

The *trans*-10, *cis*-12 isomer of conjugated linoleic acid decreases adiponectin assembly by PPAR γ -dependent and PPAR γ -independent mechanisms

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Abstract The adipocyte-derived secretory protein adiponectin functions as an insulin-sensitizing agent. In plasma, adiponectin exists as low, medium, and high molecular weight oligomers. Treatment with *trans*-10, *cis*-12 conjugated linoleic acid (*t*-10, *c*-12 CLA) reduces levels of adiponectin as well as triglyceride (TG) in mice and adipocyte cell culture models. The aim of this study was to determine whether the effects of *t*-10, *c*-12 CLA on adiponectin and TG are mediated through modulation of the transcription factor peroxisome proliferator-activated receptor γ (PPAR γ). 3T3-L1 cells were treated either during or after differentiation into adipocytes with 100 μ M *t*-10, *c*-12 CLA with or without 10 μ M troglitazone, a PPAR γ agonist, or 1 μ M GW9662, a PPAR γ antagonist, and adiponectin and TG levels were analyzed. Treatment with *t*-10, *c*-12 CLA reduced TG as well as cellular and secreted adiponectin levels and impaired the assembly of adiponectin oligomers. These changes were accompanied by decreases in PPAR γ mass. Troglitazone was able to reverse the *t*-10, *c*-12 CLA-mediated decrease in TG levels and restore the assembly of adiponectin oligomers but was unable to restore adiponectin synthesis. Conversely, treatment with GW9662 decreased TG mass and impaired adiponectin oligomer assembly but did not decrease total adiponectin mass. In a reporter assay, *t*-10, *c*-12 CLA appeared to be a partial PPAR γ agonist and prevented the stimulation of reporter activity by troglitazone. **Therefore, the *t*-10, *c*-12 CLA isomer appears to alter adipocyte adiponectin metabolism through PPAR γ -dependent and PPAR γ -independent mechanisms.**—Miller, J. R., P. Siripurkpong, J. Hawes, A. Majdalawieh, H-S. Ro, and R. S. McLeod. **The *trans*-10, *cis*-12 isomer of conjugated linoleic acid decreases adiponectin assembly by PPAR γ -dependent and PPAR γ -independent mechanisms.** *J. Lipid Res.* 2008. 49: 550–562.

Supplementary key words mouse • 3T3-L1 • adipocyte • peroxisome proliferator-activated receptor γ

Conjugated linoleic acid (CLA) refers to a group of fatty acid isomers that are related to the essential fatty acid, linoleic acid (LA; 18:2, *cis*-9, *cis*-12), but differ in both the position and the stereochemistry of their double bonds. The two isomers that have been shown to have biological activity are *trans*-10, *cis*-12 (*t*-10, *c*-12) CLA and *cis*-9, *trans*-11 (*c*-9, *t*-11) CLA (reviewed in Ref. 1). The *t*-10, *c*-12 CLA isomer has been shown to reduce obesity in animals (2–8) and triglyceride (TG) accumulation in adipocyte cell culture models (9–12), with its greatest effects, to date, having been shown in the mouse (13). This has led to the promotion of CLA as a weight-loss supplement in humans. In mice, however, the reduction in obesity is often accompanied by insulin resistance and hepatic steatosis (2, 14–16), suggesting that treatment with *t*-10, *c*-12 CLA may adversely affect TG metabolism in other tissues. In human studies, neither the large reductions in adipose tissue nor the hepatic steatosis have been observed.

Peroxisome proliferator-activated receptor γ (PPAR γ) is an essential transcription factor in adipogenesis that induces the expression of the genes necessary for the acquisition and maintenance of the mature adipocyte phenotype. These include LPL (17), the adipocyte fatty acid binding protein aP2 (18), and the glucose transport protein GLUT4 (19). Both of the CLA isomers commonly found in dietary supplement preparations have affinities for PPAR γ that are similar to LA, but of the two, *t*-10, *c*-12 CLA was shown to be a slightly better ligand (20). Compared with synthetically designed PPAR γ agonists, the thiazolidinediones (TZDs) (21), however, the affinities of CLA for PPAR γ are low.

The *t*-10, *c*-12 CLA isomer, but not the *c*-9, *t*-11 CLA isomer, appears to have profound inhibitory effects on the expression of both PPAR γ itself and PPAR γ -induced genes. When cultures of human preadipocytes were dif-

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ferentiated in the presence of individual CLA isomers, only treatment with *t*-10, *c*-12 CLA caused a marked reduction in the expression of PPAR γ as well as LPL, aP2, and GLUT4 (22).

Adiponectin (23–26) is an adipocyte-derived secretory protein (adipokine). Plasma levels of adiponectin are normally high but are reduced in obesity and correlate negatively with body fat mass (27). Additionally, low levels of adiponectin are associated with cardiovascular disease (28) and type II diabetes (29). Adiponectin monomers are assembled into large, distinct oligomeric forms that can be detected within adipocytes and in plasma as low molecular weight (LMW) trimers, medium molecular weight (MMW) hexamers, and high molecular weight (HMW) oligomers of 12–18 monomeric units (30, 31). Posttranslational glycosylation of four hydroxy-lysine residues (32) is necessary for the assembly of the HMW oligomers (33) and may be necessary for the assembly of the MMW and LMW oligomers as well (34).

Adiponectin is an insulin-sensitizing protein, and there are adiponectin receptors in both skeletal muscle and liver. In both tissues, adiponectin binding stimulates the adenosine monophosphate-activated protein kinase pathway (35). In the muscle, this serves to increase β -oxidation as well as the translocation of GLUT4 to the plasma membrane (36). In the liver, activation of adenosine monophosphate-activated protein kinase also increases β -oxidation and adiponectin binding decreases hepatic glucose output by downregulating gluconeogenesis (35).

TZDs are used to improve insulin sensitivity in type II diabetics and to correct hyperglycemia and hyperinsulinemia in animal models of obesity and diabetes, although their precise mechanism of action remains unknown. TZDs increase the conversion of preadipocytes to adipocytes, thereby increasing the number of small, insulin-sensitive adipocytes. Recent studies have shown that treatment with TZDs increased plasma levels of adiponectin in normal, obese, and type II diabetic subjects (37) and in obese-diabetic (*db/db*) mice (38, 39). In cell culture studies, adiponectin secretion from 3T3-L1 adipocytes was also increased by TZDs (39).

The aim of our study was to gain insight into the mechanisms of the effects of *t*-10, *c*-12 CLA on adiponectin metabolism in both differentiating preadipocytes and fully differentiated adipocytes using the mouse 3T3-L1 model. We also examined whether the *t*-10, *c*-12 CLA-mediated reductions in TG and adiponectin mass occur through a PPAR γ -dependent mechanism by comparison with a known PPAR γ agonist, the TZD troglitazone, and a known PPAR γ antagonist, GW9662. Finally, we used a reporter assay to examine the agonist/antagonist properties of the individual CLA isomers.

MATERIALS AND METHODS

Materials

BSA (essentially fatty acid-free), dexamethasone, methylisobutylxanthine, LA, and cycloheximide (CHX) were purchased from Sigma Aldrich Canada, Ltd. (Oakville, Ontario, Canada). Insulin

was obtained from Roche Diagnostics Canada (Laval, Quebec, Canada). Purified CLA isomers *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA (>95%, verified by gas chromatography; Matreya, Inc., Pleasant Gap, PA), troglitazone, and GW9662 (Biomol International, Plymouth Meeting, PA) were used in cell culture experiments. DMEM, trypsin, and FBS were purchased from Invitrogen Canada, Inc. (Burlington, Ontario, Canada). All chemicals were of the highest purity available. Antibodies used included mouse anti-adiponectin, mouse anti-actin (Chemicon International, Inc., Temecula, CA), mouse anti-PPAR γ (E-8; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and goat anti-mouse HRP (Bio-Rad Laboratories, Hercules, CA).

Cell culture

3T3-L1 cells (American Type Culture Collection, Manassas, VA) were maintained below confluence in DMEM containing 10% FBS at 37°C in an atmosphere of 5% CO₂. To induce differentiation (40), cells were grown to confluent monolayers in 12-well tissue culture dishes. Two days after confluence, cells were treated with MDI induction medium (0.5 μ M methylisobutylxanthine, 250 nM dexamethasone, and 5 μ g/ml insulin) in DMEM/10% FBS for 48 h. MDI was then removed and replaced with 3T3-L1 adipocyte medium (DMEM containing 10% FBS and 5 μ g/ml insulin). Adipocyte medium was removed and replaced every 2 days for up to 8 days, as indicated. Before cell harvest, microscopic images of the monolayers were captured using a Zeiss Axiovert 200 inverted microscope, as described in the figure legends.

Fatty acid and PPAR γ modulator treatment during differentiation of 3T3-L1 cells

Fatty acids were supplemented to cells and complexed to BSA (41) in a 6.9:1 molar ratio from stock solutions of 10 mM sodium fatty acid and 1.45 mM BSA. To examine the effects of fatty acid, PPAR γ agonist (troglitazone), or PPAR γ antagonist (GW9662) treatment on adipocyte differentiation, 3T3-L1 preadipocytes were induced with MDI containing no fatty acid, 100 μ M fatty acid [LA, *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, mixed isomer (MI)-CLA (50 μ M *c*-9, *t*-11 CLA + 50 μ M *t*-10, *c*-12 CLA)], 10 μ M troglitazone, or 1–10 μ M GW9662. After the induction, cells were maintained in 3T3-L1 adipocyte medium containing fatty acid or PPAR γ modulator and medium was removed and replaced every 2 days. On day 5 or 7 after induction, the medium was aspirated and replaced with serum-free medium containing insulin and fatty acid or PPAR γ modulator. Sixteen hours later, on day 6 or 8 after induction, medium was collected and cells were harvested in PBS. Cells were then disrupted by sonication for 10 s at 20% output (Branson Sonifier 250) and stored at -20° C. Cell lysate samples were analyzed for TG and total protein. Equivalent amounts of total cell protein (5 μ g for adiponectin, 40 μ g for PPAR γ) were loaded for SDS-PAGE and Western blot analysis, as described below.

Acute fatty acid and PPAR γ modulator treatment

To examine the acute effects of fatty acid, troglitazone, or GW9662 treatment, 3T3-L1 preadipocytes were first induced to differentiate as described above. On day 5 after induction, when 3T3-L1 adipocytes had attained maturity (42), adipocyte medium was removed and replaced with serum-free medium containing no fatty acid, 100 μ M fatty acid [LA, *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, MI-CLA], 1 μ M GW9662, or 10 μ M troglitazone. Medium was removed 16 h later, on day 6 after induction, and replaced with serum-free medium containing 10 μ g/ml CHX. At up to 8 h after CHX addition, medium was collected and cells were harvested in PBS. Cell lysate was prepared by sonication, as described above, and analyzed for TG and total protein. Equivalent amounts of total

protein (5 μg for adiponectin, 40 μg for PPAR γ) were loaded for SDS-PAGE and Western blot analysis, as described below.

Analysis of adiponectin mass and oligomer pattern

For analysis of total adiponectin mass, samples of cell lysate or medium were incubated with sample buffer [20 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 16% (v/v) glycerol, and 0.004% (w/v) bromophenol blue] containing 10% β -mercaptoethanol for 5 min at 100°C, to reduce oligomeric forms to monomers, and resolved on 10% (w/v) polyacrylamide gels. For analysis of adiponectin oligomers, cell lysate or medium samples were incubated for 10 min at room temperature in nonreducing sample buffer (as above, diluted 1:4 with water, without β -mercaptoethanol) and resolved on 5% (w/v) polyacrylamide gels. After electrophoresis, protein was transferred to nitrocellulose membranes and incubated overnight with mouse anti-mouse adiponectin antibody (1:5,000) and, where indicated, mouse anti-mouse actin antibody (1:1,000) followed by incubation for 1.5 h with goat anti-mouse HRP (1:5,000). Target protein was detected by chemiluminescence (Roche Diagnostics) and the signal was semiquantified, as arbitrary units (a.u.), by densitometry using Scion Image. Adiponectin mass was normalized to total cell protein. In some experiments with differentiated cells, adiponectin was normalized to cellular actin, and both methods yielded similar results. However, in the differentiation experiments, actin levels were affected by the state of differentiation (43) and did not give reliable quantitative results. Therefore, all results are expressed normalized to cell protein for consistency.

Protein and TG quantification

Total cell protein was measured using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories), based on the method of Lowry et al. (44), and adapted for microtiter plate and detergent-solubilized samples. Cell lysate samples and BSA standards were mixed with Triton X-100 to a final concentration of 1% (w/v) to remove the turbidity associated with the TG-enriched samples. Protein concentration was determined colorimetrically by comparison with a BSA standard curve, with absorbance measurements at 655 nm. For TG measurements, samples of sonicated cell lysate were incubated with Triglyceride Reagent (Diagnostic Chemical Limited, Charlottetown, Prince Edward Island, Canada) for 1 h at 37°C in a microtiter plate. Absolute TG levels were determined by comparison with a glycerol standard (0.21 $\mu\text{g}/\mu\text{l}$ glycerol = 2.5 $\mu\text{g}/\mu\text{l}$ TG), with absorbance measurements at 490 nm. Assay integrity was maintained by verification with quality controls, Precipath[®]L and Precinorm[®]L (Boehringer Mannheim). The interassay coefficient of variation was \sim 12%.

Assessment of peroxisome proliferator response element ligand activity using peroxisome proliferator response element-luciferase reporter assay

Chinese hamster ovary (CHO-K1) cells were seeded on 12-well plates (1.6×10^5 /well) in 1 ml of growth medium [5% FBS in DMEM/F12 (50:50)] and allowed to adhere overnight. Monolayers (40–80% confluent) were transfected using Polyfect[®] Transfection Reagent (Qiagen, Inc., Mississauga, Ontario, Canada; 3 μl /well) with 0.3 μg peroxisome proliferator response element (PPRE) \times 3-TK-luciferase reporter plasmid (Addgene, Inc., Cambridge, MA) and 0.2 μg of pCMV β -galactosidase control plasmid (Clontech Laboratories, Inc., Mountain View, CA). Twenty-four hours later, the transfection medium was replaced with growth medium containing 0, 10, 100, 200, or 400 μM *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, or MI-CLA. Control wells received 10 μM troglitazone, as a representative PPAR γ agonist,

or DMSO vehicle control. In some experiments, 10 μM troglitazone or 10 μM GW9662, a PPAR γ antagonist, were added together with 100–200 μM CLA isomer.

After incubation with PPAR γ ligand for 24 h, monolayers were washed with PBS (150 mM NaCl and 15 mM sodium phosphate, pH 7.3) and lysed (Reporter Lysis Buffer; Promega) for 15 min at room temperature. Lysates were clarified by centrifugation, and the supernatants were assayed for luciferase activity on 96-well plates using Luciferase Assay Reagent (Promega). Luminescence intensity was measured in a luminometer (Fluostar Galaxy; BMG Labtechnologies). β -Galactosidase activity was measured in cell lysates on a 96-well plate using 1.33 mg/ml *O*-nitrophenyl- β -D-galactopyranoside (Sigma-Aldrich, St. Louis, MO) as substrate in 200 mM sodium phosphate buffer, pH 7.3, 2 mM MgCl₂, and 100 mM β -mercaptoethanol. The assay mixture was incubated at 37°C for 30 min, and enzymatic activity was stopped by the addition of 1 M sodium carbonate. The absorbance at 450 nm was measured in a microplate reader (Bio-Rad model 3550). Luciferase activity was calculated as relative luminescence units, normalized to β -galactosidase for each well.

Statistical analysis

The results shown are from representative experiments that were each replicated at least three times. All data are presented as means \pm SD for replicate wells. Treatments were compared by one-way ANOVA using Tukey's posthoc test to identify individual differences.

RESULTS

Differentiation of 3T3-L1 cells in the presence of *t*-10, *c*-12 CLA reduces TG accumulation, adiponectin mass, and the assembly of adiponectin oligomers

To examine the effects of CLA on adipocyte metabolism, 3T3-L1 cells were differentiated in the presence of 100 μM fatty acid. In the presence of *t*-10, *c*-12 CLA, TG levels in 3T3-L1 cells were reduced to one-quarter of the level in control cells without fatty acid (0.37 ± 0.07 vs. 1.45 ± 0.14 μg TG/ μg cell protein; $P < 0.01$) or cells differentiated in the presence *c*-9, *t*-11 CLA ($P < 0.01$) (Fig. 1A). Additionally, the lipid droplets appeared to be smaller in *t*-10, *c*-12 CLA-treated cells (Fig. 1B), as reported previously (22, 45, 46). Because dietary CLA supplements contain both *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA, cells were also differentiated in the presence of an equimolar mixture of the two CLA isomers (MI-CLA) at a total fatty acid concentration of 100 μM . These cells had 40% of the cellular TG in cells without fatty acid (0.62 ± 0.24 μg ; $P < 0.05$) or cells treated with LA or *c*-9, *t*-11 CLA. The lipid droplets were also smaller in cells treated with MI-CLA (Fig. 1B). TG levels in cells treated with *t*-10, *c*-12 CLA alone, or MI-CLA, were not significantly different from one another ($P > 0.05$), suggesting that of the two CLA isomers, *t*-10, *c*-12 CLA exerted the dominant effect in the mixed isomer preparation. Moreover, because cells treated with MI-CLA received half the concentration of the *t*-10, *c*-12 CLA isomer, the results suggested that supplementation at 50 μM is sufficient for nearly maximal suppression of TG accumulation.

Because supplementation of 3T3-L1 preadipocytes with *t*-10, *c*-12 CLA during 3T3-L1 differentiation caused a

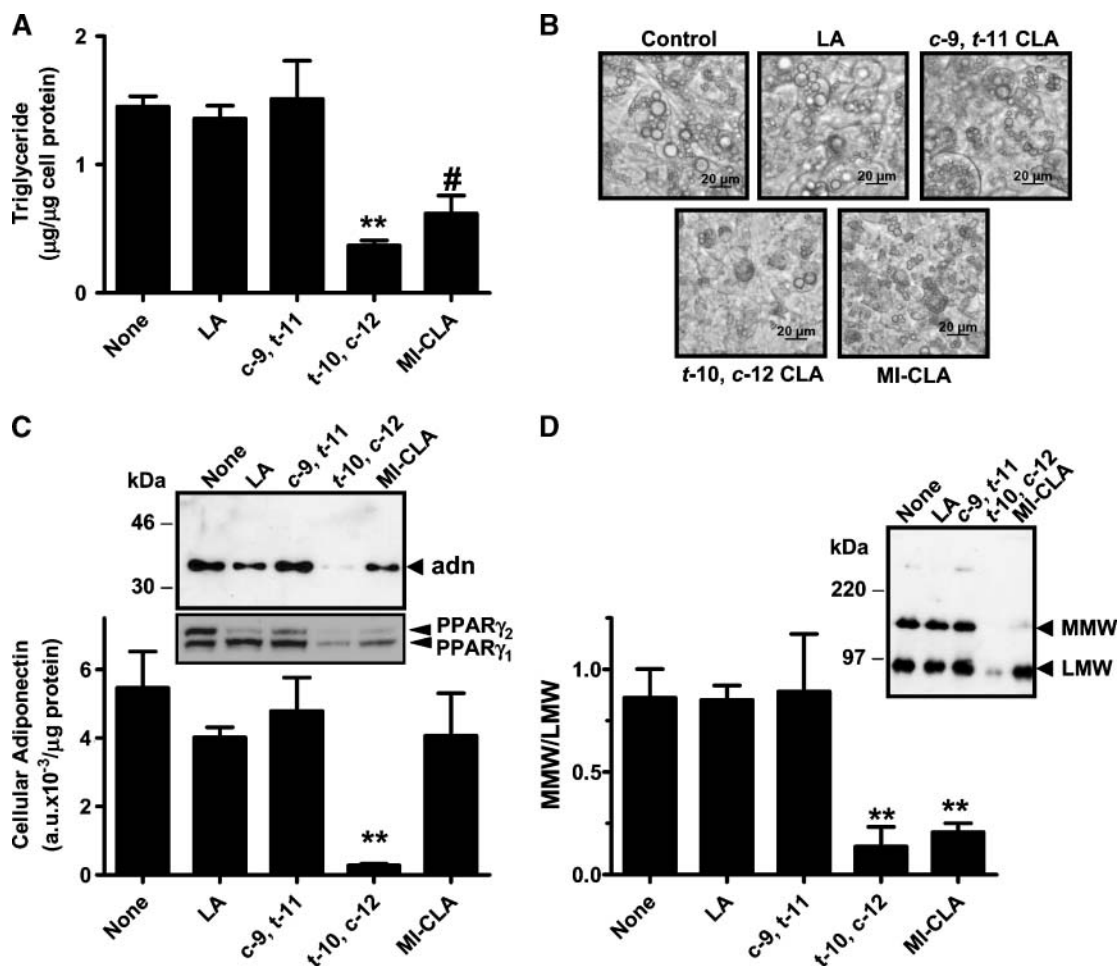


Fig. 1. Differentiation of 3T3-L1 cells in the presence of *trans*-10, *cis*-12 (*t*-10, *c*-12) conjugated linoleic acid (CLA) reduces triglyceride (TG) accumulation, adiponectin (adn) mass, and the assembly of adiponectin oligomers. **A:** 3T3-L1 preadipocytes were differentiated in the presence or absence of 100 μ M fatty acid [linoleic acid (LA), *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, or mixed isomer (MI)-CLA (50 μ M *c*-9, *t*-11 CLA + 50 μ M *t*-10, *c*-12 CLA)]. Medium was replenished every 2 days, and on day 8 after induction, the cells were harvested for the determination of TG mass. Results are shown as means \pm SD (normalized to cell protein) of replicates ($n = 3$) from a representative experiment. **B:** Representative images of differentiated 3T3-L1 adipocytes treated with or without fatty acid. Phase contrast images were collected using a Zeiss Axiovert 200 inverted microscope with an AxioCam HRC digital camera and a 20 \times Plan NEOFLUAR objective lens. Scale bar (20 μ m) is indicated in each panel. **C:** Total adiponectin and peroxisome proliferator-activated receptor γ (PPAR γ) in the cell lysate was resolved by SDS-PAGE under reducing conditions and detected by immunoblot analysis. Adiponectin was semiquantified by scanning densitometry. **D:** Adiponectin oligomers in cell lysates were resolved by SDS-PAGE under nonreducing conditions, detected by immunoblot analysis, and semiquantified by scanning densitometry, and the ratio between medium molecular weight (MMW) and low molecular weight (LMW) oligomers was calculated. Except where indicated otherwise, results are presented as arbitrary units (a.u.), means \pm SD ($n = 3$), normalized to cell protein from a representative experiment. Significant differences are shown: ** $P < 0.01$, # $P < 0.05$.

profound reduction in cellular TG mass (Fig. 1A), we hypothesized that enhanced metabolic activity may lead to a concomitant increase in adiponectin mass. However, as shown in Fig. 1C, supplementation of 3T3-L1 cells with *t*-10, *c*-12 CLA throughout differentiation resulted in a 20-fold decrease in adiponectin mass (0.28 ± 0.04 a.u. $\times 10^3$ / μ g cell protein) compared with control cells (5.47 ± 1.06 a.u. $\times 10^3$ / μ g cell protein) or cells treated with LA or *c*-9, *t*-11 CLA ($P < 0.01$). This was accompanied by a substantial loss of PPAR γ protein from the cells (Fig. 1C), as reported previously (47). Levels of adiponectin in cells differentiated in the presence of *c*-9, *t*-11 CLA or MI-CLA were not significantly different from those in cells differentiated without fatty acid. This suggested that the

c-9, *t*-11 CLA isomer may be able to compensate for the *t*-10, *c*-12 CLA-mediated effect on the production of adiponectin, despite being unable to compensate for the effect of *t*-10, *c*-12 CLA on cellular TG levels.

The HMW form of adiponectin is thought to be the most biologically active, and the ratio between HMW and total adiponectin in plasma has been shown to be a better correlate of plasma glucose and insulin levels than the level of total adiponectin (48, 49). Analysis of cellular adiponectin oligomers by nonreducing SDS-PAGE (Fig. 1D) showed that, compared with cells without fatty acid supplement, differentiation in the presence of *t*-10, *c*-12 CLA reduced the levels of LMW adiponectin by >50% (4.87 ± 1.61 vs. 11.81 ± 1.40 a.u. $\times 10^3$ / μ g cell protein; $P < 0.01$)

and reduced MMW adiponectin by >90% (0.68 ± 0.63 vs. 8.67 ± 0.84 a.u. $\times 10^3/\mu\text{g}$ cell protein; $P < 0.001$). In contrast, treatment with *c*-9, *t*-11 CLA alone did not change the distribution of cellular adiponectin oligomers (Fig. 1D). Cells treated with MI-CLA had similar levels of LMW adiponectin as cells without fatty acid (9.93 ± 1.04 vs. 11.81 ± 1.40 a.u. $\times 10^3/\mu\text{g}$ cell protein; $P = 0.07$). However, MMW adiponectin was reduced by $\sim 75\%$ by MI-CLA (2.03 ± 0.22 vs. 8.67 ± 0.84 a.u. $\times 10^3/\mu\text{g}$ cell protein; $P < 0.001$), suggesting that, in the presence of MI-CLA, assembly of the MMW oligomers in 3T3-L1 adipocytes may be impaired. The ratio of MMW to LMW adiponectin was also reduced by >4-fold ($P < 0.01$) in cells differentiated in the presence of either *t*-10, *c*-12 CLA (0.14 ± 0.10 a.u. $\times 10^3/\mu\text{g}$ cell protein) or MI-CLA (0.21 ± 0.04 a.u. $\times 10^3/\mu\text{g}$ cell protein) compared with cells without fatty acid (0.84 ± 0.14 a.u. $\times 10^3/\mu\text{g}$ cell protein). Additionally, LMW adiponectin in cells treated with *t*-10, *c*-12 CLA or MI-CLA appeared to have migrated faster than LMW adiponectin in all other treatments, suggesting that LMW adiponectin in cells receiving *t*-10, *c*-12 CLA was of a lower apparent molecular weight.

Acute supplementation of differentiated 3T3-L1 cells with *t*-10, *c*-12 CLA reduces cellular adiponectin but does not affect TG mass

To examine the acute effects of CLA on adipocyte metabolism, 3T3-L1 preadipocytes were differentiated into adipocytes in the absence of fatty acid supplement and then the differentiated cells were incubated with fatty acids. After 16 h of incubation, cellular TG levels were not affected by CLA (Fig. 2A). However, adiponectin mass (Fig. 2B, C) in cells treated with *t*-10, *c*-12 CLA (0.42 ± 0.19) or MI-CLA (0.69 ± 0.25) was reduced by 3- to 4-fold ($P < 0.01$) compared with that in untreated (1.64 ± 0.05), *c*-9, *t*-11 CLA-treated (1.70 ± 0.14), or LA-treated (1.83 ± 0.26) cells. These rapid decreases in adiponectin mass were accompanied by decreases in cellular PPAR γ mass (Fig. 2B). The differences between the effects of *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA suggested that the two CLA isomers may have opposite effects on adiponectin production. Furthermore, unlike the observations after treatment with MI-CLA during differentiation, in cells incubated with MI-CLA after differentiation, *t*-10, *c*-12 CLA appeared to be the dominant isomer and responsible for changes in adiponectin levels.

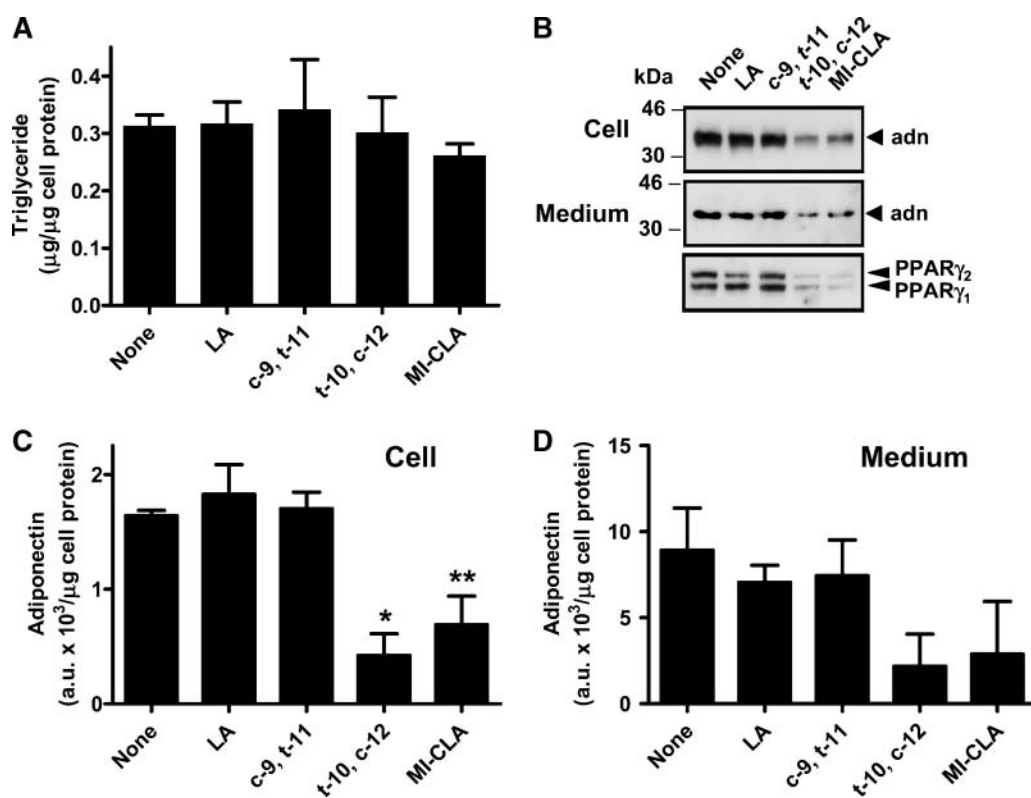


Fig. 2. Acute supplementation of differentiated 3T3-L1 cells with *t*-10, *c*-12 CLA reduces cellular adiponectin (adn) but does not affect TG mass. 3T3-L1 cells were differentiated for 5 days and then treated with or without 100 μM fatty acid [LA, *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, or MI-CLA (50 μM *c*-9, *t*-11 CLA + 50 μM *t*-10, *c*-12 CLA)] for 16 h. Cells were collected and sonicated, and TG (A) (means \pm SD normalized to total cell protein; $n = 15$) and adiponectin mass (B, C) were determined in the lysates. In separate dishes, protein synthesis was terminated after the fatty acid pretreatment by the addition of 10 $\mu\text{g}/\text{ml}$ cycloheximide (CHX), and media were collected after 8 h (B, D). Data for adiponectin are normalized to cell protein and represent replicates ($n = 3$) from a single representative experiment. Significant differences from no fatty acid supplement are shown: ** $P < 0.01$, * $P < 0.001$.

Levels of secreted adiponectin were also analyzed after 16 h of fatty acid treatment and 8 h in the presence of CHX (Fig. 2B, D), which was used to block new adiponectin synthesis. Adipocytes acutely treated with *t*-10, *c*-12 CLA secreted less adiponectin than all other treatments, indicating that treatment with the *t*-10, *c*-12 CLA isomer leads to the depletion of adiponectin from 3T3-L1 adipocytes, although these differences did not reach statistical significance. MI-CLA treatment showed similar decreases in secreted adiponectin.

Acute treatment with *t*-10, *c*-12 CLA increases the proportion of HMW adiponectin secretion

Analysis of the distribution of secreted adiponectin oligomers (Fig. 3A) showed that *t*-10, *c*-12 CLA treatment increased the ratio of HMW to total adiponectin, and the ratio was significantly different from that in all other treatments at the 8 h time point ($P < 0.05$) (Fig. 3B). However, the increased ratio was attributable to lower levels of secreted MMW adiponectin rather than to increased levels of HMW oligomers. The HMW/total adiponectin ratio was not affected by *c*-9, *t*-11 CLA or MI-CLA treatment. These experiments suggested that acute treatment with *t*-10, *c*-12 CLA may impair the assembly and/or secretion of MMW adiponectin.

Differentiation of 3T3-L1 cells in the presence of GW9662 reduces TG mass and oligomer assembly but does not affect total adiponectin levels

It has been suggested that *t*-10, *c*-12 CLA may exert its effects through the modulation of PPAR γ (12, 22). The *t*-10, *c*-12 CLA isomer has been shown to decrease the

expression of PPAR γ mRNA as well as the expression of numerous downstream target genes (22), suggesting that *t*-10, *c*-12 CLA may affect PPAR γ production directly. However, *t*-10, *c*-12 CLA and *c*-9, *t*-11 CLA have also been shown to be PPAR γ ligands (20), suggesting that the effects of the CLA isomers may be more than transcriptional. Whether CLA isomers act as agonists, antagonists, or modulators of PPAR γ , through competition with endogenous ligands, is not known. Therefore, to gain insight into *t*-10, *c*-12 CLA modulation of PPAR γ , the effects of a known PPAR γ agonist, troglitazone, and a known antagonist, GW9662, were compared with the effects of *t*-10, *c*-12 CLA on TG and adiponectin synthesis as well as on the assembly of adiponectin oligomers.

GW9662 has been shown to be a selective and potent inhibitor of PPAR γ and irreversibly binds within the ligand binding domain through covalent modification of a cysteine residue (50). If *t*-10, *c*-12 CLA exerts its effects through PPAR γ antagonism, treatment with GW9662 may elicit responses in the 3T3-L1 cells that are similar to those observed after *t*-10, *c*-12 CLA treatment. Initially, the effects of treatment with a low (1 μ M) or high (10 μ M) dose of GW9662 on TG accumulation in differentiating 3T3-L1 cells were assessed. Treatment with GW9662 reduced TG mass (Fig. 4A), and fewer lipid droplets were observed compared with untreated cells (Fig. 4B). Although some cells had accumulated lipid droplets by day 8, many cells remained lipid-deficient. TG mass in cells treated with 1 μ M (0.66 ± 0.08 μ g TG/ μ g cell protein) was not different from that in cells treated with 10 μ M (0.57 ± 0.06 μ g TG/ μ g cell protein; $P = 0.10$). Additionally, cellular TG levels were similar to those in 3T3-L1 cells differentiated

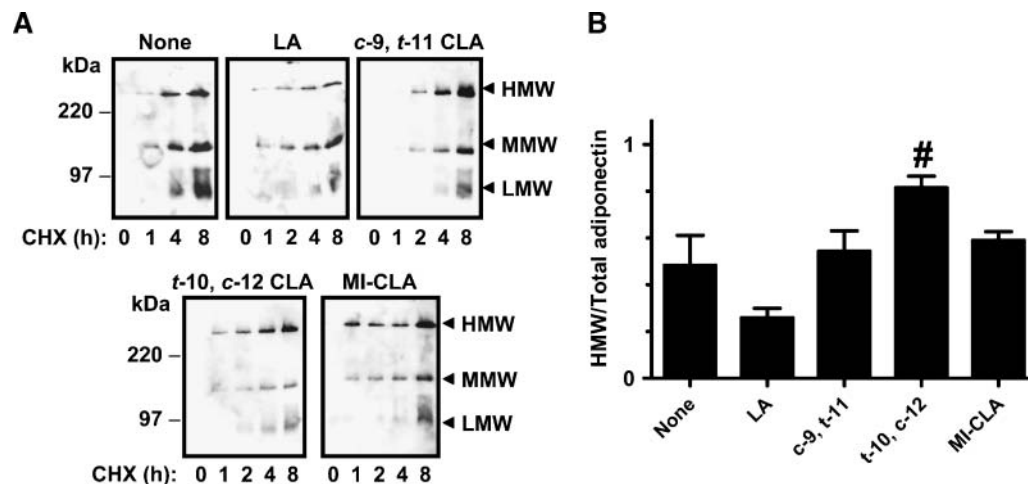


Fig. 3. Acute treatment with *t*-10, *c*-12 CLA increases the proportion of high molecular weight (HMW) adiponectin (adn) oligomer secretion. 3T3-L1 cells were differentiated for 5 days and then treated with or without 100 μ M fatty acid [LA, *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, or MI-CLA (50 μ M *c*-9, *t*-11 CLA + 50 μ M *t*-10, *c*-12 CLA)] for 16 h. Protein synthesis was terminated by the addition of 10 μ g/ml CHX, and medium was collected from independent replicate wells after up to 8 h. A: Secreted adiponectin oligomers were resolved by SDS-PAGE, under nonreducing conditions, and detected by immunoblot analysis. B: Oligomers were semiquantified by scanning densitometry, and the HMW/total adiponectin ratio was compared between treatment groups for the 8 h time point. Results are shown as means \pm SD of replicates from a single experiment ($n = 3$). [#] $P < 0.05$ (vs. all other treatment groups).

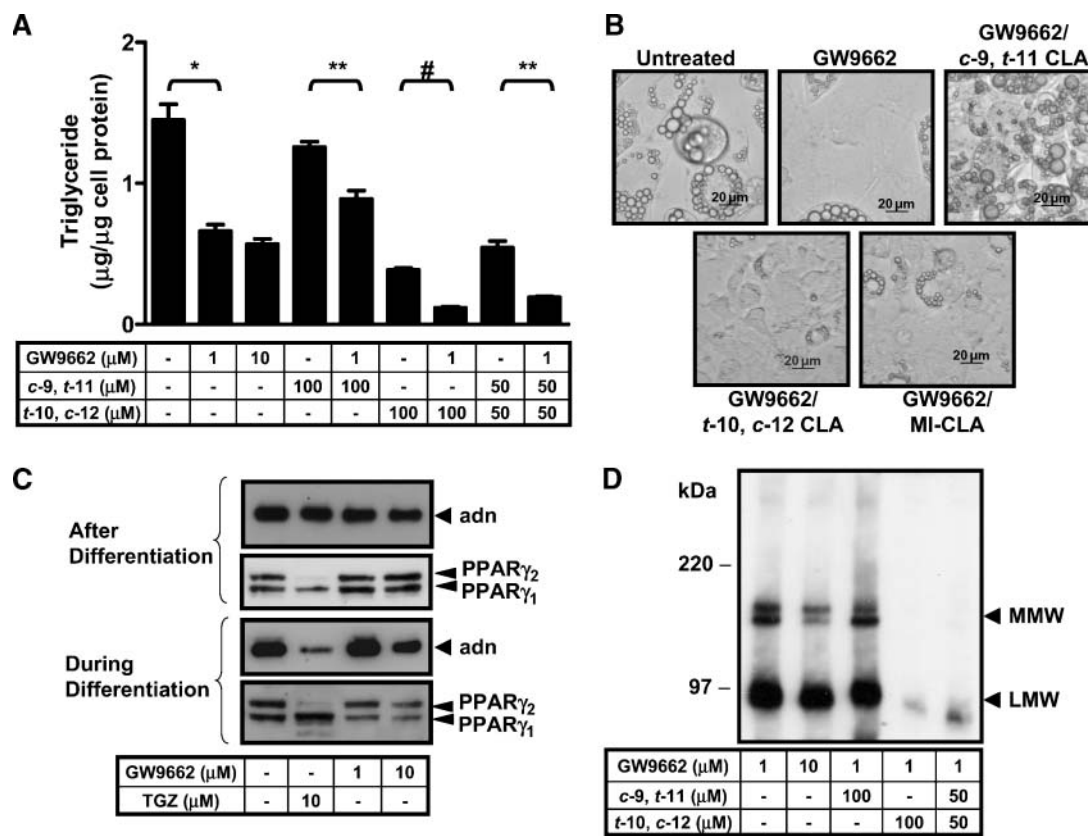


Fig. 4. Chronic treatment with GW9662 reduces TG mass and oligomer assembly but does not affect total adiponectin (adn) levels in 3T3-L1 cells. 3T3-L1 cells were differentiated in the presence 100 μM fatty acid [*c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, or MI-CLA (50 μM *c*-9, *t*-11 CLA + 50 μM *t*-10, *c*-12 CLA)] with or without 1 or 10 μM GW9662. Medium was replenished every 2 days, and on day 8 after induction, cells were harvested. **A:** TG was measured using an enzymatic colorimetric assay. Results are shown as means \pm SD normalized to cell protein of replicates from a single experiment ($n = 3$). * $P < 0.001$, ** $P < 0.01$, # $P < 0.05$. **B:** Representative images of differentiated 3T3-L1 adipocytes treated with 1 μM GW9662 with or without fatty acid were collected as described for Fig. 1. **C:** 3T3-L1 cells were differentiated in the absence (after differentiation) or presence (during differentiation) of troglitazone (TGZ) or GW9662. Medium was replenished every 2 days. On day 5 after induction, media were replaced with serum-free medium with or without PPAR γ ligand. After 16 h, the cells were collected and lysed by sonication. Total adiponectin and PPAR γ in the cell lysate were resolved by SDS-PAGE under reducing conditions and detected by immunoblot analysis. **D:** Adiponectin LMW and MMW oligomers in the cell lysate were resolved by SDS-PAGE under nonreducing conditions and detected by immunoblot analysis. For C and D, results are representative of triplicate blots from a single experiment.

in the presence of 100 μM *t*-10, *c*-12 CLA. Because both GW9662 and *t*-10, *c*-12 CLA treatment reduced cellular levels of TG, our results suggested that the *t*-10, *c*-12 CLA-mediated effects may also be through PPAR γ antagonism.

To determine whether CLA isomers could alter the effects of GW9662 on TG accumulation, cells were treated with 1 μM GW9662 and 100 μM *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, or MI-CLA during differentiation and compared with fatty acid treatment without antagonist (Fig. 4A). When 100 μM *c*-9, *t*-11 CLA was supplemented in combination with GW9662, cells accumulated significantly more TG ($P < 0.01$) than cells supplemented with GW9662 alone. The majority of cells treated with both *c*-9, *t*-11 CLA and GW9662 accumulated large lipid droplets, and few cells appeared to be lipid-deficient (Fig. 4B). Thus, *c*-9, *t*-11 CLA may act as a PPAR γ agonist, competing with GW9662 to allow normal adipogenesis. In contrast, supplementation with 100 μM *t*-10, *c*-12 CLA in addition to 1 μM GW9662 led to a significant decrease

in TG accumulation compared with treatment with either *t*-10, *c*-12 CLA or GW9662 alone ($P < 0.05$). Very few cells contained lipid droplets after 8 days of treatment, and the droplets present were very small (Fig. 4B). TG levels in cells treated with MI-CLA and GW9662 were not significantly different from those in cells treated with *t*-10, *c*-12 CLA and GW9662 and were significantly lower than those in cells treated with GW9662 or *c*-9, *t*-11 CLA plus GW9662 ($P < 0.001$). These results suggested that *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA may have distinct and opposing effects on PPAR γ . The *c*-9, *t*-11 CLA isomer was able to partially compensate for the GW9662-mediated decrease in TG accumulation and may act through PPAR γ agonism. On the other hand, *t*-10, *c*-12 CLA and GW9662 acted in an additive manner to prevent TG accumulation, and *c*-9, *t*-11 CLA was unable to overcome the effect of *t*-10, *c*-12 CLA in the mixed isomer treatment.

Despite reducing TG accumulation in 3T3-L1 cells to a similar degree as treatment with *t*-10, *c*-12 CLA, treatment

with 1 μM GW9662, either during or after differentiation, did not appear to reduce total adiponectin mass (Fig. 4C), and this was consistent with the maintenance of cellular PPAR γ levels. Although troglitazone also had no effect on adiponectin levels in the differentiated cells, the PPAR γ agonist surprisingly decreased adiponectin levels in differentiating cells. The effects of troglitazone were accompanied by marked changes in PPAR γ mass and isomer pattern.

GW9662 did not affect adiponectin oligomer pattern (Fig. 4D), in contrast to the marked changes observed with the *t*-10, *c*-12 CLA and MI-CLA preparations. This suggested that *t*-10, *c*-12 CLA may decrease adiponectin mass and oligomer pattern via a PPAR γ -independent mechanism. However, because treatment with 10 μM GW9662 did decrease total adiponectin mass (Fig. 4C) and

the levels of MMW adiponectin (Fig. 4D), it remains a possibility that the assembly of adiponectin oligomers may be a PPAR γ -dependent event, but that oligomer assembly is only reduced at higher doses of PPAR γ antagonist than those required to affect TG accumulation.

Differentiation in the presence of troglitazone restores TG mass and the assembly of adiponectin oligomers in *t*-10, *c*-12 CLA-supplemented 3T3-L1 cells

To determine whether a known PPAR γ agonist was able to reverse the *t*-10, *c*-12 CLA-mediated effects on TG and adiponectin mass and the assembly of adiponectin oligomers, 3T3-L1 cells were differentiated in the presence of the PPAR γ agonist, troglitazone, and *t*-10, *c*-12 CLA (Fig. 5). TG accumulation in cells treated with

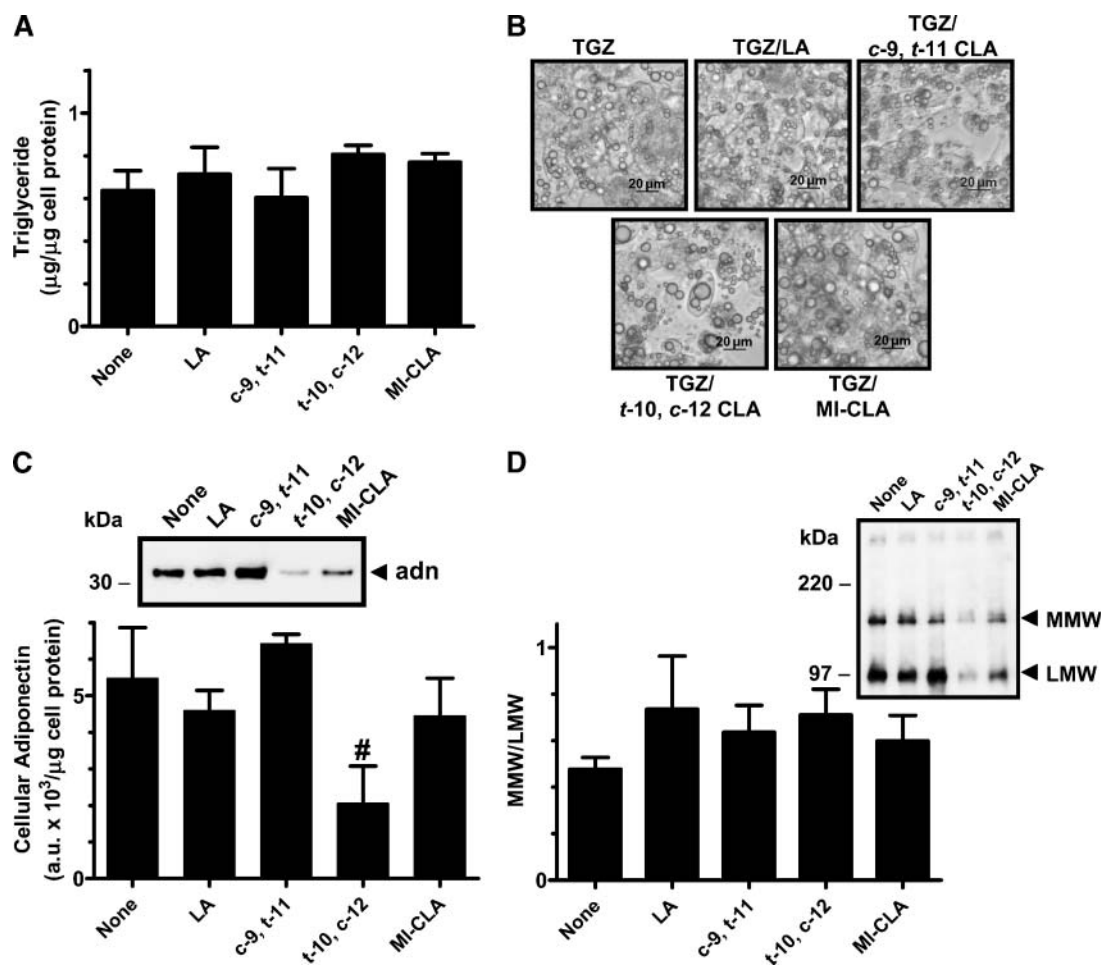


Fig. 5. Chronic treatment with troglitazone (TGZ) restores TG mass and the assembly of adiponectin (adn) oligomers in *t*-10, *c*-12 CLA-supplemented 3T3-L1 adipocytes. 3T3-L1 cells were differentiated in the presence of 10 μM troglitazone with or without 100 μM fatty acid [LA, *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, or MI-CLA (50 μM *c*-9, *t*-11 CLA + 50 μM *t*-10, *c*-12 CLA)]. Medium was replenished every 2 days, and on day 8 after induction, cells were harvested. **A:** TG was measured using an enzymatic colorimetric assay. Results are shown as means \pm SD normalized to cell protein of replicates from a single experiment ($n = 3$). **B:** Representative images (captured as described for Fig. 1) of differentiated 3T3-L1 adipocytes treated with 10 μM troglitazone with or without fatty acid. **C:** Total adiponectin in the cell lysate was resolved by SDS-PAGE under reducing conditions, detected by immunoblot analysis, and semiquantified by densitometry. Results are shown as means \pm SD normalized to cell protein of replicates from a single experiment ($n = 3$). [#] $P < 0.05$. **D:** Adiponectin oligomers in the cell lysate were resolved by SDS-PAGE under nonreducing conditions, detected by immunoblot analysis, and semiquantified by densitometry, and the ratio between MMW and LMW oligomers was calculated. For C and D, results are replicates ($n = 3$) from a single representative experiment.

troglitazone and *t*-10, *c*-12 CLA ($0.80 \pm 0.04 \mu\text{g TG}/\mu\text{g cell protein}$) was similar to that in control cells treated with troglitazone only ($0.63 \pm 0.10 \mu\text{g TG}/\mu\text{g cell protein}$). Treatment with troglitazone reversed the decrease in TG accumulation in MI-CLA treated cells (0.77 ± 0.04 vs. $0.63 \pm 0.10 \mu\text{g TG}/\mu\text{g cell protein}$). This suggested that *t*-10, *c*-12 CLA may decrease TG accumulation by PPAR γ antagonism, because a known agonist was able to reverse its effects in 3T3-L1 cells.

PPAR γ has been implicated in the activation of adiponectin expression (37, 38, 51, 52), and because treatment of differentiating or differentiated 3T3-L1 cells with *t*-10, *c*-12 CLA significantly decreased adiponectin levels, it is possible that this effect is through PPAR γ antagonism. However, troglitazone was unable to normalize the levels of adiponectin (Fig. 5C; cf. Fig. 1C), despite restoring TG mass in cells treated during differentiation with *t*-10, *c*-12 CLA. Cellular adiponectin mass after treatment with *t*-10, *c*-12 CLA and troglitazone during differentiation remained 60% lower ($P < 0.05$) than in control cells treated with troglitazone alone (2.06 ± 1.02 vs. $5.48 \pm 1.38 \text{ a.u.} \times 10^3/\mu\text{g cell protein}$). Adiponectin mass in cells treated with LA, *c*-9, *t*-11 CLA, or MI-CLA was not significantly different from that in cells without fatty acid. These results suggested that the effects of *t*-10, *c*-12 CLA on adiponectin mass could not be reversed by troglitazone and therefore are not solely the result of PPAR γ antagonism.

It has also been suggested that TZDs improve insulin sensitivity in obese and diabetic humans and animals by changing the pattern of secreted adiponectin oligomers rather than by increasing absolute mass, resulting in an increased level of the biologically active HMW oligomer in the plasma (48). This suggests that oligomer assembly may be controlled by a PPAR γ -induced mechanism. Troglitazone was tested for its ability to overcome the effects of *t*-10, *c*-12 CLA on MMW oligomer assembly (Fig. 5D). When treated during differentiation with *t*-10, *c*-12 CLA and troglitazone, levels of cellular LMW adiponectin were 2.5-fold lower than in control cells (10.82 ± 0.44 vs. $26.12 \pm 1.86 \text{ a.u.} \times 10^3/\mu\text{g cell protein}$; $P < 0.05$), but when the ratio of MMW to LMW adiponectin oligomers was calculated (Fig. 5D), no significant differences were found between treatment groups, suggesting that although troglitazone was unable to normalize adiponectin mass in *t*-10, *c*-12 CLA-treated cells, it was able to restore oligomer assembly. Additionally, the presence of troglitazone during differentiation appeared to reverse the cellular changes that caused the shift in molecular weight of LMW adiponectin observed in cells differentiated in the presence of *t*-10, *c*-12 CLA or MI-CLA alone (Fig. 5D vs. Figs. 1D, 4D). In the presence of troglitazone, LMW adiponectin in *t*-10, *c*-12 CLA- or MI-CLA-treated cells appeared to migrate at the same position as in all other treatment groups.

PPRE agonist/antagonist properties of *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA isomers

To study the effects of *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA on PPAR γ activation, transient transfection and reporter gene assays were performed in CHO-K1 cells. CHO cells

were cotransfected with PPRE-luciferase reporter plasmid and β -galactosidase plasmid and then treated with CLA isomers, troglitazone, and troglitazone with CLA isomer or the PPAR γ antagonist GW9662. As shown in Fig. 6A, 10 μM troglitazone increased the luciferase reporter activity by ~ 2.5 -fold, and 10 μM GW9662 did not increase, nor did it decrease, luciferase activity. *c*-9, *t*-11 CLA increased the expression of luciferase reporter in a dose-dependent manner, suggesting that it is a PPAR γ agonist. However, *c*-9, *t*-11 CLA is a weak agonist compared with TZDs, because 400 μM CLA increased luciferase activity to the same extent as 10 μM troglitazone. *t*-10, *c*-12 CLA also stimulated luciferase activity, but even less effectively than the *c*-9, *t*-11 CLA isomer (Fig. 6A). Even at 400 μM , *t*-10, *c*-12 CLA did not achieve the activity of 10 μM troglitazone. When *c*-9, *t*-11 CLA and *t*-10, *c*-12 CLA were mixed together (Fig. 6A, MI-CLA), the luciferase activity was the same as that with half the concentration of *c*-9, *t*-11 CLA, suggesting that in the mixture the *t*-10, *c*-12 CLA isomer does not increase or decrease reporter expression over the *c*-9, *t*-11 CLA isomer.

In Fig. 6B, we compared the effects of the individual CLA isomers as modulators of PPAR γ . When added with troglitazone, the PPAR γ antagonist GW9662 decreased the activity of the luciferase reporter by $\sim 50\%$ compared with troglitazone alone ($P < 0.05$). Conversely, when *c*-9, *t*-11 CLA was added with troglitazone, a further increase in luciferase activity was observed, suggesting that *c*-9, *t*-11 CLA has agonist properties. Conversely, addition of the *t*-10, *c*-12 CLA isomer decreased luciferase activity to the same level as the GW9662 antagonist, but at a 10- to 20-fold higher concentration. These results suggested that the two isomers of CLA may act as differential ligands for PPAR γ , *c*-9, *t*-11 CLA as an agonist and *t*-10, *c*-12 CLA as an antagonist.

The observations with the PPRE-luciferase reporter assay suggested that *t*-10, *c*-12 CLA might not have direct effects on the inhibition of PPAR γ activation but that *t*-10, *c*-12 CLA may act as a PPAR γ modulator that blocks troglitazone-induced PPAR γ activation.

DISCUSSION

The aim of our study was to gain insight into the mechanisms of the effects of *t*-10, *c*-12 CLA on adipocytes. Currently, CLA is used as a weight-loss supplement, and it will be important to establish its effects on fully differentiated adipocytes and differentiating preadipocytes, as human adipose tissue contains a mixture of cell types (53). Because both hyperplasia and hypertrophy can contribute to the onset of obesity, an understanding of the effects of CLA on both processes will be important.

In contrast to the TG-reducing effects of *t*-10, *c*-12 CLA on differentiating 3T3-L1 preadipocytes (Fig. 1A) (9–12), the levels of TG in differentiated adipocytes acutely treated with *t*-10, *c*-12 CLA were not reduced compared with those after other treatments (Fig. 2A). This suggests that the dramatic TG-lowering effects of *t*-10, *c*-12 CLA may target the differentiation process and therefore may only be

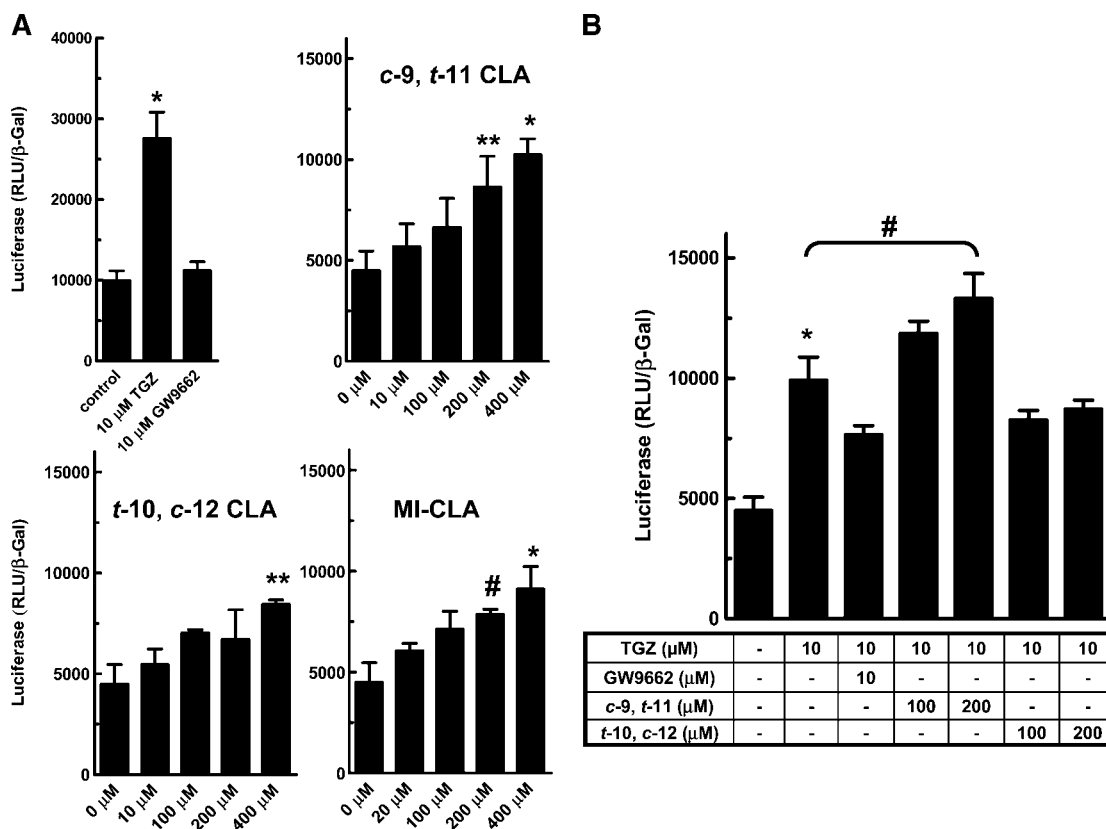


Fig. 6. PPAR γ activation by CLA isomers and PPAR γ modulators in a peroxisome proliferator response element (PPRE) reporter assay. Transcriptional activation of cellular PPAR γ was assessed using a PPRE reporter assay in CHO-K1 cells. A: Cells were transiently transfected with PPRE-luciferase expression plasmid and were then treated with troglitazone (TGZ), *c*-9, *t*-11 CLA, *t*-10, *c*-12 CLA, or MI-CLA for 24 h. B: After transfection, cells were treated for 24 h with troglitazone, troglitazone plus GW9662, troglitazone plus *c*-9, *t*-11 CLA, or troglitazone plus *t*-10, *c*-12 CLA. Cells were collected by lysis, and PPRE-luciferase activity was determined. β -Galactosidase activity, from a cotransfected constitutive expression plasmid, was used as a transfection control. Experiments were performed in triplicate wells, and results are expressed as relative luminescence units (RLU) normalized to β -galactosidase activity (means \pm SD). * $P < 0.001$, ** $P < 0.01$, # $P < 0.05$.

effective if CLA is present during the differentiation of preadipocytes into adipocytes. Alternatively, mature adipocytes may require >16 h in the presence of *t*-10, *c*-12 CLA for measurable changes in TG levels to be detected.

It has been documented that the low levels of adiponectin that are observed in obesity increase after weight loss (54). However, the *t*-10, *c*-12 CLA-mediated reduction in TG levels in 3T3-L1 cells was accompanied instead by a dramatic decrease in cellular adiponectin mass, and this was true even after treatment of differentiated adipocytes. This suggests not only that the apparent decrease in adipose tissue mass with *t*-10, *c*-12 CLA supplementation may be different from other mechanisms of weight loss but also that it may indicate compromised adipocyte function. The marked decreases in PPAR γ protein are consistent with this loss of function. Because low levels of adiponectin are associated with numerous diseases, including atherosclerosis (28) and type II diabetes (29), the reductions in adiponectin secretion with *t*-10, *c*-12 CLA supplementation cannot be considered beneficial. Depletion of adiponectin from the adipocyte may be an indication that the *t*-10, *c*-12 CLA isomer impairs adipocyte function rather than improves TG metabolism in the adipocyte.

Assessment of adiponectin oligomers after supplementation with *t*-10, *c*-12 CLA revealed that, in addition to decreasing the synthesis of adiponectin, *t*-10, *c*-12 CLA also appeared to impair the assembly of adiponectin oligomers in 3T3-L1 cells. Although the mechanism of this change has not yet been fully characterized, glycosylation has been shown to be essential for the assembly of HMW adiponectin (33) and may be necessary for the assembly of MMW adiponectin as well (34). Treatment with *t*-10, *c*-12 CLA resulted in the formation of a LMW complex with a lower apparent molecular weight, and we believe that this may be a form of LMW adiponectin that lacks appropriate glycosylation. This may explain why, in *t*-10, *c*-12 CLA-treated cells, MMW oligomers fail to assemble. Analysis of the secreted adiponectin oligomers after acute *t*-10, *c*-12 CLA treatment revealed that secretion of HMW adiponectin was maintained, but there was a significant decrease in the levels of secreted MMW adiponectin. This suggests that in differentiated 3T3-L1 adipocytes, oligomer secretion may be regulated and HMW adiponectin may be the preferred secretory form.

Treatment with *c*-9, *t*-11 CLA did not affect 3T3-L1 cells in the same manner as *t*-10, *c*-12 CLA. TG and adiponectin

mass were not reduced in *c*-9, *t*-11 CLA-treated cells, and the oligomer pattern was not different from that in cells treated with LA. When given as a mixed isomer supplement, the presence of the *c*-9, *t*-11 CLA isomer compensated for the effects of *t*-10, *c*-12 CLA on adiponectin synthesis (in differentiated 3T3-L1 cells) but was unable to normalize TG levels or restore oligomer assembly. This suggests that the effects of the *t*-10, *c*-12 CLA isomer may occur through at least two separate pathways.

To explore these differences mechanistically, we used a PPAR γ agonist, troglitazone, and a PPAR γ antagonist, GW9662, during 3T3-L1 differentiation. Addition of troglitazone with *t*-10, *c*-12 CLA during differentiation restored the accumulation of cellular TG, whereas supplementation with GW9662 alone decreased TG levels, suggesting that the reduction in TG levels mediated by *t*-10, *c*-12 CLA is through a PPAR γ -dependent mechanism. TG levels were decreased further after treatment with both GW9662 and *t*-10, *c*-12 CLA, which may indicate that *t*-10, *c*-12 CLA decreases TG levels through an additional mechanism, perhaps independent of PPAR γ antagonism. Further work is required to establish the presence and the nature of such a secondary pathway.


Direct effects of CLA isomers on PPAR γ expression were examined using a luciferase reporter assay system. This assay indicated that the *c*-9, *t*-11 CLA isomer was a PPAR γ agonist, whereas the *t*-10, *c*-12 CLA isomer appeared to be a partial antagonist or weak agonist. Many of the genes that have been shown to have decreased expression after *t*-10, *c*-12 CLA treatment are PPAR γ -activated; thus, the true *t*-10, *c*-12 CLA target may in fact be PPAR γ (20). However, the way in which *t*-10, *c*-12 CLA affects PPAR γ is still unclear. Using luciferase reporter assays, a number of studies have examined the ability of *t*-10, *c*-12 CLA and *c*-9, *t*-11 CLA to activate PPAR γ (12, 14, 20, 22). Most of the studies have used nonadipose cell lines and showed that both isomers were weak or very weak PPAR γ activators, as shown in this study. Brown et al. (22) used 3T3-L1 cells transiently transfected with a luciferase reporter construct containing a PPRE. This study showed that treatment with either *c*-9, *t*-11 CLA or *t*-10, *c*-12 CLA led to a slight decrease in luciferase activity, suggesting that these two isomers may antagonize PPAR γ activity in adipocytes. The lack of consensus in these results suggests that activation or antagonism of PPAR γ is not likely the way in which *t*-10, *c*-12 CLA exerts its effects; indeed, both isomers have been shown to act only as weak PPAR γ ligands. If *t*-10, *c*-12 CLA itself does not interact directly with PPAR γ , another way in which it might exert its effects is by influencing how other PPAR γ ligands are able to interact with this transcription factor. Two studies (12, 22) have examined how CLA isomers modulate the ability of TZDs to bind to, and activate, PPAR γ . Both studies reported that CLA isomers were able to antagonize the ligand-dependent activation of PPAR γ , with the *t*-10, *c*-12 CLA isomer having a slightly more pronounced effect. Our studies suggest that the two CLA isomers may have opposing effects on PPAR γ expression and are consistent with the changes we observed in cellular PPAR γ protein levels. Further studies

to examine the affinities, binding sites, and activating abilities of the CLA isomers are required.

In contrast to its effects on TG levels, the *t*-10, *c*-12 CLA-mediated effects on adiponectin mass do not appear to occur through a PPAR γ -dependent pathway, as adiponectin levels were not normalized by troglitazone or affected by GW9662. Although PPAR γ has been implicated in the expression of adiponectin (51), CCAAT/enhancer-binding protein α has also been reported to be necessary for maximal expression (52, 55). *t*-10, *c*-12 CLA has been shown to decrease the expression of this transcription factor as well (22).

It is thought that TZDs improve insulin sensitivity through increased assembly and secretion of adiponectin oligomers (48). Analysis of the oligomer pattern in cells treated with *t*-10, *c*-12 CLA and troglitazone showed that, despite reduced levels of both LMW and MMW adiponectin, the ratio between the two was normalized to control levels and the aberrant molecular weight of LMW adiponectin was no longer evident. Therefore, although the *t*-10, *c*-12 CLA isomer appears to impair adiponectin synthesis, reduce TG levels, and interfere with oligomer assembly, it does so via two distinct mechanisms. Adiponectin oligomer assembly and TG storage appear to be PPAR γ -dependent, whereas adiponectin mass may be PPAR γ -independent.

The mechanism by which antagonism of PPAR γ impairs the assembly of adiponectin oligomers is not clear from these studies. However, it is possible that the decrease in downstream PPAR γ -induced genes, such as GLUT4, may be involved. A lack of sufficient glucose uptake by the adipocyte may affect the normal glycosylation of adiponectin, hindering the assembly of its biologically active oligomers. Alternatively, antagonism of PPAR γ may block the expression of other, as yet unidentified, enzymes involved in oligomer assembly.

In conclusion, treatment of 3T3-L1 cells with *t*-10, *c*-12 CLA reduces TG accumulation and adiponectin production and assembly by impairing adipocyte function. The PPAR γ agonist, troglitazone, is able to normalize TG levels and adiponectin oligomer assembly in *t*-10, *c*-12 CLA-treated cells, indicating that both processes may occur through a PPAR γ -dependent mechanism. The synthesis of adiponectin, on the other hand, is not restored by troglitazone, nor is it affected by the presence of a PPAR γ antagonist, suggesting that the effects of *t*-10, *c*-12 CLA on adiponectin production are not regulated by PPAR γ . Further work is warranted to determine the nature of the PPAR γ -dependent and -independent mechanisms by which *t*-10, *c*-12 CLA alters adipocyte metabolism. However, it appears that the effects of *t*-10, *c*-12 CLA on the adipocyte may pose more risks than benefits. 

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