

**What do we have to gain?
An obesity-related study into the effects of conjugated linoleic acid
and peroxisome proliferator-activated receptors on adipocyte function**

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Abstract

The aim of this project was to examine how the conjugated linoleic acid (CLA) isomers, *cis*9,*trans*11 (9,11-CLA) and *trans*10,*cis*12 (10,12-CLA), and three different peroxisome proliferator-activated receptor (PPAR) agonists (WY14643, rosiglitazone, GW501516) influence adipocyte function. SDS-PAGE and Western blotting procedures were employed to monitor the presence of differentiation markers (PPAR γ and fatty acid synthase), and adipokines (leptin and adiponectin) in 3T3-L1 adipocytes. Lipid vesicle formation was determined from photomicrographs, as well as oil-red-O staining. It was hypothesized that CLA does not affect lipid accumulation or adipocyte differentiation via PPAR activation, but CLA does work through PPARs to positively promote adipokine expression. The cellular lipid content of vesicles was increased by the PPAR γ agonist, but none of the other PPAR agonists, nor CLAs. While PPARs and CLAs alone stimulated expression of the differentiation markers (PPAR γ and fatty acid synthase, FAS), the PPAR agonist/CLA combinations decreased PPAR γ and FAS expression. Adiponectin expression was increased for cells treated individually with 9,11-CLA and all three PPARs. The combinations also generally increased adiponectin expression. GW501516, rosiglitazone, 9,11-CLA and 10,12-CLA individually, as well as the combinations increased leptin levels. These results, as a whole, suggest CLA influences adipocyte function independent of PPARs.

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List of Abbreviations

APS	Ammonium persulfate
CLA	Conjugated linoleic acid
BSA	Bovine serum albumin
DEX	Dexamethasone
DMEM	Dulbecco's modified eagle medium
eEF2	Eukaryotic elongation factor 2
FAS	Fatty acid synthase
FBS	Fetal bovine serum
GW	GW501516, PPAR δ agonist
MIX	3-isobutyl-1-methylxanthine
PAGE	Polyacrylamide gel electrophoresis
PPAR	Peroxisome proliferator-activated receptor
Ros	Rosiglitazone, PPAR γ agonist
SDS	Sodium dodecyl (lauryl) sulfate
TBST	Tris-buffered saline with tween
TEMED	N,N,N',N'-tetramethylethylenediamine
TZDs	Thiazolidinediones
WY	WY14643, PPAR α agonist
9,11-CLA	<i>cis</i> 9, <i>trans</i> 11-CLA
10,12-CLA	<i>trans</i> 10, <i>cis</i> 12-CLA

Introduction

Overview

This study is based on adipocytes (fat cells) and three of their specific functions: (1) differentiation of pre-adipocytes into mature adipocytes, (2) fat (lipid) accumulation and (3) hormone production. What was tested was the effect that peroxisome proliferator-activated receptors (PPARs) and conjugated linoleic acids (CLAs) have on these three specific adipocyte functions, in relation to obesity and type 2 diabetes research. 3T3-L1 adipocytes were therefore used as a model and treated with agonists to each of the three different PPARs (PPAR α , PPAR γ , PPAR δ) and two different CLA isomers (*cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA) in order to determine what effect these treatments have on the three adipocyte functions listed above. There were eleven treatments in total: each of the PPAR agonists and CLAs alone, as well as the six possible PPAR agonist/CLA combinations.

The reason for focusing on PPARs and CLA was that these two factors have been found to modify adipocyte function in relation to obesity and diabetes. Studies have shown that *trans*10,*cis*12-CLA (10,12-CLA) decreases adipocyte fat accumulation *in vivo*, however, it also leads to increased insulin resistance, which leads to type 2 diabetes (Brown *et al.* 2004; Taylor and Zahradka 2004). PPAR γ is important because it acts as a transcription factor that mediates adipocyte differentiation, the process that leads to production of new adipocytes (Chae and Kwak 2003). The purpose of this study was to give a general overview of the effects PPARs and CLA have on adipocyte function, to determine whether or not PPARs mediate the effects of CLA and to relate the results to obesity and/or type 2 diabetes.

Obesity/Diabetes/Insulin Resistance

Obesity is a disease that is very prevalent in our society today (Cowherd *et al.* 1999). It is now realized that the accumulation of a few extra pounds can lead to a number of fairly severe conditions including heart disease, increased blood pressure, cancer, inflammation and insulin resistance (Cowherd *et al.* 1999). Insulin resistance, which occurs when the body becomes resistant to the effects of insulin, is of particular concern because this phenomenon is a precursor to type 2 diabetes (Bergman and Mittelman 1998; Song *et al.* 2002).

Type 2 diabetes mellitus is growing so rapidly worldwide, it is estimated that the disease will affect approximately 250 million people by the year 2020 (Ravussin and Smith 2002). Diabetes generally results when glucose is not being taken up into cells and instead remains in the blood, resulting in hyperglycaemia. Hyperglycaemia occurs when tissues such as skeletal muscle and liver fail to respond to physiological doses of insulin and the pancreatic beta cells eventually fail to secrete adequate amounts of insulin in response to elevated plasma glucose (Ravussin and Smith 2002).

Adipose tissue (fat tissue) is one of the primary tissues involved with insulin resistance and diabetes. When fat is stored in locations other than adipose tissue such as the liver, skeletal muscle and pancreatic beta cells, minimal adipose tissue is developed and this leads to insulin resistance and diabetes (Erol 2005; Ravussin and Smith 2002). Storage of fat in ectopic locations is commonly referred to as lipotoxicity.

Adipocytes

Adipocytes play an important role in regulating energy homeostasis and are extremely sensitive to insulin (Erol 2005). They are able to draw excess glucose and fat from the

bloodstream, store them as triglycerides (fat) and then release stored fat to other body tissues as free fatty acids and glycerol when necessary (Erol 2005). In addition, an increase in fatty acid oxidation by adipocytes contributes to maintaining lipid homeostasis (Erol 2005).

Because adipocyte function provides the framework for this study, each of the three functions are described below.

Differentiation

An understanding of adipogenesis has been enhanced by the availability of cell culture models that undergo this process. In the case of the adipocytes used in this study, mouse 3T3-L1 adipocytes, the differentiation process begins with proliferation of pre-adipocytes (immature adipocytes) (Cowherd *et al.* 1999). Pre-adipocytes can be distinguished from mature adipocytes because they tend to look more like fibroblasts, rather than mature adipocytes (Ailhaud 1982). They are flat, elongated cells with extending processes and an oval nucleus (Camelliti *et al.* 2005). During pre-adipocyte proliferation, dexamethasone (DEX) and 3-isobutyl-1-methylxanthine (MIX) can be added to accelerate the differentiation process (Ailhaud 1982). These compounds are used *in vitro* to mimic absent hormonal cues that stimulate differentiation *in vivo*. Insulin is also required for differentiation since it promotes lipid formation and accumulation. This phase is referred to as the hormonal induction/clonal expansion phase (Cowherd *et al.* 1999).

Eventually, the cells reach confluence, which is the stage when proliferating adipocytes have grown to the maximum capacity within their growth space and growth starts to become inhibited due to surface-to-surface contact with other cells (Georgia Institute of Technology 2001). During this time, the adipocytes begin to change shape, becoming larger and more

rounded (Ailhaud 1982). The cells start to accumulate lipid droplets that are stored in their cytoplasm and the cells eventually reach the growth arrest/terminal differentiation stage (Cowherd *et al.* 1999). The adipocytes stop growing and are considered to be permanently differentiated mature adipocytes.

The two key markers of adipocyte differentiation that were studied in this project are the transcription factor, PPAR γ , and the enzyme, fatty acid synthase (FAS). PPAR γ promotes adipocyte differentiation and FAS is the enzyme present in mature adipocytes that catalyzes fatty acid synthesis (Erol 2005). These markers may be used as markers of differentiation because they are both present in mature adipocytes, but not pre-adipocytes.

Fat Accumulation

Once adipocytes mature, they become able to store fat as droplets that take up most of the cell. There is, however, a limit to how much fat can accumulate in each adipocyte. Once the cell has stored as much fat as it is able to, adipogenesis is triggered and more fat cells are produced (Erol 2005). It is in this way that excess lipid is delegated to smaller, younger and more capable adipocytes (Erol 2005). Lipid accumulation in adipocytes is important because it prevents ectopic lipid accumulation leading to lipotoxicity, and potentially hazardous effects on other non-adipocyte cells (Erol 2005).

Adipokine Production

Although adipose tissue largely functions to store fat, it has recently been discovered that this tissue plays a vital role as an endocrine organ as well (Havel 2004). Adipocytes secrete many hormones, which are commonly referred to as adipokines. Some of these

adipokines include leptin, adiponectin, angiotensinogen, plasminogen activator inhibitor-1, resistin and tumor necrosis factor- α . These hormones, many of which are regulated by insulin, have been found to act as regulators of energy homeostasis, glucose and lipid metabolism, the immune response, reproduction and vascular homeostasis (Arner 2003; Werner and Nickenig 2004). The two adipokines that were used as markers of adipokine production were leptin and adiponectin.

Leptin

Leptin is a 16kDa hormone that is found in adipose tissue, blood and cerebrospinal fluid (Rajala and Scherer 2003). Leptin binds to a receptor in the region of the hypothalamus that controls food intake (Rajala and Scherer 2003). In general, low levels of circulating leptin can lead to hunger, a lowered metabolic rate and weight gain, thus leading to obesity (Havel 2004). To combat this, leptin production increases in obese patients, which increases energy expenditure and decreases food intake by causing the patient to feel full (Gregoire *et al.* 1998; Rajala and Scherer 2003). Studies indicate that administering extra leptin to obese patients reduces appetite and that administering leptin to leptin-deficient rodent models has reversed insulin resistance (Havel 2004). This suggests that an increase in circulating leptin levels may serve to curb obesity.

The peripheral metabolic influences that leptin exerts over the liver, muscle and pancreas are also important for maintaining energy homeostasis (Erol 2005). Without the ability of leptin to stimulate the oxidation of peripheral fatty acids, lipid can accumulate in peripheral tissues, causing damaging effects to those tissues and leading to insulin resistance (Erol 2005).

Although leptin has many beneficial roles in the body, obese individuals with high leptin levels often become leptin insensitive at the receptor level (Werner and Nickenig 2004). A high plasma level of leptin is an independent predictor for cardiovascular morbidity and mortality and is often associated with insulin resistance (Werner and Nickenig 2004).

Adiponectin

Adiponectin (Adipocyte Complement-Related Protein) is a 30kDa adipose specific, insulin-stimulated, secreted protein that circulates in human serum (Erol 2005; Rajala and Scherer 2003). Elevated circulating adiponectin levels increase insulin sensitivity, and have also been found to decrease atherosclerosis (Havel 2004). Adiponectin appears to increase insulin sensitivity by improving glucose and lipid metabolism, as well as lowering circulating levels of glucose without stimulating insulin secretion (Arner 2003; Havel 2004).

In obese patients and patients with type 2 diabetes, adiponectin levels are low and the beneficial anti-atherosclerotic and insulin sensitizing effects are absent (Havel 2004). Because PPAR γ is able to stimulate adiponectin levels, persistently low adiponectin expression may be due to a mutation present that inactivates PPAR γ (Havel 2004; Rajala and Scherer 2003).

Peroxisome Proliferator-Activated Receptors (PPARs)

PPARs are members of the nuclear receptor superfamily that contain a signature type II zinc finger DNA binding motif and a hydrophobic ligand-binding pocket (Lee *et al.* 2003). There are three subtypes of PPARs, each found in distinct tissues and with varying biological roles (Lee *et al.* 2003). PPAR α is expressed mostly in the liver, heart, muscle and kidney where it regulates fatty acid catabolism (Lee *et al.* 2003). PPAR γ is found in adipocytes and

macrophages and is involved in lipid storage and glucose homeostasis (Lee *et al.* 2003). Along with these two functions, PPAR γ operates as a transcription factor essential for adipogenesis, as well as causing an increase in adipocyte adiponectin expression (Havel 2004, Chae and Kwak 2003). PPAR δ is expressed ubiquitously and its function is less well defined. It may aid in keratinocyte differentiation and wound healing and/or as a mediator of very low density lipoprotein signaling in the macrophage (Lee *et al.* 2003).

It is important to note that adipocytes in this study were not treated with actual PPARs, but with PPAR agonists. The PPAR agonists used to treat the adipocytes were WY14643 (WY) for PPAR α , rosiglitazone (Ros) for PPAR γ and GW501516 (GW) for PPAR δ . The role of PPAR agonists is to activate the PPARs in adipocytes and stimulate their gene transcription function.

Conjugated Linoleic Acid (CLA)

CLA is a group of positional and geometric fatty acid isomers that are derived from 18:2 linoleic acid (Evans *et al.* 2000). They are found naturally in ruminant meats, pasteurized cheeses and dairy products and have shown a variety of health benefits, including anticarcinogenic, antiatherogenic, antidiabetic, and antiobesity actions (Evans *et al.* 2000). The isomers focused on in most obesity related CLA studies include the *cis*9,*trans*11-CLA (9,11-CLA) and *trans*10,*cis*12-CLA isomers (10,12-CLA) (McLeod *et al.* 2004). These two isomers were used to treat adipocytes in this study.

The Importance of CLA and PPARs

One of the main reasons for investigating CLA with regard to obesity studies is that CLA has been shown to reduce body fat deposition and decrease atherosclerosis by modulating fatty acid oxidation and utilization, which results in lower serum lipids (Peters *et al.* 2001). PPARs are also important because, PPAR γ is able to act as a transcription factor in adipogenesis, as well as an activator of adipocyte determination and differentiation factor 1, which is another transcription factor that prevents the accumulation of excess lipid in non-adipocyte tissues (Chae and Kwak 2003; Erol 2005).

Much work is still being done to determine the role that PPARs and CLA play in adipocyte function and the mechanism by which they operate. Because CLA is a fatty acid, and PPARs are known to respond to fatty acid ligands, it is possible that CLA may even work through PPARs to affect adipocyte functions (Weldon *et al.* 2004). This project aims to discover the effects CLA and PPARs have on fat accumulation in adipocytes, the differentiation markers PPAR γ and FAS, and production of the adipokines leptin and adiponectin.

Hypothesis

I propose that Western blotting with antibodies to the differentiation markers, PPAR γ and FAS, will show that CLA works independently of PPARs in promoting adipocyte differentiation. I propose that oil-red-O staining of lipid droplets in adipocytes will show that CLA works independently of PPARs to affect fat accumulation in adipocytes. I also propose that Western blotting with antibodies to leptin and adiponectin will show that PPARs do mediate the beneficial actions of CLA on adipokine production.

Methods

Cell Line

The cells used in this project were mouse 3T3-L1 adipocytes. L1 is a continuous substrain of 3T3 (Swiss albino) developed through clonal isolation (Green and Kehinde 1975). Adipocytes were allowed to differentiate from pre-adipocytes to adipocytes according to an established protocol, which involved plating 3T3-L1 adipocyte cells onto 12 well plates (day 0) and incubating at 37 °C in a standard CO₂ incubator (Student *et al.* 1980). Two days later (day 2), the growth media was replaced with 2mL/well Dulbecco's Modified Eagle Medium (DMEM), 10⁻⁷M insulin, 0.5mM 3-isobutyl-1-methylxanthine (MIX) and 0.25µM dexamethasone (DEX). On day 4, the media was replaced with 2mL/well DMEM + 10% fetal bovine serum (FBS) and 10⁻⁷M insulin. Individual treatments were added either on day 2 with the MIX and DEX, or on day 4. If treatments were added on day 2, they were re-added on day 4 upon the switch from DMEM to DMEM + 10% FBS. After day 4, the plates were incubated for another 5 days, refreshing the DMEM + 10% FBS, insulin and treatments once on either day 6 or 7. The cells were then harvested on day 9 and the samples placed at -20° C until used.

It should be noted that on occasion, due to time restrictions, a heavier volume of cells was sometimes plated into the wells on day 0. This allowed the cells to be ready for treatment with MIX and DEX one day earlier. When this was the case, initial treatment days occurred on either day 1 or day 3, as opposed to day 2 or day 4.

Treatments

Unless indicated otherwise, the following final concentrations of various compounds were used individually and in combinations to treat adipocytes: 250µM WY14643 (WY,

PPAR α agonist), 10 μ M rosiglitazone (Ros, PPAR γ agonist), 10 μ M GW501516 (GW, PPAR δ agonist), 50 μ M *cis*9,*trans*11-CLA (9,11-CLA) and 50 μ M *trans*10,*cis*12-CLA (10,12-CLA).

Harvesting Adipocytes

Once the adipocytes were ready for harvesting, the media was removed and the wells were rinsed twice with phosphate-buffered saline. After rinsing, the solution was replaced with 200 μ L 2 \times sample buffer (20% glycerol, 2% SDS, 0.03125M Tris-HCl, pH 6.8) in each well. The plate was rotated on a shaker for approximately 5 minutes. Once the adipocytes lysed, the contents of each well were transferred to labeled sample tubes, sonicated for approximately 15 seconds and then frozen at -20° C until required.

Protein Assay

After harvesting, a Bicinchoninic Acid protein assay was used to determine the protein concentration of each sample. These values ensured that an equal amount (5-10mg protein) of each sample was analyzed during the Western blot procedure. Standards and samples were pipetted into the wells of a 96-well microtitre plate in triplicate. Each well received 10 μ L of either a bovine serum albumin (BSA) standard dilution, or 10 μ L of sample. The standard dilutions of BSA were 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0mg/mL, diluted with the appropriate amount of 2 \times sample buffer. Reagents A and B from a Pierce Bicinchoninic Acid protein assay kit were mixed in a 50:1 ratio and 200 μ L of that mixture was added to each well. The plate was then incubated at 37° C for 30 minutes. After incubation, the plate was allowed to cool for approximately 2 minutes and then the absorbance was measured with a Molecular Devices Thermomax microplate reader at 550nm.

SDS-PAGE and Transfer

The samples were initially subjected to sodium dodecyl (lauryl) sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Resolving gels of 7.5% or 15% acrylamide were prepared containing 0.1% SDS, 0.34M Tris-HCl buffer pH 8.8, 0.08% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.1% ammonium persulfate (APS). A 5% stacking gel (0.1% SDS, 0.13M Tris-HCl pH 6.8, 0.3% TEMED and 0.1% APS) was poured on top of the resolving gel. One microlitre 14.3M β -mercaptoethanol and 1 μ L 10% bromophenol blue were added to each sample, which were then heated, for 2 minutes in a microwave, by placing the tubes into a preheated container of water (95° C). Samples were loaded along with a BenchMark pre-stained protein ladder and the gels were run in 1 \times SDS-PAGE running buffer (5 \times buffer = 0.125M Tris base, 0.959M glycine, 1% SDS) for approximately 1 hour at 50mA.

Once the electrophoresis was completed, the protein was transferred to a polyvinylidene difluoride membrane. The gel and membrane were placed face to face with a sheet of white blotting paper on either side, and one green scrub pad on the outside of each piece of blotting paper. The entire “sandwich” was prepared in transfer buffer solution (20% methanol, 0.025M Tris base, 0.129M glycine) and then placed into the transfer apparatus along with the buffer and an ice pack. The transfer was conducted at 100V for 1 hour. When the transfer was complete the membrane was stored in 1 \times Tris-buffered saline with Tween (TBST) (5 \times buffer = 0.1M Tris-HCL, pH 7.4, 0.75M NaCl, 0.25% Tween 20) at 4° C.

Immunoblotting

The membrane was incubated in 10mL 3% BSA-TBST, on a rotator, for 1 hour. 10 microlitres of the desired primary antibody were then added (1:1000) and incubation continued for 1 hour. The primary antibody solution was then removed and the membrane was washed for a minimum of 20 minutes in 1×TBST, refreshing the TBST at least 4 times. The TBST was removed and 10mL 1% BSA-TBST was added to the membrane along with 1µL of the appropriate horseradish peroxidase-conjugated secondary antibody (1:10 000) for 1 hour. The membrane was then washed again in 1×TBST, for a minimum of 20 minutes, until everything was ready to develop.

In preparation for developing films, each membrane was dipped in ECL Plus Western Blotting Detection System solution, which provided the substrate for the horseradish peroxidase enzyme. The product of the reaction was luminescent and its intensity was captured by exposing the membrane to film. The membrane was placed between two acetate sheets, which were placed into the developing cassette. In the dark room, a sheet of Kodak scientific imaging film (X-OMAT LS) was placed on top of the membrane and exposed for an allotted period of time. The film was then placed into developer for approximately 30 seconds, rinsed off in water and then placed into the fixer. Once dry, the film was labeled and bands analyzed.

Antibodies

The primary antibodies used during immunoblotting and the respective molecular mass of the protein they detect include: 16kDa leptin, 30kDa adiponectin, 55kDa PPAR γ , 265kDa fatty acid synthase and 100kDa eukaryotic elongation factor 2 (eEF2).

Densitometry

Densitometry was performed on films showing bands of interest using a model GS800 imaging densitometer (Bio-Rad Laboratories, Hercules, CA). Using the background subtraction method, an area of absorbance of equal-size surrounding each band was subtracted from the absorbance of the band. Data was analyzed based on the adjusted volumes of each band.

Oil-Red-O Staining and Photography

To stain lipid droplets present in the adipocytes, the media was removed and wells were washed twice with 1×Tris-buffered saline. The bottom of each well was then covered with Streck tissue fixative and allowed to sit on the bench for 30 minutes. Wells were then washed twice with distilled water and oil-red-O stain was added for one hour. The stain was prepared by mixing 0.5% oil-red-O solution and distilled water (6:4); particulates were subsequently removed with a syringe filter. After 1 hour, the stain was poured off and the wells were washed twice with distilled water.

Pictures of the adipocytes were taken with an Olympus C-5050 zoom digital camera through an Olympus CK-2 inverted microscope, magnified 150 times.

Data Analysis

Films from the differentiation and adipokine Western blot procedures were produced and bands were analyzed using densitometry. In each case, the membrane was blotted with eEF2 to provide a control for loading variability. The changes in adipokine expression and differentiation marker expression, induced by PPAR agonists and CLA, were determined based

on the following calculations. Adjusted volume readings for each of the bands were obtained using densitometry. These values were then divided by the eEF2 control values to normalize for loading. The band/eEF2 ratios were then divided by the value for the untreated control in each experiment. This manipulation defined the fold increase in the levels of hormone or differentiation markers produced by each treatment compared to the control. The treatment/control ratios for the individual PPAR agonist and CLA treatments were plotted for each trial. In order to compare the combination effects to the individual PPAR agonists, the treatment/control ratios for the combinations were divided by the individual PPAR agonist treatment/control ratio and then plotted.

There are no error bars plotted on the bar graphs, as Figures 1 and 2 represent one trial and Figures 5-12 were plotted with each coloured bar representing an individual trial. Individual trials were plotted in order to observe the general trend. This experiment aimed to look specifically at whether treatments caused an increase or decrease in differentiation/adipokine marker expression and not the degree to which each of the markers increased or decreased.

Results

Adipocyte Differentiation

Adipocytes were treated on day 4 during the differentiation period, cell extracts were prepared at the end of the incubation period and subjected to gel electrophoresis. Proteins were then transferred from the gel to a membrane and probed with antibodies to the differentiation markers PPAR γ and FAS. Band intensities were subsequently quantified by densitometry and the values normalized to eEF2 as described in Methods.

PPAR γ

The alterations in PPAR γ expression due to PPAR agonist and CLA treatments are presented in Figure 1 and based on a single trial. PPAR γ expression in adipocytes seemed to increase by treatment with each of the PPAR agonists and CLA isomers alone. The increase was just under 2-fold. The combination of WY+9,11-CLA produced the largest increase in PPAR γ expression, showing an increase just over 2.5-fold. The rest of the PPAR agonist/CLA combinations had decreased expression compared to the PPAR agonists and CLAs alone, with the 10,12-CLA combinations showing PPAR γ expression even slightly lower than the control.

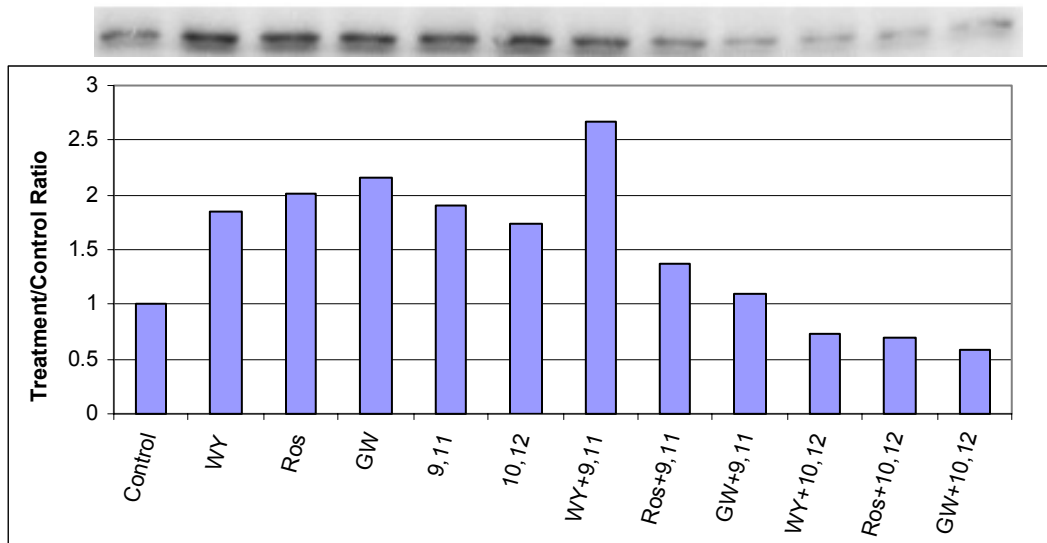


Figure 1. The treatment/control ratios showing PPAR γ expression in 3T3-L1 adipocytes. Cells were treated with various PPAR agonists, *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA on day 4. PPAR γ protein levels were determined by Western blotting (representation shown above graph), followed by densitometry. The specific treatments were 250 μ M WY14643 (WY, PPAR α agonist), 10 μ M rosiglitazone (Ros, PPAR γ agonist), 10 μ M GW501516 (GW, PPAR δ agonist), 50 μ M *cis*9,*trans*11-CLA (9,11) and 50 μ M *trans*10,*cis*12-CLA (10,12). The results for a single trial are shown.

Fatty Acid Synthase

As seen in Figure 2, the individual PPAR agonists and 10,12-CLA increased FAS expression, with WY producing the largest increase in FAS expression. In contrast, 9,11-CLA had an opposite effect to those observed with the other treatments. The 9,11-CLA isomer actually caused FAS expression to decrease compared to the control. More trials will have to be completed to determine if this is statistically significant.

All of the PPAR agonist/CLA combinations exhibited a decrease in FAS expression. The levels of expression were quite a bit lower than the control.

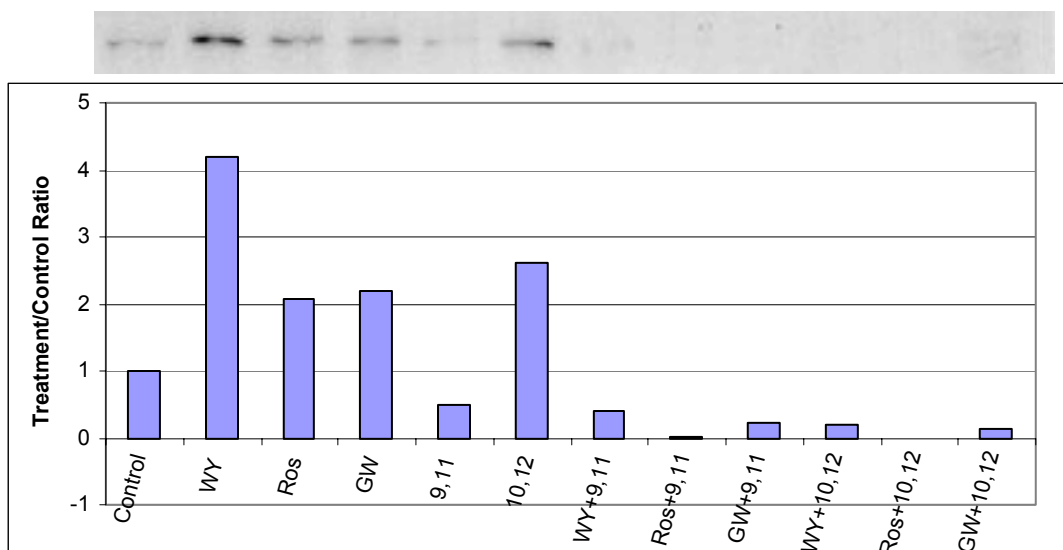


Figure 2. The treatment/control ratios showing fatty acid synthase (FAS) expression in 3T3-L1 adipocytes. Cells were treated with various PPAR agonists, *cis*9,*trans*11-CLA and *trans*10,*cis*12-CLA on day 4. FAS protein levels were determined by Western blotting (representation shown above graph), followed by densitometry. The specific treatments were 250μM WY14643 (WY, PPARα agonist), 10μM rosiglitazone (Ros, PPARγ agonist), 10μM GW501516 (GW, PPARδ agonist), 50μM *cis*9,*trans*11-CLA (9,11) and 50μM *trans*10,*cis*12-CLA (10,12). The results for a single trial are shown.

Lipid Accumulation

The degree of lipid accumulation was determined based on photomicrographs of the oil-red-O stained triglyceride (lipid) droplets found in the 3T3-L1 adipocytes. Adipocytes were treated with each of the PPAR agonists and CLA isomers alone, as well as the six PPAR agonist/CLA combinations. Preliminary data suggested that with the exception of Ros (PPARγ agonist) and the Ros combinations, none of the PPAR agonist or CLA treatments visually increased or decreased the number of adipocytes that accumulated lipid. Figure 3 shows a comparison of the 10,12-CLA treated well (3c) to the control well (3a). It can be seen that there is no increase in lipid accumulation in the 10,12-CLA treated cells.

Treatment of adipocytes with Ros alone and Ros in combination with both CLA isomers did substantially increase fat accumulation. A representative picture is shown in Figure 3, comparing a Ros treated well (3b) to the control well (3a). The larger red blotches on the bottom left area of Figure 3a are free floating dye droplets that have not been incorporated into adipocytes.

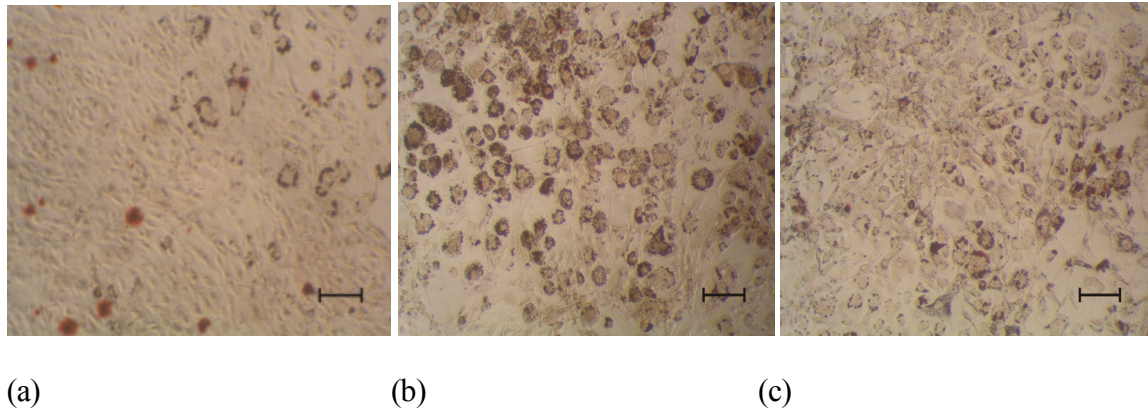


Figure 3. Staining of triglyceride-rich droplets in 3T3-L1 adipocytes with oil-red-O to observe lipid accumulation caused by PPAR agonists and CLA. Cells were allowed to differentiate according to the protocol described in Methods. Each panel is covered with pure adipocytes; those with red have accumulated lipid droplets in their cytoplasm. Cells were treated on day 4 with the following: (a) control; (b) 10µM rosiglitazone (PPAR γ agonist); (c) 50µM *trans*10,*cis*12-CLA. It is evident that more adipocytes in the rosiglitazone treated panel have accumulated lipid (red colour), than adipocytes in the control and *trans*10,*cis*12-CLA panels. Scale bars in (a), (b) and (c) = 100µm. (Individual adipocytes are approximately 50µm on average).

Cell Death

Two twelve well plates were seeded with 3T3-L1 adipocytes and subjected to the same treatments as stated above for lipid accumulation. The sole difference between these plates was that one plate was treated on day 2 after seeding and the other being treated on day 4. At the end of the treatment period, representative pictures were taken of each well and observations were noted. The adipocytes grew to confluence and accumulated lipid droplets in most of the wells to varying degrees. It was observed, however, that cells seemed to lift off the day two-

treated plate in all wells that had been subjected to the PPAR agonist and CLA isomer combinations. In fact, cells were lifting off the bottom of the plate as early as day 4 (two days after treatment). Representative photographs are shown in Figure 4. Adipocytes in Figure 4a and 4b, which were treated with WY (PPAR α agonist) and 9,11-CLA respectively, remained viable. This is indicated by the abundance of adipocytes that stayed attached to the plate, either with accumulated lipid as evidenced by the red colouring, or without accumulated lipid, producing a granular appearance in the photograph. In contrast, almost all adipocytes treated with the WY+9,11-CLA combination (panel c), lifted off the plate and therefore became unviable. This is evidenced in Figure 3c, which shows a photograph of the WY+9,11-CLA treated well that is completely clear of adipocytes.

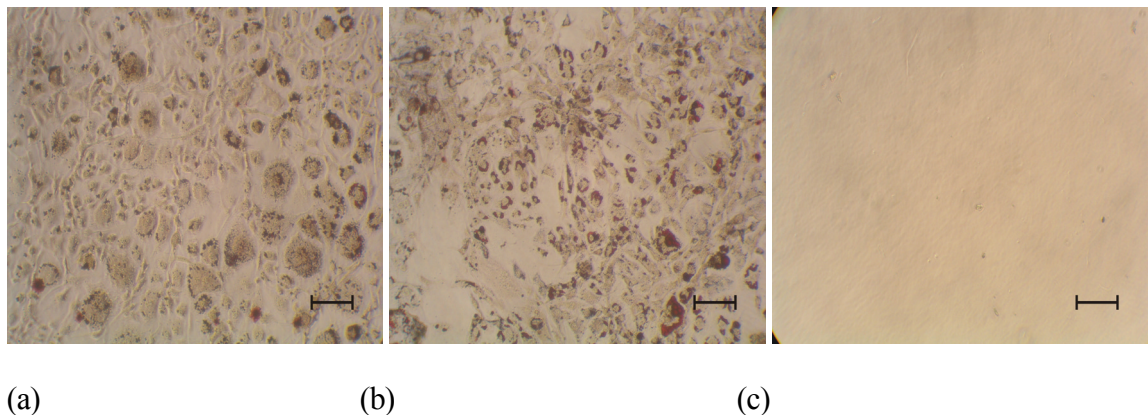


Figure 4. An assessment of 3T3-L1 adipocyte viability by phase contrast microscopy. The adipocytes were treated on day 2 with (a) 50μM WY14643 (PPAR α agonist); (b) 50μM *cis*9,*trans*11-CLA; (c) 50μM WY14643 + 50μM *cis*9,*trans*11-CLA, then stained with oil-red-O dye on day 10. Panels (a) and (b) are covered with adipocytes (shown by red colour and grainy appearance) and panel (c) lacks adipocytes. Scale bars in (a), (b) and (c) = 100μm. (Individual adipocytes are approximately 50μm on average).

Adipokine Production

Adipokine expression was examined by Western blot analysis as previously described for the markers of differentiation. In this case, the membranes were blotted with antibodies to adiponectin and leptin. Results from the films of various blots are presented below.

Adiponectin

Variation in adiponectin expression was measured based on adipocyte treatments with individual PPAR agonists and CLA isomers, as well as the six combinations of the two. Preliminary results examining changes in adiponectin levels due to the individual PPAR agonists and CLA isomers are shown in Figure 5, with each coloured bar representing a specific trial.

The results indicated that WY, Ros, GW and 9,11-CLA all caused a general increase in adiponectin expression. Ros seemed to have the largest effect on adiponectin expression based on both the degree of expression shown, as well as the number of trials where an increase was observed. A few trials showed extremely high levels of expression compared to the control, while treatment with 10,12-CLA was the only case where an individual agent did not produce much change in adiponectin expression.

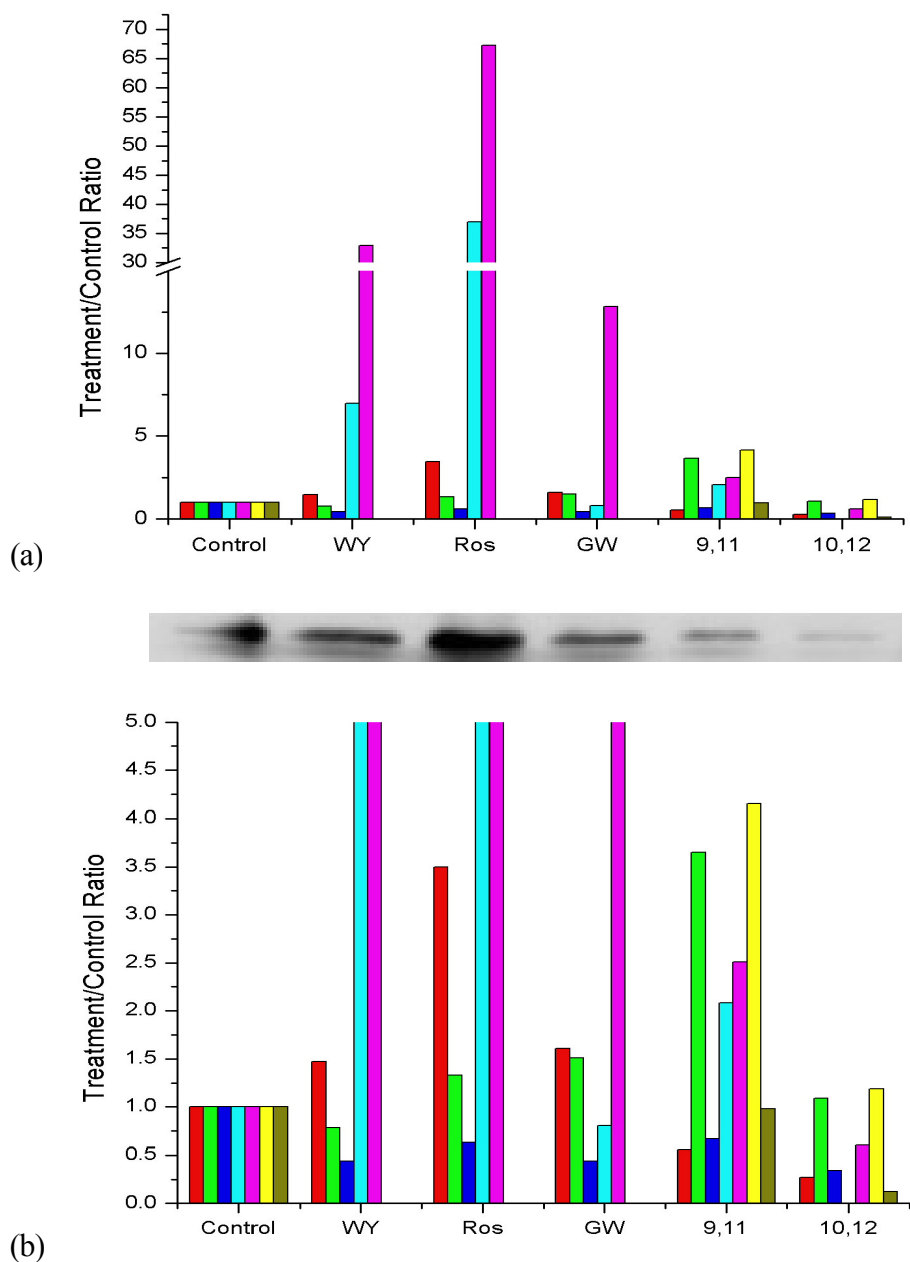


Figure 5. The treatment/control ratios for adiponectin expression in 3T3-L1 adipocytes plotted in (a) full view and (b) magnified view. Results for all trials are plotted, with each coloured bar representing adiponectin expression of an individual trial compared to its control. Cells were treated with various PPAR agonists and either *cis9,trans11* or *trans10,cis12*-CLA on day 4, and adiponectin protein levels determined by Western blotting (representation shown between the two graphs) and densitometry. The specific treatments were 250µM WY14643 (WY, PPAR α agonist), 10µM rosiglitazone (Ros, PPAR γ agonist), 10µM GW501516 (GW, PPAR δ agonist), 50µM *cis9,trans11*-CLA (9,11) and 50µM *trans10,cis12*-CLA (10,12).

In addition to monitoring the effect of individual PPAR agonists and CLA on adiponectin expression, the six combinations of the two were also tested. Results for the changes due to the PPAR agonist/CLA combinations, with respect to the individual PPAR agonist, are presented in Figure 6-8 below, in relation to the respective PPAR agonist.

Figure 6 shows the results for the combination of 9,11-CLA or 10,12-CLA with WY. As can be seen, the WY+9,11 and WY+10,12 combinations both showed relative increases in adiponectin expression compared to WY alone.

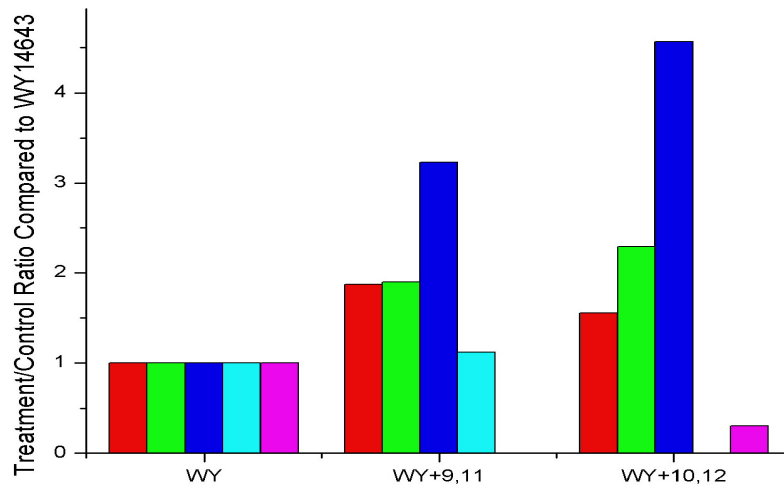


Figure 6. A comparison of the WY treatment/control ratios for adiponectin expression in 3T3-L1 adipocytes. Results for all trials are plotted, with each coloured bar representing adiponectin expression of an individual treatment/control ratio compared to WY (set to 1). Cells were treated with 250µM WY14643 (WY, PPAR α agonist) alone or in combination with either 50µM *cis*9,*trans*11-CLA (9,11) or 50µM *trans* 10,*cis*12-CLA (10,12) on day 4. Adiponectin protein levels were determined by Western blotting and densitometry.

A similar pattern was observed with Ros (PPAR γ agonist), as shown in Figure 7. Both PPAR agonist/CLA combinations showed a general increase in adiponectin expression compared to Ros alone.

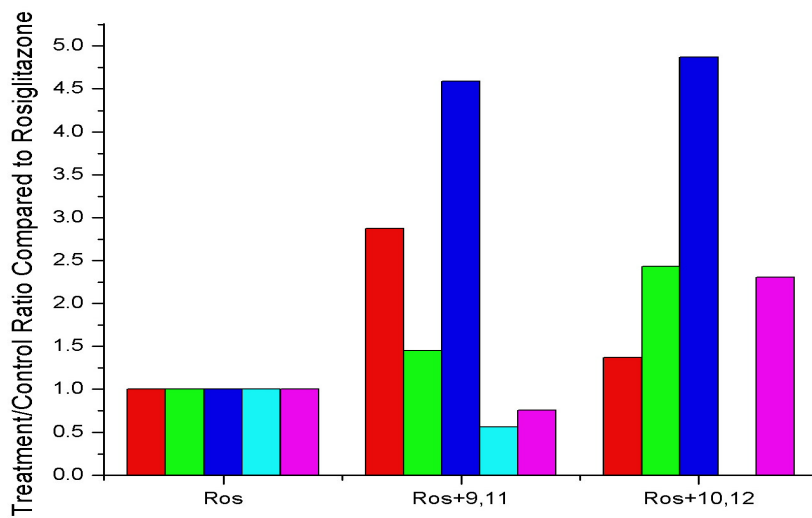


Figure 7. A comparison of the Ros treatment/control ratios for adiponectin expression in 3T3-L1 adipocytes. Results for all trials are plotted, with each coloured bar representing adiponectin expression of an individual treatment/control ratio compared to Ros (set to 1). Cells were treated with 10 μ M rosiglitazone (Ros, PPAR γ agonist) alone or in combination with either 50 μ M *cis*9,*trans*11-CLA (9,11) or 50 μ M *trans*10,*cis*12-CLA (10,12) on day 4. Adiponectin protein levels were determined by Western blotting and densitometry.

Interestingly, the effect of GW in combination with the CLA isomers produced an increase in adiponectin expression for the GW+9,11-CLA combination, but no change was observed with the GW+10,12-CLA treatment. There was one trial that produced a very intense band with GW+10,12-CLA treatment, but this could potentially be an outlier. The rest of the trials for that combination treatment did not change much from the control. These results are shown in Figure 8.

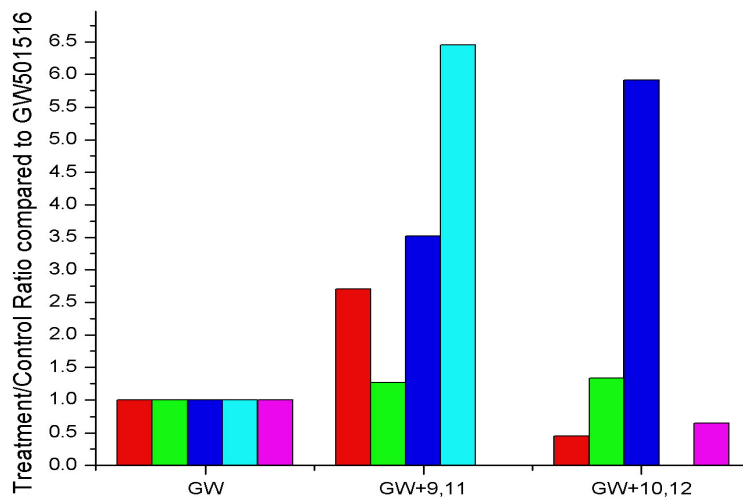


Figure 8. A comparison of the GW treatment/control ratios for adiponectin expression in 3T3-L1 adipocytes. Results for all trials are plotted, with each coloured bar representing adiponectin expression of an individual treatment/control ratio compared to GW (set to 1). Cells were treated with 10 μ M GW501516 (GW, PPAR δ agonist) alone or in combination with either 50 μ M *cis*9,*trans*11-CLA (9,11) or 50 μ M *trans*10,*cis*12-CLA (10,12) on day 4. Adiponectin protein levels were determined by Western blotting and densitometry.

Leptin

Leptin levels were also monitored in adipocytes treated with the PPAR agonists and CLA. Figure 9 shows the affect that individual PPAR agonists and CLA have on leptin levels. There seemed to be a general increase in leptin expression produced upon treating the adipocytes with Ros, GW, 9,11-CLA and 10,12-CLA. The same trend was not seen for WY treatment. WY did not generally appear to change leptin expression, with some trials increasing leptin expression and others decreasing it.

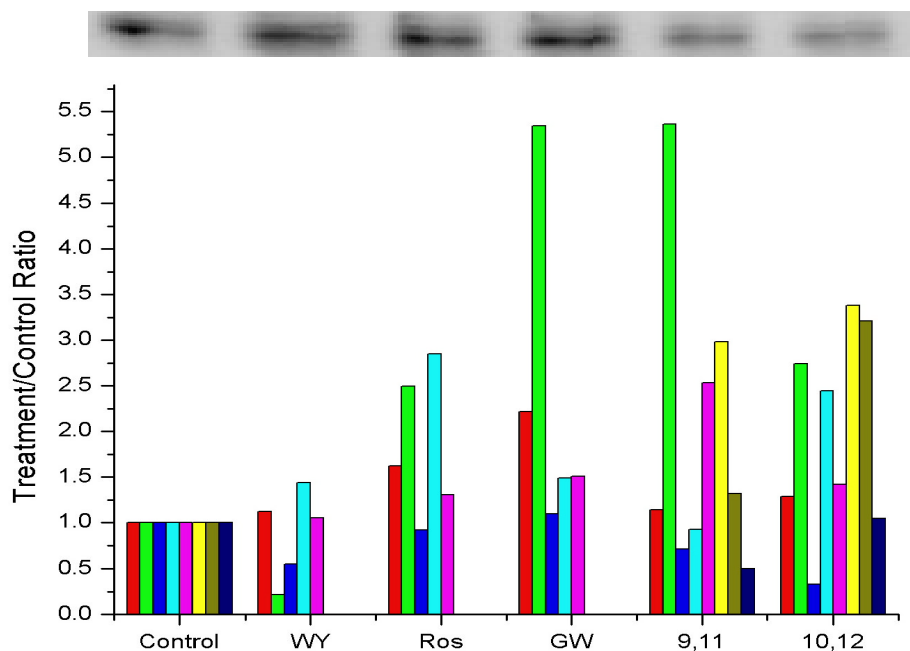


Figure 9. The treatment/control ratios for leptin expression in 3T3-L1 adipocytes. Results for all trials are plotted, with each coloured bar representing leptin expression of an individual trial compared to its control. Cells were treated with various PPAR agonists and either *cis9,trans11* or *trans10,cis12*-CLA on day 4, and leptin protein levels determined by Western blotting (representation shown above graph) and densitometry. The specific treatments were 250µM WY14643 (WY, PPAR α agonist), 10µM rosiglitazone (Ros, PPAR γ agonist), 10µM GW501516 (GW, PPAR δ agonist), 50µM *cis9,trans11*-CLA (9,11) and 50µM *trans10,cis12*-CLA (10,12).

As was done with adiponectin, leptin levels were examined in cells treated with combinations of PPAR agonists and CLA as well. Figure 10 shows the effect of combining CLA with WY. The WY+9,11-CLA combination did not increase leptin expression much compared to WY. Most of the trials gave approximately equal expression to WY. Only one trial showed a distinctively high level of leptin expression. This could be an outlier. The WY+10,12-CLA combination, on the other hand, did show a general increase in leptin expression. All of the trials increased leptin expression compared to the control.

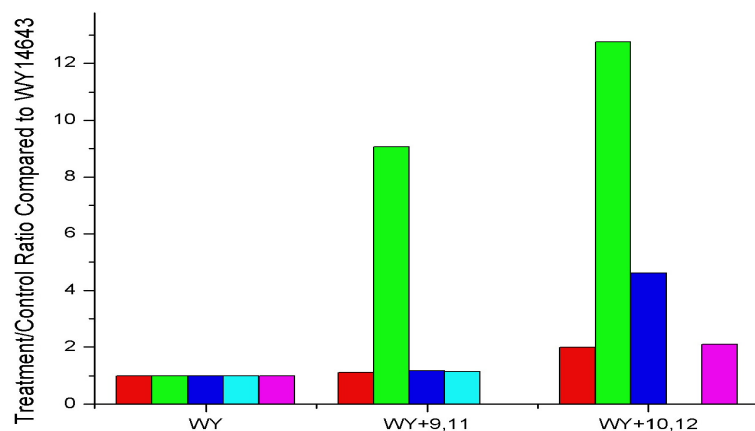


Figure 10. A comparison of the WY treatment/control ratios for leptin expression in 3T3-L1 adipocytes. Results for all trials are plotted, with each coloured bar representing leptin expression of an individual treatment/control ratio compared to WY (set to 1). Cells were treated with 250 μ M WY14643 (WY, PPAR α agonist) alone or in combination with either 50 μ M *cis*9,*trans*11-CLA (9,11) or 50 μ M *trans*10,*cis*12-CLA (10,12) on day 4. Leptin protein levels were determined by Western blotting and densitometry.

The combination of Ros plus each of the CLA isomers produced an increase in leptin levels. Three out of four trials for each combination showed an increase compared to Ros alone (Figure 11).

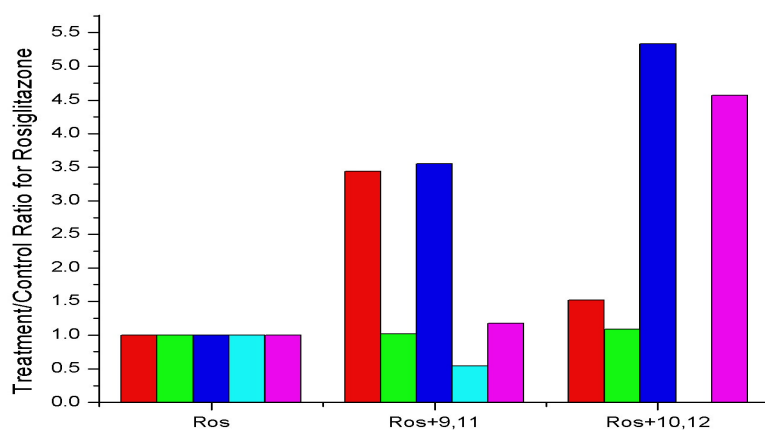


Figure 11. A comparison of the Ros treatment/control ratios for leptin expression in 3T3-L1 adipocytes. Results for all trials are plotted, with each coloured bar representing leptin expression of an individual treatment/control ratio compared to Ros (set to 1). Cells were treated with 10 μ M rosiglitazone (Ros, PPAR γ agonist) alone or in combination with either 50 μ M *cis*9,*trans*11-CLA (9,11) or 50 μ M *trans*10,*cis*12-CLA (10,12) on day 4. Leptin protein levels were determined by Western blotting and densitometry.

Finally, leptin expression was also tested with the combination of GW+9,11-CLA or 10,12-CLA. The results are shown in Figure 12. There did not appear to be much change in leptin levels in the presence of 9,11-CLA compared to GW alone. There was, however, an increase seen with the GW+10,12-CLA combination.

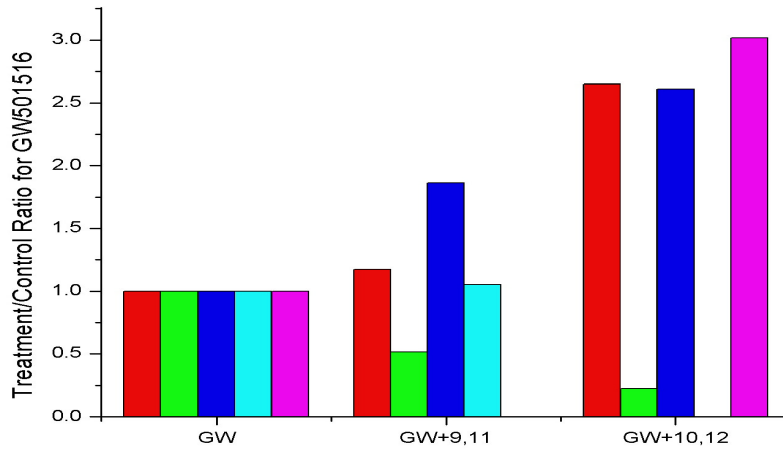


Figure 12. A comparison of the GW treatment/control ratios for leptin expression in 3T3-L1 adipocytes. Results for all trials are plotted, with each coloured bar representing leptin expression of an individual treatment/control ratio compared to GW (set to 1). Cells were treated with 10 μ M GW501516 (GW, PPAR δ agonist) alone or in combination with either 50 μ M *cis*9,*trans*11-CLA (9,11) or 50 μ M *trans*10,*cis*12-CLA (10,12) on day 4. Leptin protein levels were determined by Western blotting and densitometry.

Discussion

Adipocyte Differentiation

This section of the project aimed to determine what effect PPAR agonists and CLA might play in affecting the expression of PPAR γ and FAS and how this might apply to adipocyte differentiation.

PPAR γ

In general, treatment with all three PPAR agonists and both 9,11- and 10,12-CLA isomers alone caused an increase in PPAR γ expression to relatively the same level. These results suggest that in the presence of PPAR agonists and CLA, new adipocyte differentiation is likely occurring. This would produce more adipocytes and might explain the increased fat accumulation effects seen in this study by Ros, the PPAR γ agonist. By accumulating fat in adipocytes, lipotoxicity may be prevented, which is caused by harmful accumulation of fat in other organs of the body, such as the kidney and liver.

In general, the increase in differentiation caused by the PPAR agonists and CLA might be beneficial if there is excess glucose in the blood. The glucose could be converted to fat and then stored in the increasing number of new adipocytes being produced. This would decrease the plasma glucose and potentially help to prevent impending insulin resistance. The fact that an increase in fat accumulation was not seen alongside the increase in PPAR γ expression for treatments other than Ros, seems to negate the hypothesis that increased adipocyte differentiation produces more adipocytes to accumulate fat and prevent lipotoxicity. Also, since CLA did not show an increase in fat accumulation along with increased differentiation, as

was seen for the PPAR γ agonist, this suggests CLA works independently of PPAR γ . More studies and trials will need to be completed to verify these results and hypotheses.

Currently, thiazolidinediones (TZDs), which are a group of PPAR γ agonists, are being used as a treatment for type 2 diabetes. TZDs decrease insulin resistance, even though they do not decrease the degree of obesity usually found in the patient population (Arner 2003). This indicates that new adipocytes are being formed and fat is accumulating alongside the improvements in insulin sensitivity. The effects of TZDs have been to decrease hyperglycaemia by directly reducing insulin resistance, which leads to increased glucose uptake into skeletal muscle and adipose tissue (Arner 2003). Based on these reports, it is obvious that stimulation of PPAR γ with an agonist is beneficial.

PPAR agonist/CLA combinations

The PPAR agonist/CLA combinations, with the exception of WY+9,11-CLA, seemed to decrease PPAR γ expression in relation to the PPAR agonists and CLA alone. Although the WY+9,11-CLA combination actually showed the highest level of PPAR γ expression, over and above the individual PPAR agonists and CLA isomers, it is difficult to say whether this result is significant because only one trial was completed.

The rest of the PPAR agonist/CLA combinations decreased the level of PPAR γ expression. This is very interesting because on their own, each of the PPARs and CLAs induced PPAR γ expression, but together there appeared to be a dampening effect. Perhaps CLA alone stimulated factors that promoted PPAR γ expression, which would explain the elevation in PPAR γ expression seen by CLA treatment, but then also acted as an antagonist that prevented PPAR activation when agonists, such as Ros, and CLA were added in combination.

Consequently, addition of CLA, together with a PPAR agonist would prevent adipocyte differentiation, producing fewer adipocytes and thereby decreasing fat accumulation. In the more general picture, this may not be a desirable effect because if the PPAR agonist/CLA combinations were preventing adipocyte differentiation, the excess lipid might not be stored in adipocytes and could accumulate instead in other organs. This lipotoxicity is undesirable because it can lead to insulin resistance and diabetes (Ravussin and Smith 2002). It is nevertheless very interesting that in combination these compounds had a very different effect on adipocytes than when they were added individually. Understanding this process may provide considerable insight into how these agents work. As such, however, their distinct actions support the hypothesis that CLA does not produce its effects through stimulation of PPARs.

Fatty Acid Synthase

Fatty acid synthase (FAS) is another marker of adipocyte differentiation. The pattern that the PPAR agonists and CLA alone produced for FAS was somewhat similar to that of PPAR γ . The fact that the individual treatments with each of the PPAR agonists and CLAs increased FAS expression indicated again that adipocytes differentiated and should have been accumulating lipid. As for PPAR γ expression, the PPAR agonist/CLA combinations also showed a significant decrease in FAS expression.

The two conditions to note especially were WY and 9,11-CLA. WY showed an exceptionally large increase in FAS expression, indicating an increased production of new adipocytes. As stated above, an increase in the number of adipocyte cells could be beneficial for preventing lipotoxicity.

The 9,11-CLA isomer caused the only contrasting result, if the FAS expression results were being compared to the PPAR γ expression results. Fatty acid synthase expression was decreased compared to the control when treated with 9,11-CLA, as opposed to the increase it caused in PPAR γ expression. Perhaps 9,11-CLA still allowed some differentiation of adipocytes (as evidenced by increased PPAR γ expression), but interfered with production or functioning of the FAS enzyme. This could have affected lipid accumulation and is more congruent with the lack of fat accumulation seen in 9,11-CLA treated cells.

PPAR agonist/CLA combinations

If 9,11-CLA truly does decrease FAS expression, as Evans *et al.* 2000 suggest, perhaps 9,11-CLA was somehow inhibiting the PPAR agonists when the two were applied in combination. The 9,11-CLA+PPAR combinations showed decreased FAS expression. Perhaps the PPAR agonists, which stimulated PPAR activation and induced differentiation, were inhibited by the addition of 9,11-CLA. If either the PPAR agonist itself, or the PPAR activation step was somehow inhibited, this might be an explanation for the decrease in the FAS expression seen by the combinations. CLA may be exhibiting antagonistic behavior. This still does not explain why unlike 9,11-CLA, 10,12-CLA promoted increased FAS expression alone, but like 9,11-CLA, in combination with PPARs, seemed to inhibit differentiation. These conflicting results provide evidence that the regulation of PPAR γ and FAS expression, by CLA and PPARs, occurs via different routes.

In general, PPAR agonists and CLA stimulated differentiation markers on their own, but the CLA/PPAR agonist combinations decreased expression of the differentiation markers. Although it cannot be concluded that CLA is not working through PPARs based solely on this

set of experiments, when the results for fat accumulation and adipokine production are taken into consideration, these divergent results suggest that CLA operates by a different mechanism than the PPAR agonists.

Lipid Accumulation

The hypothesis for this thesis was based on unpublished results from Hung (2004), which indicated that PPAR γ agonists promoted lipid droplet accumulation above controls, while 10,12-CLA had no effect on this process. This data indicates that CLA does not operate by stimulating PPARs.

While the stimulation of PPARs, especially PPAR γ , is closely linked to adipocyte maturation and therefore increased capacity to store lipid, the literature is conflicting regarding how CLA affects fat accumulation. Evans *et al.* (2000), as well as Mashek and Grummer (2004), indicate that 10,12-CLA seems to decrease fat accumulation (fewer adipocytes accumulated fat). This conflicts with the results described by Hung (2004).

My results showed that most of the PPAR agonists and CLA did not affect fat accumulation substantially. Treatment with Ros alone and in combinations, were the only treatments that caused a visual increase in the amount of lipid present in the cell population. Because CLA did not increase the amount of accumulated fat, but the PPAR γ agonist did, CLA apparently does not operate by activating one or more of the PPAR agonists. If CLA was working through PPARs, CLA should have stimulated PPAR γ , just as Ros did, to increase fat accumulation.

Cell Death

One of the most interesting results from this project was the observation that the adipocytes treated with PPAR agonist/CLA combinations, on day 2, caused the cells to lift off the plate. The evidence indicated that these treatments seemed to have perturbed the cells in such a way that cell death occurred. How exactly the combinations caused the cells to lift off the plates is unknown and could be the beginning of a new research direction.

Interestingly, a similar phenomenon was described by Evans *et al.* (2000). Their research group obtained results that seem to suggest that 10,12-CLA attenuates pre-adipocyte proliferation. This is similar to the results observed in this study for the combination treatments because if adipocytes were lifting off the plate and dying, there would be fewer adipocytes available to differentiate. This does not, however, explain why treatment of adipocytes with 10,12-CLA alone did not cause similar cell death. The study by Evans *et al.* (2000) also does not give any indication why, if it is 10,12-CLA that attenuates differentiation, did the 9,11-CLA/PPAR agonist combinations cause cell death along with the 10,12-CLA/PPAR agonist combinations.

Although there is no data to support this claim, cell death may serve as the mechanism whereby 10,12-CLA exerts antiobesity effects in mice (Evans *et al.* 2000). The presence of fewer adipocytes to collect fat could certainly decrease the amount of fat accumulation. This hypothesis would only work if an excess of lipid was not present. If there was still a lot of lipid in the body, lipotoxicity due to the accumulation of lipid in non-adipose tissue could result.

Because there were again differences seen in cell death between the PPAR agonist/CLA combinations and individual treatments, this further supports the premise that CLA and PPARs operate through distinct mechanisms.

Adipokine Production

The goal for this section of the study was to determine what effect PPAR agonists and CLA have on adiponectin and leptin expression in 3T3-L1 adipocytes.

Adiponectin

The adiponectin results indicate that all of the PPAR agonists, as well as *cis9,trans11*-CLA (9,11-CLA) caused an increase in adiponectin expression. These results, especially for Ros, are supported by literature reports (Rajala and Scherer 2003). The elevated adiponectin level due to Ros seems to also be indirectly supported by studies presented in the literature, which show that an inactivation of PPAR γ leads to a decrease in adiponectin expression (Havel 2004). The conclusion logically follows that if a lack of PPAR γ activation decreases adiponectin expression, then an increase in PPAR γ activation should increase adiponectin expression. Since adiponectin leads to increased insulin sensitivity, treatment with WY and Ros may be very beneficial. Putz *et al.* (2004) also found that adiponectin might reduce intrahepatic and muscle triglyceride content, thereby helping to prevent lipotoxicity. The mechanism suggested for this reduction in lipid is an increase in muscle fat oxidation and activation of genes important for fatty acid transport and oxidation (Putz *et al.* 2004).

There has also been literature support for dietary CLA causing an increase in adiponectin levels in rats, leading to beneficial results such as alleviation of hypertension and insulin resistance (Inoue *et al.* 2004; Nagao *et al.* 2003).

PPAR agonist/CLA Combinations

In comparing each of the PPAR agonist/CLA combinations to WY, Ros or GW alone, it is evident that CLA must have produced some effects with the PPAR agonists in order to increase the expression of adiponectin. All of the PPAR agonist/CLA combinations, except GW+10,12-CLA increased adiponectin expression compared to the PPAR agonist alone.

It would be interesting to further investigate the mechanism that allows the PPAR agonist/CLA combinations to cause even more of an increase in adiponectin expression compared to the individual PPAR agonists. It was seen that both WY and 9,11-CLA stimulated adiponectin expression compared to the control. The even greater increase caused by the combination could be due to additive effects produced by the WY (PPAR α agonist) and CLA together. This additive hypothesis neither supports nor rejects the original hypothesis that CLA works through PPARs to affect adipokine levels. CLA and PPAR agonists could both be additively stimulating expression of the PPAR to increase adiponectin levels, but the two could also be working additively through separate pathways to induce adiponectin expression.

While the additive effect was seen with WY and 9,11-CLA, this may not have been the case with the PPAR agonist+10,12-CLA combinations. The difference in these results suggests that CLA works independently of PPARs. While WY stimulated adiponectin, 10,12-CLA did not cause an increase on its own; and yet the combination of the two increased adiponectin expression above that of the PPAR agonist. There must be more happening here than just additive effects. CLA may have activated some other pathway or intermediate that may also increase adiponectin, and this putative pathway must be independent of the PPARs.

In summary, it appears that all of the combination treatments, except one, increased adiponectin expression compared to the PPAR agonist alone. The individual PPAR agonists and 9,11-CLA also stimulated adiponectin. These treatments, therefore, may be very beneficial for decreasing insulin resistance. Because 10,12-CLA did not seem to change adiponectin expression, this could be an indication that CLA does not mediate beneficial antiobesity and antidiabetic effects through the adipokine adiponectin.

Leptin

As was seen with adiponectin, treatment with each of the individual PPAR agonists and CLA isomers seemed to increase leptin expression. The only treatment that did not really change leptin expression was WY alone. The amount of leptin produced by the cells stayed approximately the same after treatment with WY as it did with the control. These data imply that PPAR α has no role in controlling leptin expression. With regard to CLA, there is some literature that supports the increase in leptin levels produced by 10,12-CLA (Brown *et al.* 2004). Unfortunately, this report shed no light on how to interpret my results and identify a suitable mechanism to explain these results.

PPAR agonist/CLA Combinations

Results for the effects of PPAR agonist/CLA combinations on leptin were very similar to those seen with adiponectin expression. Almost all of the combinations increased leptin expression, except for the WY+9,11-CLA treatment and the GW+9,11-CLA treatment.

It is interesting to note that WY did not show an increase in leptin expression compared to the control on its own, but 10,12-CLA did. The fact that the WY+10,12-CLA combination

stimulated leptin expression relative to WY alone, indicates that 10,12-CLA was able to work in combination with PPAR agonists to lift leptin levels.

In contrast, a closer look at the WY+9,11-CLA combination reveals some interesting results. Just like with WY and 10, 12-CLA, WY on its own did not show an increase in leptin expression compared to the control, but the CLA, 9,11-CLA, did. The observation that the combination of the two did not increase expression compared to WY, seems to indicate then that 9,11-CLA, in combination with WY, becomes unable to stimulate leptin expression. This is surprising since 9,11 stimulates leptin on its own. The fact that one combination increased leptin levels, while the other combination did not, seems to indicate that CLA and PPARs work through different mechanisms.

The Ros combinations and the GW+10,12-CLA combination, again, left the door open for the possibility that PPAR agonist/CLA combinations cause an increase in leptin due to an additive effect. Both the PPAR agonists and the CLA isomers increased leptin levels on their own, compared to the control, and then together, the combinations increased leptin even more than the PPAR agonists alone.

The hypothesis that CLA may work through PPARs to positively effect adipokines cannot be fully determined based on these results. At the same time, it is not possible to refute the hypothesis either. The fact that the CLA isomers were able to increase leptin levels on their own may be an indication that CLA is able to activate a PPAR, which would then cause the observed increase in leptin expression. However, there could be other mechanisms involved. Since the combinations seemed to provide the variable results discussed above, this could be an indication that CLA and PPARs work through separate pathways.

Experimental Variability

While up to five trials were conducted to test the effects of PPAR agonists and CLA isomers on adipokine levels, it was difficult to statistically assess the data due to the nature of the methods. Indeed, although the exact same procedures and conditions were employed for each experiment, there was still inherent variability within the procedure, which means more trials will be needed for a better quantitative analysis. The solutions were one point of variability in the system. New batches of solutions were made when old ones ran out and even slight changes in their composition (error in preparation, new lots of chemicals) could have affected binding of the blocking solution or antibodies during the Western blot procedure. Similarly, new lots of antibodies were purchased during the course of this work. Since most antibodies were polyclonal, a different lot meant it came from a different animal. It is therefore very likely each lot exhibited different characteristics that would affect the eventual result. However, the most likely source of variation was the immunoblotting portion of the procedure. It is necessary to produce an image on film to detect the location of antibody binding, and the clarity of the bands on the final film can depend on many things: loading the samples onto the gel, transferring the proteins to the membrane, the Western blotting process, application of the ECL plus and film exposure time. Some films turned out very nicely, with dark, clear bands, while other turned out less defined and very pale. All of these factors are very difficult to control and replicate exactly, therefore, in order to explicitly state specific trends within the results, more trials will have to be run. Consequently, the analysis used for this set of experiments was to compare the pattern seen in each trial and not the raw numbers. Since all the conditions were relatively constant, a comparison between treatments within each

experiment was possible. Interestingly, a statistical analysis is being developed to identify quantifiable differences in patterns even with the degree of variability seen herein.

Summary and Further Study

What this project has accomplished is a preliminary look into the effects of CLA and PPAR agonists on lipid accumulation, adipocyte differentiation and adipokine production. Looking at the results as a whole, it appears that CLA did not work through PPARs to affect any of the discussed adipocyte functions. This conclusion is based primarily on the fact that the CLA isomers consistently did not affect the measured processes the same way as the PPAR agonists. From here it will be very interesting to do more trials to confirm results and then to look into mechanisms that might be causing the changes. Follow-up research could look into one of the adipocyte functions more closely and investigate other markers of differentiation or adipokines. It would also be interesting to determine the mechanism whereby PPAR agonist/CLA combinations attenuate adipocyte growth on day 2. It is unclear why the combination treatments killed the adipocytes. Finally, it might be worth examining the possibility that the CLA isomers are actually functioning as PPAR antagonists, as well as looking at combinations of PPAR agonists to see if a similarity with CLA can be seen.

Conclusions

1. The combination of PPAR agonists and CLA together decrease expression of FAS and PPAR γ , while individual PPAR agonists and CLA isomers stimulate expression of these differentiation markers.
2. The PPAR γ agonist, rosiglitazone, stimulates fat accumulation alone and in combination, while CLA does not affect fat accumulation.
3. Rosiglitazone (PPAR γ agonist), GW501516 (PPAR δ agonist), WY14643 (PPAR α agonist) and *cis* 9,*trans* 11-CLA stimulate adiponectin expression.
4. All the PPAR agonist/CLA combinations stimulate adiponectin expression compared to the individual PPAR agonists, except GW+10,12.
5. Leptin expression is increased in adipocytes treated with rosiglitazone, GW501516, c9,t11-CLA and t10,c12-CLA.
6. All the PPAR agonist/CLA combinations stimulate leptin expression compared to the individual PPAR agonists, except WY+9,11 and GW+9,11.
7. Thus, CLA does not work through PPARs to affect fat accumulation, adipocyte differentiation or adipokine production.

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