysis assays

Fatty acid biosynthesis was measured in differentiated adipocytes 24 h after starting treatments by removing the treatment media and incubating the adipocytes in Hanks' Balanced Salt Solution (HBSS; Invitrogen, Carlsbad, CA) containing 37 KBq [14C]-acetate [specific activity 2.1 GBg/mmol, (PerkinElmer Radioisotopes, Waltham, MA)] for 30 min (incorporation was linear for 60 min, data not shown). Cells were washed in PBS three times, pelleted, and then resuspended in 100 µl PBS and 0.1% SDS. Neutral lipids were extracted in 1 ml of 2:1 chloroform:methanol [153] and measured by scintillation counting. Cells briefly exposed to 37 KBq [14C]-acetate in media, followed by immediate washing and extraction as above, were used to determine background levels, which were subtracted from sample values.

Fatty acid oxidation was measured in differentiated adipocytes in 3.5 cm culture plates 12 h after starting treatments by adding 37 KBq [14C]-oleic acid [specific activity

2.2 GBg/mmol, (PerkinElmer Radioisotopes, Waltham, MA)] to the treatment media for 2 h and then collecting [^{14}C]-CO$_2$ for 1 hr in collection jars as reported [138].

For lipolysis assays, the TG pool of differentiated adipocytes was labeled by adding 37 KBq [^{14}C]-acetate [specific activity 2.1 GBg/mmol, (PerkinElmer Radioisotopes, Waltham, MA)] to the media for 4 h, after which time the plates were washed four times with PBS, and specific experimental media treatments were initiated. Media (0.1 ml) was collected after 12 h, neutral lipids extracted in 1 ml of 2:1 chloroform:methanol [153] and measured by scintillation counting. The use of labeled [^{14}C]-acetate and the 2:1 chloroform:methanol extraction step considerably reduced non-specific background to 50 dpm, as determined by using the above protocol on cells that had been briefly exposed to 37 KBq [^{14}C]-acetate in media.

siRNA transfection

3T3-L1 adipocytes, 5 d post differentiation, were transfected by siQUEST transfection reagent (Mirus, Madison, WI), using final concentrations of 2 μl of siQUEST reagent per ml of media and siRNA at 40 nmol/L, 24 hours before adding fatty acids .

Immunoblot analysis

Nuclear and cytosolic extracts were isolated using a nuclear extract kit (Active Motif, Carlsbad, CA). Equal amounts of proteins were separated by SDS-PAGE, transferred to Immun-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA), probed with the indicated primary antibodies, and detected with secondary antibodies. Enhanced chemiluminescence (Pierce, Rockford, IL) was used for detection. Band intensities were determined from digital images from exposures in the linear range using

software (Quantity One, Biorad, Hercules, CA). All western blot analyses were repeated at least three times.

Immunoprecipitation

Immunoprecipitations were performed according to the procedure described [146]. In brief, the collected 3T3-L1 adipocytes were sonicated, lysates were centrifuged, and aliquots of the supernatants were immunoprecipitated overnight with specific antibody or control nonspecific IgG serum. Protein A agarose beads were used to bind the specific or non-specific antibody complexes, washed five times, and the bound proteins were eluted in SDS sample buffer for immunoblot analysis.

Quantification of TG content.

Cell isolation and TG measurements were performed according to the manufacturer' instructions using TG reagent (T2449; Sigma, St. Louis, MO) and free glycerol reagent (F6428; Sigma, St. Louis, MO). TG data are expressed as nmol of TG per mg of protein.

Measurement of mRNA levels.

Total RNA was extracted by TRIzol (Invitrogen) following the manufacturer's protocol. Total RNA (2 μg) was used for cDNA synthesis. Real-time PCR was performed by a Bio-Rad iCycler using iQ SYBR Green Supermix reagent (Bio-Rad, Hercules, CA) using PCR primers for MCP1 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [158], ANGPTL4 [167], CPT1a [168], and PDK4 [169]. Target mRNA levels were normalized to GAPDH, which showed no significant variation in microarray analyses between linoleic acid and $t10c12$ CLA treatments. Experiments were repeated three times, and each sample was analyzed using one RNA control and two replicates of each cDNA

pool, and the relative amounts of each target mRNA and GAPDH were calculated using the comparative C_T method [88], according to the manufacturer's software (Bio-Rad, Hercules, CA). Cycle numbers were used to calculate gene expression levels in the log linear amplification range.

Statistical Analysis

Two-way ANOVA was used to analyze the data as follows: for Figures 1, 2b, 3, 4a, 5 and Table 1, the main effects of CLA (LA or CLA) and GW0742 (- or +) or GSK0660 (- or +), and their interaction (CLA x GW0742 or CLA x GSK0660) were analyzed. One-way ANOVA was used to analyze the data in Figures 2a, and 4b. Pairwise comparisons were calculated using Tukey's test (criterion for significance, $\alpha \leq 0.05$, except for Table 1 for which $\alpha \leq 0.1$ was used). All analyses were performed using JMP8 software (SAS, Cary, NC).

Results

The possible involvement of PPARδ in the response to $t10c12$ CLA was first tested using an agonist or antagonist of PPARδ. Treatment with GW0742, an agonist, produced TG levels that were not significantly different at 86% of the LA control, but the combination of GW0742 and 50 μM $t10c12$ CLA had significantly lower TG levels at 46% of the LA control (Fig. 1A; We generally used 50 μM $t10c12$ CLA if assaying chemicals that increased TG loss, but otherwise used 100 μM $t10c12$ CLA to obtain large changes in the molecular responses of interest). GSK0660, a PPARδ antagonist, had no significant effect on TG levels when used alone (101% of LA control), but significantly interfered with the ability of 100 μM $t10c12$ CLA to reduce TG levels at 72% of LA control as compared to a 50% level for 100 μM $t10c12$ CLA alone (Fig 1B). The effects

of the combination treatments of $t10c12$ CLA with PPARδ siRNA or random siRNA were significantly different, with TG levels of 77% or 55%, respectively, relative to the control treatment of LA and random siRNA (Fig 1 C). PPARδ protein levels were reduced by 47% by the treatment with siRNA against PPARδ relative to samples treated with a control random siRNA (Fig 1D). This siRNA-mediated attenuation of TG loss was consistent with the PPARδ antagonist result above, and collectively these results indicated that PPARδ facilitated TG loss in the response to $t10c12$ CLA. We next determined whether three PPARδ regulated genes [angiopoietin-related protein 4 (ANGPTL4); carnitine palmitoyltransferase Ia (CPT1a); and pyruvate dehydrogenase kinase 4 (PDK4)] had increased mRNA levels, as an indicator of increased PPARδ transcriptional activity [170]. Their mRNA levels were significantly increased in $t10c12$ CLA treated adipocytes by 108%, 78%, or 111% for ANGPTL4, CPT1a, or PDK4, respectively (Fig. 2 A). Taken together, these results indicated PPARδ was activated and facilitated TG loss in the response to $t10c12$ CLA.

We next determined the effects of PPARδ on specific pathways involved in the response to $t10c12$ CLA. We measured the effect of GW0742 and GSK0660 on the $t10c12$ CLA-dependent induction of monocyte chemotactic protein-1 (MCP1), an inflammatory mRNA induced by $t10c12$ CLA [158]. Treatment with $t10c12$ CLA and GW0742 did not significantly change MCP1 mRNA levels (Fig. 2B). However, treatment with $t10c12$ CLA and GSK0660 significantly reduced MCP1 mRNA levels to 32% of the $t10c12$ CLA control value (Fig. 2B). The effect of PPARδ on fatty acid biosynthesis, lipolysis, or oxidation was determined using $t10c12$ CLA and GSK0660. Fatty acid biosynthesis in adipocytes treated with $t10c12$ CLA alone was 45% of the LA control,

while the combination of $t10c12$ CLA and GSK0660 was moderately but significantly

higher at 55% of the LA control value (Fig 3a). Fatty acid lipolysis with $t10c12$ CLA

alone or in combination with GSK0660 was 159% and 99% of LA control levels,

respectively (Fig 3b). Fatty acid oxidation with $t10c12$ CLA alone or in combination with

GSK0660 was 311% and 128% of LA control levels, respectively (Fig 3c). These results

indicated that PPARδ significantly attenuated the inflammatory pathway, as well as fatty

acid lipolysis and oxidation, but only moderately affected inhibition of fatty acid

biosynthesis in $t10c12$ CLA treated adipocytes.

Activated AMPK plays an essential role in the response to $t10c12$ CLA [158].

Therefore, the ability of PPARδ to affect AMPK activity was measured with $t10c12$ CLA

alone or in combination with GW0742 or GSK0660. The amount of activated AMPK

increased in the presence of $t10c12$ CLA, and this amount was further increased by 13%

in combination with GW0742, or decreased by 46% in combination with GSK0660 (Fig

4A). A possible mechanism for this effect was via a physical interaction between AMPK

and PPARδ [163], and we hypothesized this interaction might be affected by $t10c12$ CLA

treatment. We measured this interaction using a coimmunoprecipitation assay and

determined that the amount of AMPK bound to PPARδ increased by 84% in $t10c12$ CLA

treated adipocytes (Fig 4B). These results indicated a PPARδ agonist or antagonist

affected AMPK activity, and that $t10c12$ CLA treated adipocytes had an increased

amount of PPARδ bound to AMPK.

PPARγ protein levels are reduced in $t10c12$ CLA treated adipocytes [60, 158], and

we tested whether activated PPARδ could compete more effectively with PPARγ for

RXR. Therefore, the amount of PPARδ or PPARγ present in a protein complex with

RXR was measured by coimmunoprecipitation with RXR. The amount of PPARγ bound to RXR was significantly reduced to 32% of control LA levels in the presence of $t10c12$ CLA, and this amount was further reduced to 20% when both $t10c12$ CLA and GW0742 were present (Fig 5). The amount of PPARδ bound to RXR significantly increased by 105% in $t10c12$ CLA treatments, and this amount increased further by 215% when both $t10c12$ CLA and GW0742 were present (Fig 5). These results indicated PPARδ competed more effectively for RXR in the presence of $t10c12$ CLA.

The above results suggested the combination of GW0742 and $t10c12$ CLA should be tested in mice for its ability to reduce body and WAT weight. Mice were fed diet with or without $t10c12$ CLA, and with or without GW0742. After two weeks body weight, retroperitoneal, and epididymal fat pads were measured (Table 1). The reductions in body weight were significant for $t10c12$ CLA ($p=0.02$) and GW0742 ($p=0.08$). The effects on retroperitoneal mass were significant for $t10c12$ CLA ($p<0.01$) and approached significance for GW0742 ($p=0.14$). The effects on epididymal mass were significant for $t10c12$ CLA ($p<0.01$) and GW0742 ($p=0.06$). None of the interactions between GW0742 and $t10c12$ CLA were significant (Table 1).

Discussion

Here we demonstrated PPARδ played a positive role in reducing TG levels in $t10c12$ CLA treated adipocytes. In support of increased PPARδ activity, three PPARδ target genes showed increased mRNA levels in the response to $t10c12$ CLA. Inhibiting PPARδ protein activity or amounts with an antagonist or siRNA, respectively, attenuated the TG loss response caused by $t10c12$ CLA, while addition of a PPARδ agonist increased the TG loss. Functional analyses of PPARδ in several systems support its

involvement in TG loss [160, 163]. Overexpressed PPARδ decreased TG levels, and reduced levels of PPARδ increased TG levels, in 3T3-L1 adipocytes [165]. PPARδ was required for fatty acid transport and oxidation in human adipocytes [171]. Mice expressing constitutively active PPARδ had increased expression of genes involved with fatty acid oxidation and uncoupling protein 1 (UCP1), and had reduced adiposity [160]. PPARδ and AMPK are both required for efficient fatty acid oxidation in muscle cells [171]. Our results were in agreement with these studies, as PPARδ strongly affected *t*10*c*12 CLA's ability to increase fatty acid oxidation and lipolysis, but only moderately affected the reduced rate of fatty acid biosynthesis in *t*10*c*12 CLA treated adipocytes.

PPARδ had increased binding to AMPK in *t*10*c*12 CLA treated adipocytes, consistent with the ability of AMPK to bind to PPARδ in muscle cells [163]. A PPARδ agonist or antagonist affected AMPK activity, as treatment with GW0742, an agonist of PPARδ, increased the amount of activated AMPK, while treatment with GSK0660, a PPARδ antagonist, reduced the amount of activated AMPK. The physical interaction of PPARδ and AMPK might be a mechanism by which PPARδ activity increased AMPK activity as constitutively-activated PPARδ resulted in activated AMPK in transgenic mice [163], and the PPARδ agonist GW501516 activated AMPK in muscle cells [171]. The ability of PPARδ to affect AMPK activity might explain observations that fatty acids activate AMPK [172, 173], as fatty acids are natural agonists of PPARδ [174]. As a fatty acid, *t*10*c*12 CLA is a potential ligand for PPARδ, but our data did not resolve whether it activated AMPK through direct binding to PPARδ or via an unknown indirect mechanism. Although the detailed mechanism by which PPARδ activates AMPK is unproven, it is clear that PPARδ's ability to affect AMPK activity was predominantly

mediated by a non-transcriptional mechanism as total AMPK protein levels were not significantly changed. Non-genomic regulation of proteins involved in non-transcriptional processes such as metabolic regulation is an emerging trend for the family of PPAR transcription factors, as similar behavior has been observed for PPARγ [143, 157].

Activated AMPK results in reduced amounts of PPARγ protein [60, 158] and increased PPARγ phosphorylation [157], which reduces its transactivation activity [142]. Therefore, PPARδ's ability to activate AMPK indirectly facilitated reduced amounts of PPARγ activity in the response to $t10c12$ CLA. Reduced amounts of PPARγ activity are important for $t10c12$ CLA-mediated TG loss, as activated PPARγ opposes $t10c12$ CLA effects [110, 157]. In addition to these indirect effects on PPARγ activity via changes in AMPK activity, PPARδ also reduced PPARγ activity via direct competition for RXR, the required heterodimeric partner for PPARγ and PPARδ [166, 175, 176]. The amount of the PPARγ/RXR heterodimeric protein complex decreased, and the amount of PPARδ/RXR increased, in the presence of $t10c12$ CLA. The amount of PPARγ/RXR heterodimeric protein complex was reduced further when both $t10c12$ CLA and GW0742 were present, providing additional support for the ability of activated PPARδ to compete for RXR. Thus, PPARγ activity was reduced by a combination of AMPK-mediated and PPARδ-mediated mechanisms. Additionally, $t10c12$ CLA treatment increases the amount of Sirtuin 1 (SIRT1) bound to PPARγ [157], which represses PPARγ transactivation activity [146]. The inflammatory response is an important component of the TG loss caused by $t10c12$ CLA [60, 158], and both PPARγ and PPARδ are considered to have anti-inflammatory roles in many cell types [177]. However, we observed that inhibition

of PPARδ reduced the inflammatory response in *t*10*c*12 CLA treated adipocytes. This was consistent with PPARδ's activation of AMPK, as activated AMPK is required for the inflammatory response to *t*10*c*12 CLA [158]. The combined effects of AMPK, PPARδ, and SIRT1 on reducing PPARγ activity facilitates an inflammatory response, as PPARγ plays an anti-inflammatory role in adipocytes [115].

The effects of *t*10*c*12 CLA and GW0742 in mice were promising as this combination produced the smallest average fad pads and the most reduction in the average body mass. The effects of *t*10*c*12 CLA and GW0742 were significant for decreases in body mass and epididymal fat pads, and approached significance for retroperitoneal fat pads, but indicated trials with larger numbers of animals are needed to obtain more significant data. In support of our results, concurrent administration of the AMPK activator AICAR and a PPARδ agonist was more effective than a PPARδ agonist alone for increased running endurance in muscle tissue and reduced epididymal tissue mass in mice [163]. Our results indicate combinations of *t*10*c*12 CLA and a PPARδ agonist have potential for reducing adiposity in mammals.

Tables and figures

Table 1. The effect of GW0742 and t10c12 CLA on body weight or adipose tissue weight in mice[1]

BW change (g/2 wk) or Tissue (g)[3]	CLA		GW0742		P value		
	-CLA	+CLA	-CLA	+CLA	GW0742	CLA	GW0742 x CLA
BW	0.44 ± 0.94^a	-0.06 ± 0.96^{ab}	0.07 ± 0.59^{ab}	-0.52 ± 0.43^b	0.08	0.02	0.84
EPI	0.34 ± 0.12^a	0.23 ± 0.07^b	0.30 ± 0.12^a	0.20 ± 0.06^b	0.06	<0.01	<0.83
RP	0.18 ± 0.08^a	0.07 ± 0.03^b	0.15 ± 0.08^a	0.05 ± 0.02^b	0.14	<0.01	0.83

[1] Data are presented as mean ± SEM, n = 11 for BW and RP, n=22 for EPI. Means in a row not sharing a common superscript differ, $P < 0.1$.

[2] C57Bl/6J male mice were fed 0.2% $t10c12$ CLA with or without GW0742 for two weeks.

[3] The changes in body weight (BW) or the final weights of the epididymal (EPI) or retroperitoneal (RP) were measured after 14 d of treatments.
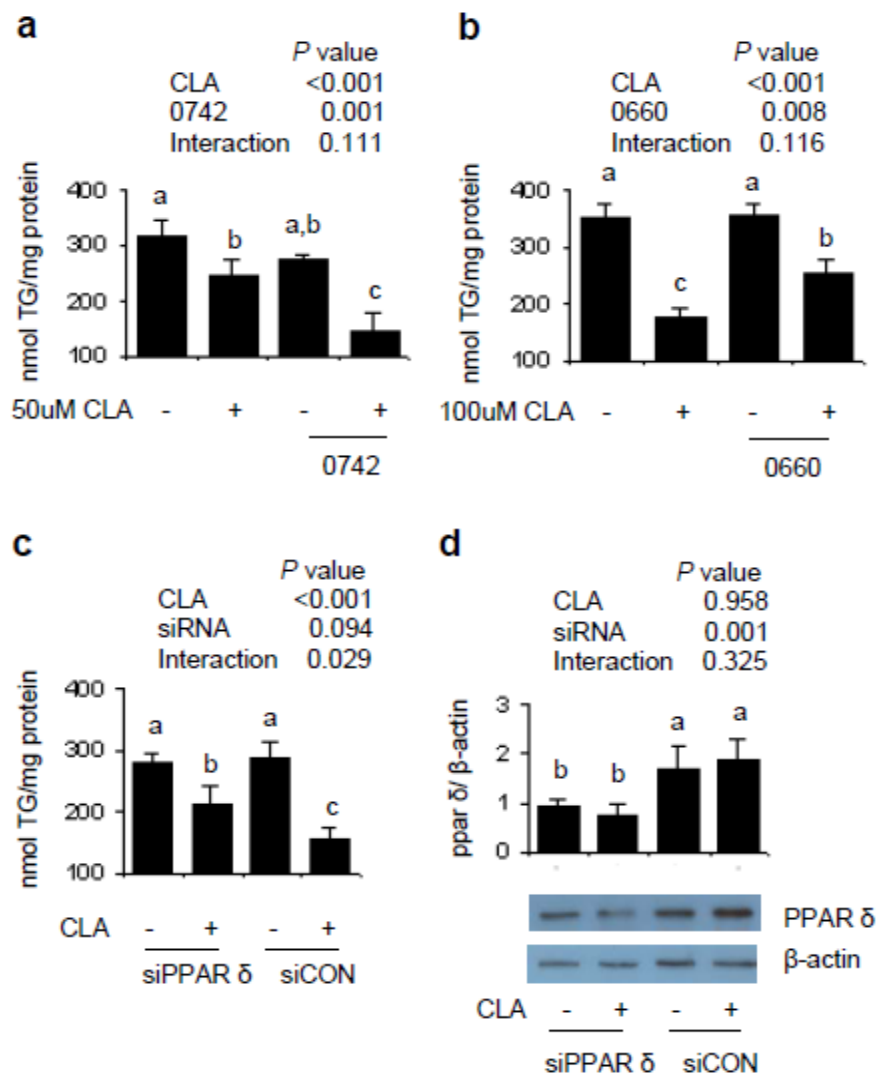
Figure 1. PPARδ activation, inhibition, or siRNA knockdown affects TG loss in $t10c12$ CLA treated adipocytes. A-C) Triglyceride (TG) levels were measured in 3T3-L1 adipocytes after incubation with LA (-) or $t10c12$ CLA (+), with or without GW0742, GSK0660, or siRNA [control (CON) or specific for PPARδ (siPPARδ)] for 24 h. D) A representative western blot of the siRNA knockdown of PPARδ is shown. Each bar represents the mean + SEM, n=3, and is representative of three independent experiments for A-C, or represents three independent experiments for D. Means not sharing a common letter differ, $P \leq 0.05$.
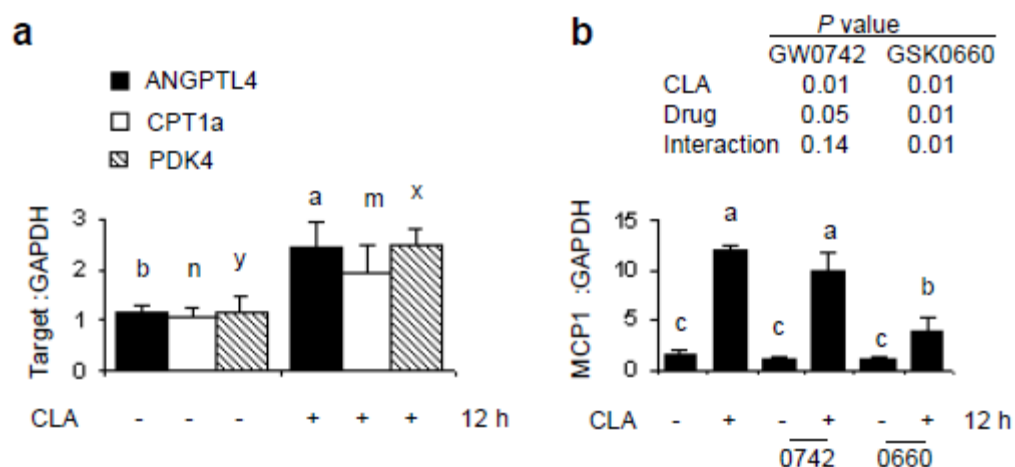
Figure 2. PPARδ regulated genes and MCP1 have increased expression, and MCP1 expression is affected by a PPARδ agonist or antagonist, in *t*10*c*12 CLA treated adipocytes. A-B) The mRNA from 3T3-L1 adipocytes treated with LA (-) or *t*10*c*12 CLA (+) for 12 h [with or without GW0742 (0742) or GSK0660 (0660)], and was then isolated and analyzed by real time PCR using primers for the following genes: MCP1, ANGPTL4, CPT1a, and PDK4. Each bar represents the mean + SEM, n=3 independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.
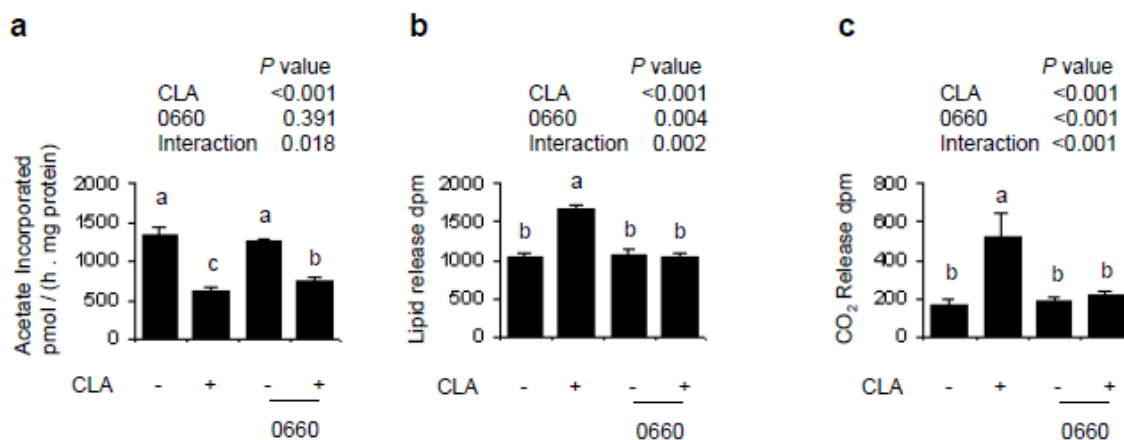
Figure 3. Rates of fatty acid biosynthesis, lipolysis, and oxidation are affected by $t10c12$ CLA and GSK0660. A) Adipocytes were treated with LA (-) or $t10c12$ CLA (+), with or without GSK0660 (0660), for 24 h and then labeled with [^{14}C]-acetate to determine the rate of fatty acid biosynthesis. B) As in A except that the amount of lipid released into the media at 12 h was measured. C) As in A except cells were labeled with [^{14}C]-oleate at 12 h after treatment and the amount of [^{14}C]-CO$_2$ released was measured. Each bar represents the mean + SEM, n=3, and is representative of three independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.
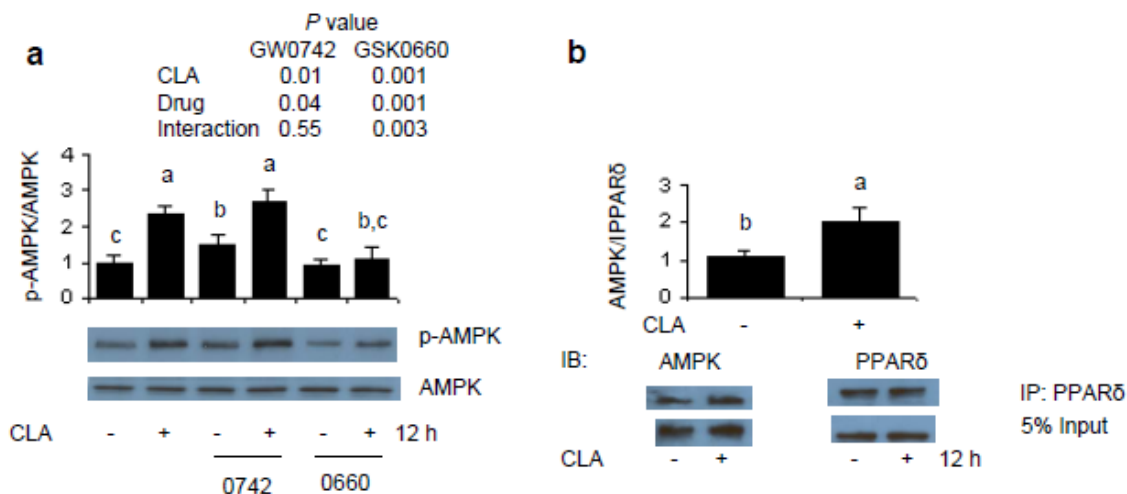
Figure 4. $T10c12$ CLA, with or without a PPARδ agonist or antagonist, affects AMPK activity and AMPK's interaction with PPARδ. A) 3T3-L1 adipocytes were treated with LA (-) or $t10c12$ CLA (+) for 12 h, with or without GW0742 (0742) or GSK0660 (0660). A representative immunoblot indicates the AMPK protein detected with antibody to phosphorylated AMPK (p-AMPK) or total AMPK (AMPK), respectively. The ratio of p-AMPK to AMPK is indicated in the bar graph, and each bar corresponds to the treatment described below the immunoblots. B) Cell extracts from adipocytes treated with LA (-) or $t10c12$ CLA (+) for 12 h were immunoprecipitated with antibody to PPARδ (IP: PPARδ). Input (5% Input) or immunoprecipitated proteins were detected by immunoblot analysis using antibody to total AMPK or PPARδ (IB: AMPK or PPARδ). The ratios of AMPK to PPARδ for the immunoprecipitated samples are shown in the bar graph according to the CLA (-) or (+) designation. Each bar represents the mean + SEM, n=3 independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.
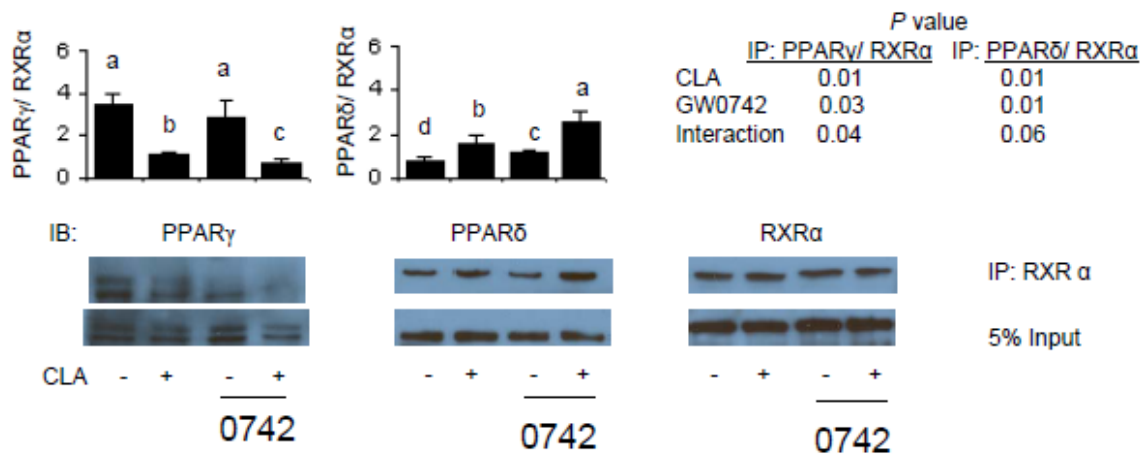
**Figure 5.** PPARγ and PPARδ bind to RXRα in *t*10*c*12 CLA treated adipocytes. Adipocytes were treated with LA (-) or *t*10*c*12 CLA (+), with or without GW0742 (0742) and extracts were immunoprecipitated using antibody to RXRα (IP: RXRα). Representative immunoblots demonstrate the amounts of protein detected in the input (5% Input) and immunoprecipitated samples for PPARγ, PPARδ, and RXRα. The ratios of PPARγ or PPARδ to RXRα are shown in the bar graph according to the treatment described below the immunoblots. Each bar represents the mean + SEM, n=3 independent experiments. Means not sharing a common letter differ, $P \leq 0.05$.

Chapter VI. Implications

Obesity has reached epidemic proportions in the United States and other developed countries, and is increasing even in the developing countries. T10c12 CLA causes fat loss in several mammalian models, particularly mice, and in human adipocytes. However, t10c12 CLA has not been demonstrated to be effective for TG loss in humans. Deciphering the mechanisms by which t10c12 CLA reduces fat in mammals might lead to therapies to prevent obesity. Currently, the mechanisms utilized by t10c12 CLA to cause fat loss are not known. The goal of this research was to discover some of the mechanisms utilized by t10c12 CLA to cause TG loss in adipocytes.

Here we demonstrated that the strong activation of AMPK induced by t10c12 CLA explained much of its isomer-specific delipidation effect in adipocytes. AMPK is an evolutionarily conserved protein kinase that regulates energy balance in cells. AMPK is a master regulator that reduces anabolic biosynthesis and increase catabolic processes to increase cellular energy levels. The inhibited lipid synthesis and enhanced fatty acid oxidation triggered by activated AMPK helps explain the dramatic fat loss and variety of physiological and molecular responses observed in t10c12 CLA-treated 3T3-L1 adipocytes and WAT in mice. The AMPK inhibitor Comp.C was able to attenuate changes in several key metabolic or regulatory proteins known to be inhibited by AMPK phosphorylation, including ACC, SREBP1c, PPARγ, C/EBPα, FAS, HMGR, ChREBP [Figure 1]. This results support our proposed role of AMPK as a master regulatory node in the response to t10c12 CLA. Our results showed AMPK was activated within 30 minutes, which positioned AMPK upstream of the later ISR and inflammatory responses in t10c12 CLA treatments [Figure 1]. Comp.C treatment also eliminated the ISR indictors ATF4 and ATF3 proteins and also reduced the amount of NF-κB translocated to

the nucleus, and inhibited MCP-1 mRNA levels. In conclusion, AMPK inhibitor comp.C inhibits all of the key indicators we tested in the response to t10c12 CLA.

We also demonstrated that activated AMPK could mimic many of the responses observed in t10c12 CLA treated adipocytes. We demonstrated this in several ways. T10c12 CLA's delipidation effects were enhanced by metformin in 3T3-L1 adipocytes and ML mice. More convincingly, phenformin, a stronger AMPK activator than metformin, caused TG losses similar to those caused by t10c12 CLA, and had a very similar transcriptome response as measured by microarray analysis. This provides strong support for the hypothesis that AMPK activation is sufficient to explain the majority of the responses to t10c12 CLA. The inability of phenformin to cause fat loss in humans is likely due to the uptake, distribution, and metabolism of the drug in the body. The combination of t10c12 CLA and metformin has therapeutic potential in humans and needs to be tested in human trials.

The inflammatory response was reported to be induced in t10c12 CLA-treated adipocytes. To answer the question whether an inflammatory response is required for effective delipidation by CLA, we explored which aspects of the inflammatory response were required. We found that the inhibition of NFkB attenuated TG loss in CLA treated adipocytes [Figure 1]. In support of the role of the inflammatory response, TNFα, a strong inflammatory cytokine, enhanced CLA's ability to cause fat loss [see model in Figure 1]. Interestingly, we found TNFα activated AMPK and TNFα's ability to delipidate was blocked by compound C. A comparison of AMPK activation, COX induction, and TG loss led to a model that suggested both AMPK and COX induction

were required for TG loss, and led us to more specifically investigate the role of prostaglandins in the CLA-meidated TG loss.

Our inhibitor studies indicated prostaglandins, Cox2, and PLA2 mediate at least part of the inflammatory response required for t10c12 CLA-mediated delipidation [Figure 1]. Specifically, previous studies in the Miner laboratory found treating mice with a delta-6 desaturase inhibitor (SC-26196) to reduce the production of arachidonic acid, the substrate for prostaglandin biosynthesis by Cox1/ 2, weakly inhibited t10c12 CLA-induced delipidation . In our studies in 3T3-L1 adipocytes, we used an alternative inhibitor delta-5, 6-desaturase inhibitor (CP-24879) because SC-26196 was no longer available, and found it inhibited t10c12 CLA-mediated delipidation [Figure 1]. Both ibuprofen and indomethacin, non-selective Cox1/2 inhibitors with different chemical structures, also inhibited t10c12 CLA-mediated delipidation [Figure 1]. Increased phosphorylation of PLA2, which regulates PG biosynthesis by cleaving membrane lipids to generate arachidonic acid, in t10c12 CLA-treated adipocytes further indicates the important role of prostaglandins [Figure 1].   These inhibitor studies suggested prostaglandins were required for efficient TG loss. We next used prostaglandin "add-back" treatments to better define their role.

Our hypothesis was that activated AMPK and prostaglandins were sufficient to cause TG loss. To test this, we used metformin to activate AMPK without creating a strong inflammatory response. We then added individual prostaglandins or arachidonic acid to test our hypothesis. Metformin alone causes a modest delipidation because it moderately activated AMPK. Individual prostaglandins had little effect when added alone. However, we found PGH2 (precursor to the PG family), or PGF2α caused strong

delipidation when used in combination with metformin. This supports the idea that a major component of the inflammatory response responsible for TG loss in 3T3-L1 adipocytes are the prostaglandins. However, the prostaglandins are probably less important in the animal as COX2 mRNA levels are low and not strongly induced, Most likely some other aspect of the inflammatory response plays an equivalent role in WAT in animals. One possible mechanism utilized by PGF2α's for its delipidation effects is that it results in phosphorylation of PPARγ [31] or inhibition of PPARγ expression [32]. Our data indicates an alternative to a stronger activator of AMPK in adipocytes might be to use an AMPK activator and a second compound that leads to higher prostanoid levels.

Phenformin causes an inflammatory response and TG loss, while little inflammation and TG loss are observed with metformin. This supports the premise that inflammation is needed for TG loss. The microarray analysis of 3T3-L1 adipocytes treated with t10c12 CLA or phenformin demonstrated similar changes in transcript profiles, suggesting the similar abilities of t10c12 CLA and phenformin to strongly activate AMPK and to cause similar reductions in TG levels were due to activation of similar pathways. These results suggest strong AMPK activation in adipocytes is sufficient for a robust delipidation response. This implies t10c12 CLA only needs to activate AMPK in adipocytes to account for the majority of its effects. However, phenformin appears to act at muscles in humans as it was reported to cause toxic lactic acidosis, which limited its application in humans, and explains why it does not cause significant fat loss in animals.

 Our further studies showed t10c12 CLA is selective in the cellular response. We found that t10c12 CLA strongly induced an ISR in 3T3-L1 adipocytes, but fibroblasts did not show this response. Our microarray analysis of liver tissues of mice treated with

t10c12 CLA showed a weak ISR, and lacked a significant delipidation or inflammatory response. Most drugs or nutritional supplements do not show strong tissue specificity and generate undesirable side effects in non-target tissues. This provides t10c12 CLA potential advantage as an adipocyte specific chemical for obesity therapeutics. To extend our knowledge of CLA's cell-type selectivity, a microarray study of other types of tissues in mice responding to t10c12 CLA is worth performing in the future. If the t10c12 CLA response is preferentially selective for adipocytes, it provides an excellent platform for adding additional less-selective nutritional molecules for reducing adiposity that collectively provide a more adipocyte specific effect, and improve CLA's advantage in clinical trials.

Our studies demonstrated cross regulation of AMPK and SIRT1 was occurring in t10c12 CLA treatment [Figure 1]. Activated AMPK affected the amount of SIRT1-related deacetylation of NF-κB in the molecular response to $t10c12$ CLA, possibly via changes in the fatty acid oxidation dependent $NAD^+/NADH$ ratio [151]. SIRT1 affected the amount of activated AMPK, as SIRT1 inhibitors attenuated AMPK activation at 12 h. SIRT1 inhibitors did not affect AMPK activation in the first 2 h of $t10c12$ CLA treatment, indicating early AMPK activation did not depend on SIRT1 activity. The combined data support the hypothesis that both AMPK and SIRT1 positively regulate each other in the response to $t10c12$ CLA-treated adipocytes. To our surprise, SIRT1 utilized mechanisms that both promoted and inhibited the inflammatory response in $t10c12$ CLA treatments. Our study showed that SIRT1 had a pro-inflammatory bias in the response to $t10c12$ CLA. It could be mediated through facilitating activation of AMPK as phenformin, a

strong AMPK activator, can cause an inflammatory and TG reduction response in adipocytes. The understanding on the detailed mechanism is still unclear.

Our chemical studies suggested that both AMPK activation and reduced PPARγ activity are important for reducing TG levels in the response to t10c12 CLA [Figure 1]. The key outcome is determined by whether AMPK and PPARγ becomes activated and inhibits the activity of the other. In opposition to AMPK activity, PPARγ is required for differentiation and maintenance of the adipocytes. Activation of PPARγ by thiazolidinediones (TZDs) strongly attenuates t10c12 CLA effects in adipocytes. PPARγ is also antagonistic NF-κB in t10c12 CLA-treated adipocytes and is considered anti-inflammatory. In contrast, PPARγ antagonist GW9662 and t10c12 CLA are synergistic for delipidation. This suggests an approach combining t10c12 CLA with nutritional supplements that further inhibit PPARγ in adipocytes is promising for reducing adiposity.

PPARδ plays a positive role in reducing TG content in t10c12 CLA treated adipocytes [Figure 1]. PPARδ facilitates TG loss in CLA treated adipocytes by increasing fatty acid oxidation. PPARδ had increased binding to AMPK in CLA treated adipocytes, subsequently affected AMPK activity. PPARδ reduces PPARγ activity by a second mechanism that appears to depend on the reduced amounts and activity of PPARγ caused by AMPK. This second mechanism appears to affect the competition for RXR, the required heterodimeric partner for PPARγ, PPARδ, LXR and RAR [175, 176, 178]. This suggested the low levels of PPARγ activity, caused by the presence of t10c12 CLA, can be further decreased by competition for RXR by other ligand-activated nuclear receptors. In conclusion, activated AMPK [112] and, activated PPARδ, inhibit PPARγ activity. This provides a promising platform for combinations of t10c12 CLA with less tissue-specific

drugs such as metformin [111] or PPARδ agonists to attain more effective reduction of adiposity.

Currently, CLA is used in much lower dosage in human studies than in animal studies [22-23]. Some side effects of CLA were reported in the presence of extreme reduction of fat content in fat tissues when CLA was used at high dosage, including lipodystrophy, insulin resistance, elevated oxidative stress, hyperinsulinemia and liver steatosis in mice. For enhancing the delipidation of CLA in human trials, it is preferable to have a lower dose of CLA. Our goal is to accomplish this by looking for mechanism-based synergistic effects of drugs or nutritional supplements. Therefore, the modest fat loss in the fat tissues would be attained at a lower dosage of CLA. Our studies discovered several mechanisms which play important roles in CLA effects and provide good targets for synergy. This provides us an excellent platform to figure out the important factors involved in CLA effects, and subsequently test if their agonists or antagonists are able to enhance CLA delipidation when CLA is used at the low dosage in human trials. Our studies also help figure out how these side effects occur in CLA treatment. This also helps reveal the nutritionally regulated sensors and regulatory networks that can be utilized to reduce adiposity.

One of the most novel aspects of our findings are that PPARγ and PPARδ appear to regulate AMPK activity. This finding contrasts with the vast majority of current publications that consider PPARγ and PPARδ to act as transcription factors, capable of both activation or repression. Very little has been published on the non-genomic effects of these two proteins. Our results demonstrate PPARγ and PPARδ can affect AMPK activity levels without apparent changes in transcription of this gene, as AMPK protein

levels do not change. Our demonstration that CLA affects the PPARγ/SIRT1 interaction and the PPARδ/AMPK interaction, together with the known ability of SIRT1 to interact LKB1, an upstream kinase of AMPK, provides a remarkably intimate view of this cast of metabolic regulators and their ability to cross regulate each other's behavior.

The question of how t10c12 CLA causes these changes in the SIRT1/AMPK/ PPARδ/ PPARγ axis remains unknown. AMPK is affected by a large diversity of stimuli, as is appropriate for its central role in cell energy homeostasis. This diversity of potential stimuli precludes a simple exploration of how AMPK is activated by t10c12 CLA. The whole area of how fatty acids signal to key regulatory proteins such as AMPK is poorly understood and this lack of knowledge makes deciphering the mechanisms used by t10c12 CLA more difficult. T10c12 CLA is known to preferentially accumulate in adipocytes in animals and humans. Our results indicate adipocytes are particularly sensitive to its effects for unknown reasons. The combination of preferential accumulation and increased sensitivity are ideal for a tissue specific effect. The challenge is to find other drugs or nutritional molecules that have similar profiles and are capable of causing synergistic effects in the key SIRT1/AMPK/ PPARδ/ PPARγ axis.
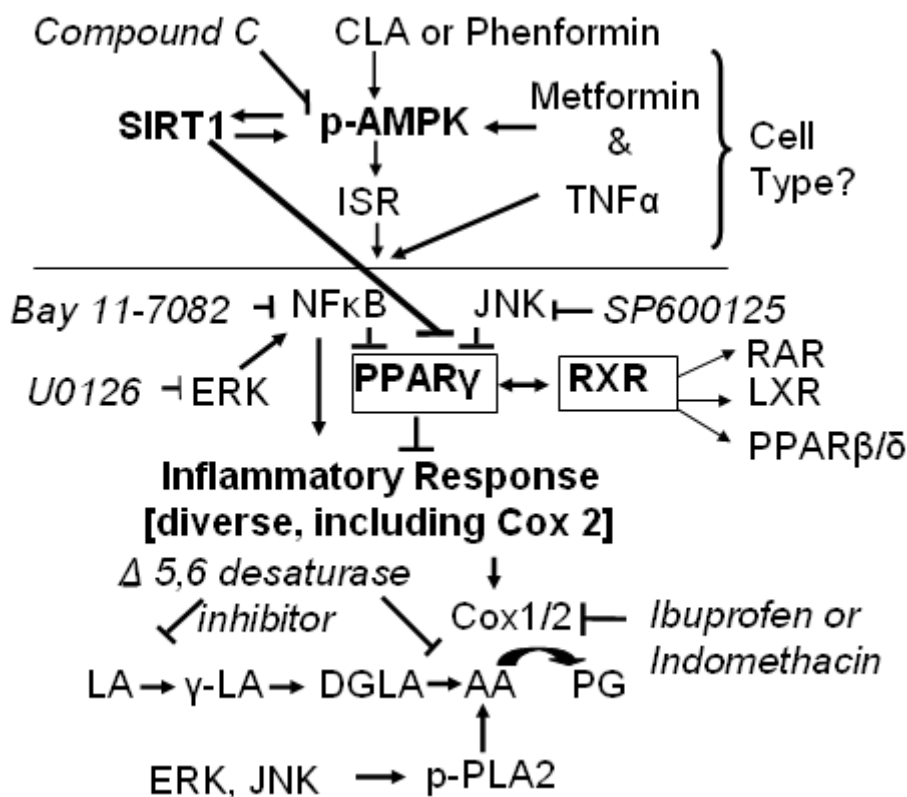
Figure 1. Working model for t10c12 CLA signaling. CLA or phenformin strongly activates AMPK to a phosphorylated state (p-AMPK), which initiates an integrated stress response (ISR): either step might be cell-type specific for t10c12 CLA. The ISR activates the NF-κB, JNK, and ERK axis, which together initiate the inflammatory response and delipidation. Metformin with TNFα produces a similar delipidation. AMPK, NF-κB, JNK, or ERK directly inhibit PPARγ, which normally inhibits inflammation. RAR, LXR or PPARβ/δ compete with PPARγ for RXR. Either activated AMPK or SIRT1 affects the other. Phospholipase A2 (PLA2) is activated by phosphorylation (p-PLA2) by either JNK or ERK, increasing the amounts of arachidonic acid (AA), the substrate for cyclo-oxygenase 1 or 2 (Cox1/2), for increased prostaglandins (PG), which are inhibitory to lipogenesis. The fatty acid precursors to AA are indicated as LA, γ-LA, and DGLA. Inhibitors we have been found to reduce the effects of t10c12 CLA are italicized.

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