# *Trans*10, *cis*12-conjugated linoleic acid prevents triacylglycerol accumulation in adipocytes by acting as a PPARγ modulator

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**Abstract A group of polyunsaturated fatty acids called conjugated linoleic acids (CLAs) are found in ruminant products, where the most common isomers are *cis*9, *trans*11 (*c*9,*t*11) and *trans*10, *cis*12 (*t*10,*c*12) CLA. A crude mixture of these isomers has been shown in animal studies to alter body composition by a reduction in body fat mass as well as an increase in lean body mass, with the *t*10,*c*12 isomer having the most pronounced effect. The objective of this study was to establish the molecular mechanisms by which *t*10,*c*12 CLA affects lipid accumulation in adipocytes. We have shown that *t*10,*c*12 CLA prevents lipid accumulation in human and mouse adipocytes at concentrations as low as 5 μM and 25 μM, respectively. *t*10,*c*12 CLA fails to activate peroxisome proliferator-activated receptor γ (PPARγ) but selectively inhibits thiazolidinedione-induced PPARγ activation in 3T3-L1 adipocytes. Treatment of mature adipocytes with *t*10,*c*12 CLA alone or in combination with Darglitazone down-regulates the mRNA expression of PPARγ as well as its target genes, fatty acid binding protein (aP2) and liver X receptor α (LXRα). Taken together, our results suggest that the *trans*10, *cis*12 CLA isomer prevents lipid accumulation in adipocytes by acting as a PPARγ modulator.**—Granlund, L., L. K. Juvet, J. I. Pedersen, and H. I. Nebb. ***Trans*10, *cis*12-conjugated linoleic acid prevents triacylglycerol accumulation in adipocytes by acting as a PPARγ modulator. *J. Lipid Res.* 2003. 44: 1441–1452.**

**Supplementary key words** fatty acid binding protein • liver X receptor • Darglitazone

Adipocytes play a central role in maintaining lipid homeostasis and energy balance in vertebrates by storing triacylglycerols (TAGs) or releasing free fatty acids in response to changes in energy demands (1). The increase in incidence of obesity and Type II diabetes has focused attention on all aspects of adipocyte biology (2). In this regard, transcription factors have been identified that directly influence adipogenesis, in which peroxisome proliferator-activated receptor γ (PPARγ) has been shown to play a crucial role (3).

PPARγ is a member of the nuclear hormone receptor super family (4), which forms heterodimers with the retinoid X receptor (RXR), and regulates gene expression by binding to a PPAR-responsive element (PPRE) of the direct repeat 1 type in the promoter region of a variety of target genes (5, 6). PPARγ2 is found almost exclusively in adipose tissue and has been linked to adipocyte differentiation (7, 8). Natural high-affinity ligands for PPARγ have not been identified, but polyunsaturated fatty acids and 15-deoxy-12,14-prostaglandin J2 show micromolar affinity for the receptor, in line with their serum levels (9, 10). Interest in the PPARγ receptor field increased when a new class of synthetic antidiabetic drugs, the thiazolidinediones (TZDs), were shown to act as high-affinity ligands for PPARγ (4). The effects of these ligands are mediated by changes in the transcriptional rate of PPARγ target genes (11). Even though TZDs are used in the treatment of Type II diabetes, they are shown to induce adiposity and body weight gain in rodents (12) as well as weight gain in human patients (13). On the other hand, Mukherjee and coworkers have described a synthetic PPARγ modulator, LG100641, which blocks adipocyte differentiation but stimulates glucose uptake in 3T3-L1 adipocytes (14).

Conjugated linoleic acids (CLAs) are a group of positional and geometric isomers of linoleic acid (C18:2n-6), produced by bacterial biohydrogenation in the ruminant gut (15). The best sources for CLAs in the human diet are ruminant meat and dairy products (16, 17), in which the two predominant isomers are *cis*9, *trans*11 (*c*9,*t*11) and *trans*10, *cis*12 (*t*10,*c*12) CLA. In rodents, a crude mixture of CLA isomers is shown to have anticarcinogenic (18), antiatherogenic (19, 20), antidiabetic (21), and antiobe-

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Abbreviations: aP2, fatty acid binding protein (also known as aFABP); CLA, conjugated linoleic acid; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SGBS, Simpson-Golabi-Behmel syndrome; TAG, triacylglycerol, TZD, thiazolidinediones.

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sity (21, 22) effects. With regard to the antiobesity effect observed in mice, pigs, and hamsters fed CLA isomers, the body composition is altered by a reduced body fat mass and an increased lean body mass (22–27).

Several in vitro studies have shown that treatment with CLA isomers attenuates lipid content in adipocytes (22, 24, 28, 29). A reduced intracellular TAG content in mature 3T3-L1 adipocytes was found to be due to the *t*10,*c*12 isomer (24, 28). This also has been observed in primary cultures of stromal vascular cells from human adipose tissue (30).

The mechanisms of action regarding CLA's effects on lipid accumulation in adipocytes are still not known. It has been shown, however, that CLA isomers are ligands for PPARα, with *c*9,*t*11 CLA as a better ligand than *t*10,*c*12 CLA, but with a lower affinity than the high-affinity PPARα agonist, WY 14,643 (31).

The purpose of this study was to better understand the molecular mechanisms by which *t*10,*c*12 CLA affects lipid accumulation in adipocytes. In this study, we have shown that *t*10,*c*12 CLA prevents lipid accumulation in both mouse and human adipocytes. Furthermore, we have demonstrated that even though *t*10,*c*12 CLA is not a PPARγ ligand, it is able to reduce Darglitazone-induced PPARγ activation and down-regulate Darglitazone-induced gene expression of PPARγ and the target genes fatty acid binding protein (aP2) and liver X receptor α (LXRα) in mature adipocytes. Taken together, our data indicate that *t*10,*c*12 CLA prevents lipid accumulation in adipocytes by acting as a PPARγ modulator.

## MATERIALS AND METHODS

### Materials

Dulbecco's Modified Eagle's Medium (DMEM), DMEM/Nutrient mixture F12 medium, penicillin, streptomycin, L-glutamine, WY 14,643, linoleic- and α-linolenic (linolenic) acid were from Sigma (St. Louis, MO). Darglitazone was kindly provided by Medicinal Chemistry, AstraZeneca R and D, Mølndal, Sweden. The CLA isomers *c*9,*t*11 and *t*10,*c*12 (purity >90%) were a gift from Natural ASA, Hovdebygda, Norway. Other chemicals were obtained from Sigma. Agarose was purchased from Bio-Rad Laboratories, Inc. (Richmond, CA). Multiple DNA labeling systems and radiolabeled [α-$^{32}$P]deoxy-CTP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Bio-Trans nylon filter was from ICN Bio-chemicals, Inc. (Irvine, CA). Dual luciferase assay was obtained from Promega Corp. (Madison, WI).

### Cell culture

The 3T3-L1 adipocyte cell line [American Type Culture Collection (ATCC), Manassas, VA] was maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin/streptomycin at 37°C. 3T3-L1 cells were grown to confluence and then exposed to adipogenic reagents for 3 days, followed by culturing for 3 more days in a medium containing insulin, as described elsewhere (32). The cells were then grown 5 more days before staining with Oil Red O to visualize lipid content at Day 11 (D11) of differentiation. Insulin at a concentration of 1 μg/ml, methylisobutylxanthin at 0.5 mM, and dexamethasone at 0.1 μM were used as adipogenic reagents.

Human Simpson-Golabi-Behmel syndrome (SGBS) adipocytes were cultured and differentiated as described by Wabitsch and coworkers (33).

Fatty acids were added as a 6 mM stock solution dissolved in 6% fatty acid-free bovine serum albumin (BSA). Linoleic and linolenic acids were used at concentrations of 100 μM, Darglitazone at 1 μM, and WY 14,643 at 5 μM unless otherwise stated in the figure legends. *C*9,*t*11 CLA and *t*10,*c*12 CLA were used in concentrations as stated in the figure legends.

### Constructs

The reporter construct containing 1,500 bp of the 5′-flanking region of the mLXRα gene in front of luciferase as a reporter [pLXRα(−1500/+1800)LUC] was made as described earlier (34). pGL3-basic luciferase reporter vector was obtained from Promega Corp. pCMV-RXRα and pSG5-mPPARα expression vectors were gifts from Dr. Jan-Åke Gustafsson, whereas pSG5-mPPARγ2 expression vector was a gift from Dr. Johan Auwerx. pSG5-GAL4-PPARα and pSG5-GAL4-PPARγ chimera expression constructs containing the ligand binding domain (LBD) of mouse PPARα or PPARγ and the $(UAS)_5$-tk-LUC reporter construct were generous gifts from Dr. Krister Bamberg.

### Transfection and luciferase assay

Transient transfections of COS-1 cells were performed in 6-well plates with $2 \times 10^5$ cells per well after the calcium phosphate precipitation method essentially as described by Graham and van der Eb (35). For full-length PPAR transfection studies, each well received 5 μg reporter construct [pLXRα(−1500/+1800)LUC)], 2.5 μg pSV-β-galactosidase as an internal control, 0.4 μg pCMV-RXRα, and 0.4 μg pSG5-PPARα or pSG5-PPARγ2. For LBD transfection studies, each well received 0.5 μg of the reporter construct $(UAS)_5$-tk-LUC, 1 μg pSV-β-galactosidase as an internal control, and 0.5 μg pSG5-GAL4-PPARα or pSG5-GAL4-PPARγ. The cells were harvested after 72 h, and the luciferase activity was measured according to the protocol (Promega). The luciferase activity was normalized against β-galactosidase activity measured by incubating 10 μl extract with 0.28 mg o-nitrophenyl-β-D-galactopyranoside in 50 mM phosphate buffer, pH 7.0, 10 mM KCl, 1 mM $MgCl_2$ for 30 min at 30°C and reading absorbance at 405 nm.

3T3-L1 preadipocytes were grown to confluence in 6-well plates and differentiated as described above. The adipocytes were transfected at D11 of differentiation using 16 μl Lipofectamine Plus reagent, 4 μl Lipofectamine (Life Technologies, Inc.), 1 μg pLXRα(−1500/+1800)LUC, 0.2 μg pCMV-RXRα, 0.2 μg pSG5-PPARγ, and 100 ng pTK Renilla luciferase as a control of transfection efficiency. Three hours after transfection, cells were cultured in serum containing medium and incubated for 48 h in the same medium containing appropriate agents, as indicated in the figure legends. The luciferase activities were measured as recommended by the manufacturer (Dual Luciferase assay, Promega). All transfections were performed in triplicate.

### Protein content

Protein was measured using the bicinchoninic acid assay (Uptima, Interchim, France).

### Intracellular TAG content

3T3-L1 adipocytes were harvested and sonicated after addition of water at 20% output for 10 s. Intracellular TAG content was measured using a colorimetric assay (Triglycerides Enzymatique PAP 150, bioMerieux) that quantifies the glycerol content of the samples. Each sample was transferred to a 96-well plate, and absorbance was measured at 492 nm on a microtiter plate reader (Titertek Multiskan Plus, Labsystems, Helsinki).



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### Cell viability

To determine if adherent cell number was altered by CLA treatment, the cell monolayer, after spent media was discarded, was harvested, and total number of adherent cells was counted after trypan blue staining. In short, cells were seeded in 6-well plates and stimulated as described in the figure legends. The medium was removed and adherent cells harvested and centrifuged at 1,200 *g* for 2 min and redisolved in cell medium. The cell suspension was treated with 0.4% filtered trypan blue stain dissolved in an isotonic solution. Total number of adherent cells, as well as number of living and dead adherent cells, was calculated and expressed as cell number in each well.

### Preparation and analysis of RNA

Total RNA from mature 3T3-L1 adipocytes was extracted using Trizol, as recommended by the manufacturer (Life Technologies, Inc., Gaithersburg, MD), and 20 μg total RNA was used for Northern blotting, as described earlier (36). Probes used were PPARγ (Dr. Johan Auwerx), aP2 (37), and LXRα (38). cDNA probe for human ribosomal protein L27 (ATCC-107385) was purchased from ATCC and used as control for equal RNA loading.

### Oil Red O staining

Oil Red O staining was used to monitor lipid accumulation during adipocyte differentiation, essentially as described by Wu, Bucher, and Farmer (39).

### Statistical analysis

Data from these studies were analyzed by Student's *t*-test. For all analyses, the acceptable level of significance was $P \leqslant 0.05$. Statistical analysis was conducted using SPSS 9.0 (SPSS Inc., Chicago, IL).

## RESULTS

### *t*10,*c*12 CLA inhibits lipid accumulation in adipocytes

Mouse 3T3-L1 adipocytes were stimulated with 25 μM CLA from D0 until D11 of differentiation, whereas human SGBS adipocytes were stimulated with 5 μM CLA from D0 until D12, at which days lipid content was visualized by Oil Red O staining (**Fig. 1A**). A marked difference in lipid accumulation between *c*9,*t*11 CLA- and *t*10,*c*12 CLA-treated cells was observed. 3T3-L1 and SGBS adipocytes treated with 25 μM and 5 μM *c*9,*t*11 CLA, respectively, had nearly the same lipid content as the control, whereas *t*10,*c*12 CLA stimulation resulted in a pronounced reduction in lipid content.

To confirm the Oil Red O staining observations, intracellular TAG content was quantified in 3T3-L1 and SGBS cells at D11 and D12 of differentiation (Fig. 1B). For both cell types, a significantly lower TAG content was observed with 25 μM and 5 μM *t*10,*c*12 CLA compared with controls and *c*9,*t*11 CLA-treated cells.

Cell viability, evaluated as number of living and dead adherent cells compared with total number of adherent cells, was calculated for both cell lines after CLA treatment at D11 and D12 of differentiation. In 3T3-L1 adipocytes, no significant differences in adherent cell numbers were observed (Fig. 1C). In human adipocytes, there was a significant reduction in total cell number as well as living cells in the *t*10,*c*12 CLA-treated cells compared with the control. However, there were no significant differences between the two CLA treatments. Concentrations of *t*10,*c*12 CLA as high as 50 μM or above were cytotoxic in the mouse adipocytes, whereas concentrations up to 20 μM were not toxic for the human cells (data not shown). Treatment with both CLA isomers resulted in an increased protein content of adherent 3T3-L1 cells compared with the control; however, this was only significant for the *c*9,*t*11 isomer (data not shown). In SGBS cells, both CLA isomers resulted in a reduced protein content compared with the control. However, there was no significant difference in protein content between the two CLA treatments for either of the cell lines (data not shown).

### *t*10,*c*12 CLA acts as a PPARγ modulator

To study the mechanisms underlying the lipid-reducing effect of *t*10,*c*12 CLA in adipocytes, we tested whether the CLA isomers affected PPARα and PPARγ activity differently. We first examined the effect of CLA on the transcriptional activity of PPARα and PPARγ by utilizing the 5′-flanking region of LXRα, a well-known PPAR target gene (40, 41). COS-1 cells were cotransfected with the LXRα-PPRE-LUC-reporter gene and PPARα or PPARγ expression vectors. The cells were treated with different agonists for PPARα and PPARγ for 72 h (**Fig. 2A**). For both PPARα and PPARγ, the CLA isomers only slightly induced reporter gene activity at concentrations as low as 5 μM to 25 μM, compared with cotransfected cells treated with vehicle (BSA). Treatment with CLA isomers above 50 μM was toxic to the cells, as demonstrated by trypan blue exclusion test (data not shown). Compared with the two CLA isomers, 100 μM linoleic and linolenic acid induced the luciferase activity by 2- and 5-fold, respectively, in PPARα cotransfected cells. Linolenic acid did not induce reporter gene activity in PPARγ cotransfected cells. WY 14,643 and Darglitazone are well-known PPARα and PPARγ activators, respectively, and were used in these transactivation systems as positive controls.

To examine whether the CLA isomers are able to activate the LBD of PPARα or PPARγ, another transactivation system was used. COS-1 cells were cotransfected with the pSG5-GAL4-PPARα or pSG5-GAL4-PPARγ chimera expression constructs as well as the $(UAS)_5$-tk-LUC reporter construct (Fig. 2B). For both PPARα and PPARγ, the CLA isomers failed to induce reporter gene activity.

In the same transfection system, we tested whether the CLA isomers could compete with WY 14,643 and Darglitazone in activating PPARα and PPARγ, respectively. CLA failed to antagonize the effect of WY 14,643 on reporter gene activity (data not shown). However, CLA competitively reduced the Darglitazone-induced transactivation of PPARγ in a dose-dependent manner after stimulation with increasing concentrations of CLA (**Fig. 3A**). The inhibition of Darglitazone-induced reporter gene activity was more pronounced for the *t*10,*c*12 isomer compared with the *c*9,*t*11 isomer. However, the difference between the two isomers was only significant at 1 μM. This observation indicates that CLA might act as a PPARγ modulator, with



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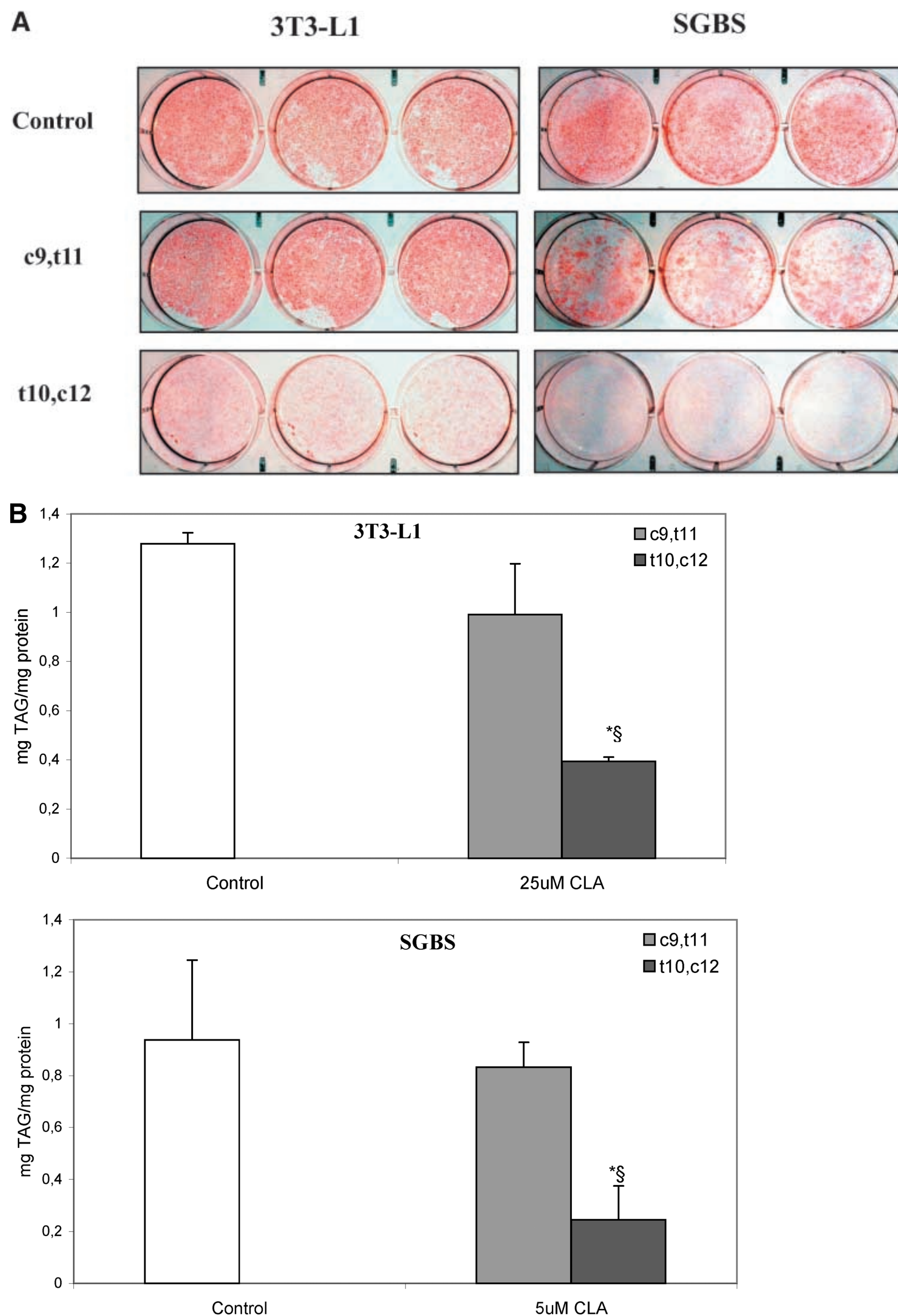


**Fig. 1.** Triacylglycerol (TAG) accumulation in adipocytes. Mouse 3T3-L1 and human Simpson-Golabi-Behmel syndrome (SGBS) preadipocytes were treated with either of the two conjugated linoleic acid (CLA) isomers from Day 0 (D0) until D11 (3T3-L1) or D12 (SGBS). The adipocytes were treated with 25 μM and 5 μM of *cis*9, *trans*11 (*c*9,*t*11) and *trans*10, *cis*12 (*t*10,*c*12) CLA, or vehicle [bovine serum albumin (BSA)] as control in 3T3-L1 and SGBS cells. A: Adipocytes were stained with Oil Red O to visualize lipid content on D11 (3T3-L1) and D12 (SGBS) of differentiation. B: TAG level in the cells at D11 (3T3-L1) (upper panel) and D12 (SGBS) (lower panel) of differentiation. The results are given as mean ± SD (n = 3). Each experiment was performed in triplicate. Significantly different ($P \leqslant 0.05$) from control* and from *c*9,*t*11 CLA-treated[§] cells. C: Total number of adherent cells, as well as adherent living and dead cells on D11 (3T3-L1) (upper panel) and D12 (SGBS) (lower panel) of differentiation. The results are given as mean ± SD (n = 3). Each experiment was performed in duplicate. *Significantly different ($P \leqslant 0.05$) from control cells.



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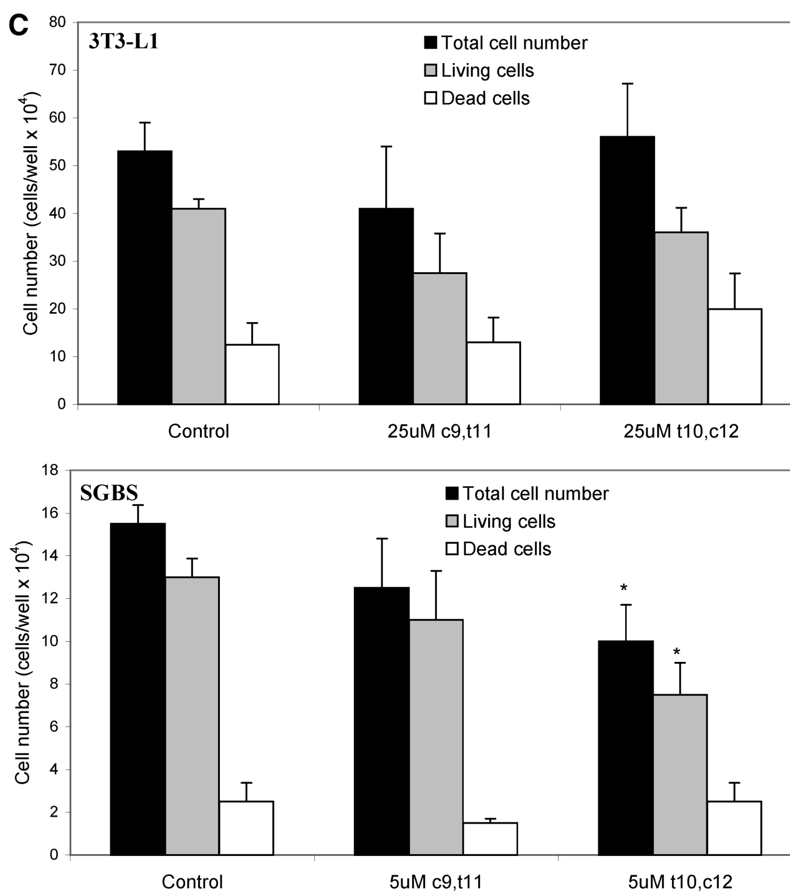


**Fig. 1.** *Continued.*

*t*10,*c*12 CLA as the most effective competitor. This modulatory effect was also observed using the LXRα-PPRE-LUC reporter construct in COS-1 cells cotransfected with full-length mouse PPARγ expression plasmid (Fig. 3B). Using the full-length PPARγ, there was an even more pronounced difference between the two CLA isomers in the modulatory effect of Darglitazone-induced transactivation. The difference between the two CLA isomers was significant for the concentrations 1 μM, 15 μM, and 25 μM. This modulatory effect was also observed when human PPARγ was cotransfected in the same transfection system (data not shown).

To confirm these data in a more physiological system, mature 3T3-L1 adipocytes were cotransfected with the LXRα-PPRE-LUC reporter construct and mouse PPARγ expression plasmid. The adipocytes were treated with Darglitazone either alone or in combination with increasing concentrations of CLA (Fig. 3C). Both CLA isomers resulted in a dose-dependent attenuation of Darglitazone-induced reporter gene activity. The *t*10,*c*12 isomer had a more pronounced effect, one that was not, however, significantly different from that of the *c*9,*t*11 isomer.

To examine whether *t*10,*c*12 CLA also functions as a PPARγ modulator in adipocytes by inhibiting Darglitazone-induced differentiation, 3T3-L1 cells were stimulated with 1 μM Darglitazone either alone or in combination with 25 μM of either of the two CLA isomers from D0 until D11 of differentiation (**Fig. 4**). The combination of *t*10,*c*12 and Darglitazone resulted in a marked reduction in lipid content compared with Darglitazone stimulation alone, as observed by Oil Red O staining (Fig. 4A). To confirm the observations by Oil Red O staining, intracellular TAG content was measured. Darglitazone treatment in combination with either of the two CLA isomers resulted in a lower TAG content, compared with Darglitazone treatment alone (Fig. 4B). However, the effect was more pronounced for the *t*10,*c*12 isomer, significantly lower than for Darglitazone treatment in combination with *c*9,*t*11 CLA.

### *t*10,*c*12 CLA down-regulates PPARγ gene expression as well as its target genes, aP2 and LXRα

To study whether the expression of PPARγ, aP2, and LXRα was affected by CLA treatment, 3T3-L1 cells were stimulated with 1 μM Darglitazone, 25 μM *c*9,*t*11 CLA, 25 μM *t*10,*c*12 CLA, and 1 μM Darglitazone in combination with either of the two CLA isomers (**Fig. 5**). PPARγ mRNA expression was markedly down-regulated after *t*10,*c*12 CLA treatment, whereas *c*9,*t*11 CLA had no effect compared with vehicle (Fig. 5A). However, Darglitazone in combination with *c*9,*t*11 CLA resulted in an up-regulation of the PPARγ mRNA expression compared with Darglitazone treatment alone.

For both aP2 and LXRα, *t*10,*c*12 CLA resulted in a clear down-regulation of gene expression compared with vehi-



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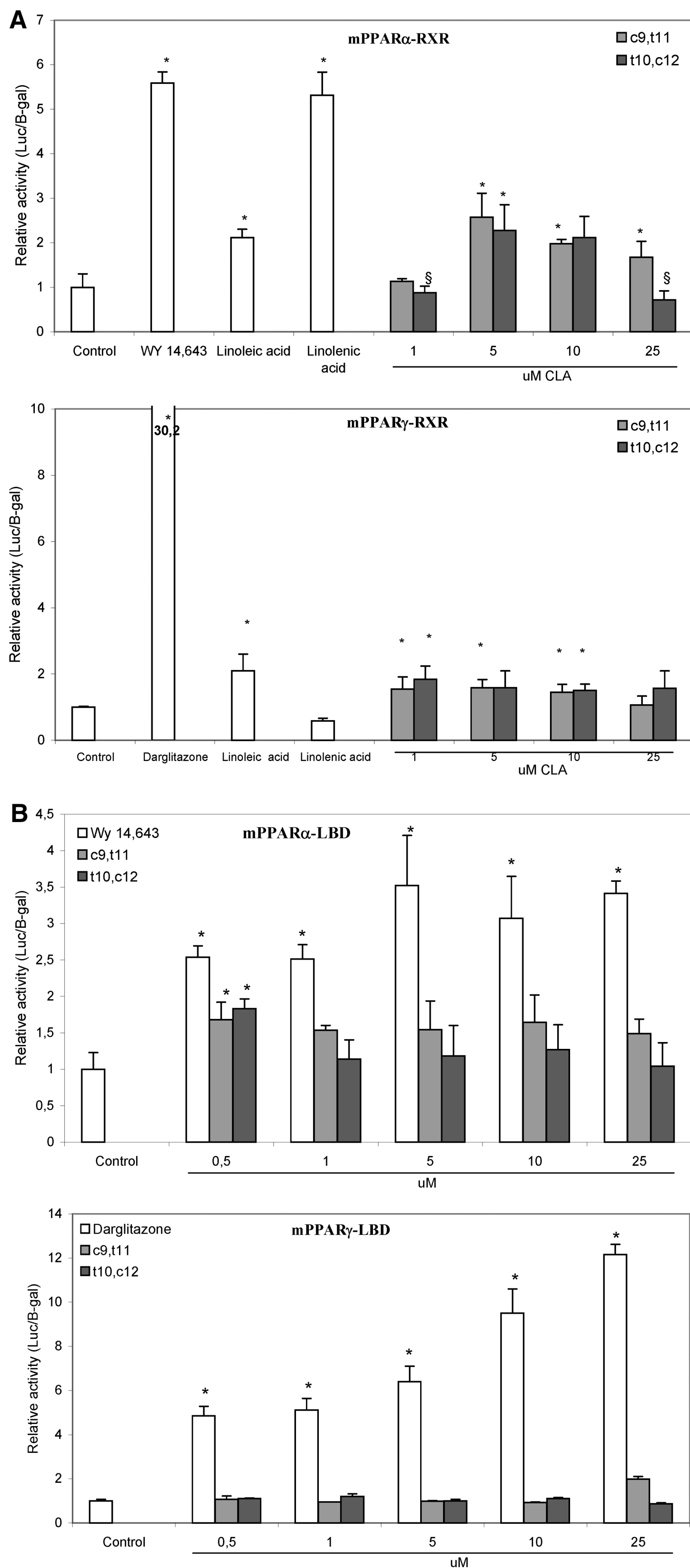


**Fig. 2.** CLA isomers as weak ligands for peroxisome proliferator-activated receptor α (PPARα) and PPARγ. A: A construct containing 1,500 bp of the 5′-flanking region of the mLXRα gene in front of a luciferase reporter [pLXRα(−1,500/+1,800)LUC] was cotransfected with 0.4 μg of an expression plasmid of retinoid X receptor α (RXRα) (pCMV-RXRα) and 0.4 μg of an expression plasmid encoding mPPARα (pSG5-PPARα) (upper panel) or mPPARγ (pSG5-PPARγ) (lower panel) into COS-1 cells. The cells were treated with 5 μM WY 14,643 (PPARα), 1 μM Darglitazone (PPARγ), 100 μM linoleic acid, 100 μM linolenic acid, and increasing concentrations of *c*9,*t*11 and *t*10,*c*12 CLA (1–25 μM) and harvested after 72 h. β-Galactosidase activity was used as an internal control. The values are presented relative to cotransfected cells stimulated with vehicle (BSA) as control, and given as the mean ± SD (n = 3). Each experiment was performed in triplicate. Significantly different ($P \leqslant 0.05$) from control* and from *c*9,*t*11 CLA-treated§ cells. B: COS-1 cells were cotransfected with a chimeric receptor expression plasmid, pSG5-GAL4-mPPARα (upper panel), or pSG5-GAL4-mPPARγ (lower panel), and the reporter plasmid $(UAS)_5$-tk-LUC, treated with increasing concentrations of WY 14,643 (PPARα), Darglitazone (PPARγ), and *c*9,*t*11 and *t*10,*c*12 CLA (0, 5–25 μM), and harvested after 72 h. β-Galactosidase activity was used as an internal control. The values are presented relative to cotransfected cells stimulated with vehicle (BSA) as control, and are given as mean ± SD (n = 3) Each experiment was performed in triplicate. * Significantly different ($P \leqslant 0.05$) from control.

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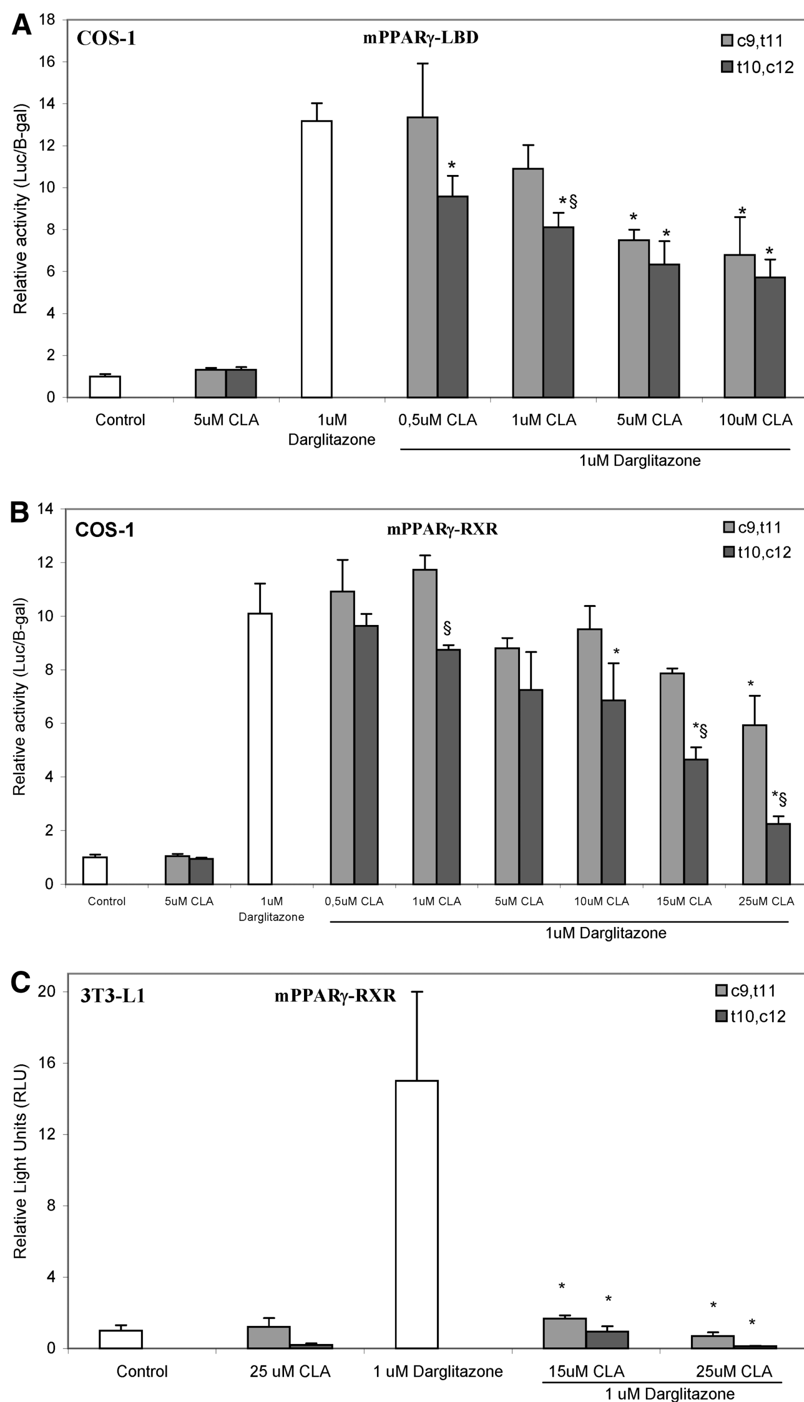


**Fig. 3.** Modulating effect of CLA on Darglitazone-induced PPARγ activation. A: COS-1 cells were cotransfected with a chimeric receptor expression plasmid, pSG5-GAL4-mPPARγ, and the reporter plasmid $(UAS)_5$-tk-LUC. The cells were treated with 5 μM *c*9,*t*11 CLA, 5 μM *t*10,*c*12 CLA, or 1 μM Darglitazone, either alone or in addition to increasing concentrations of CLA (0, 5–10 μM) and harvested after 72 h. β-Galactosidase activity was used as an internal control. The values are presented relative to cotransfected cells stimulated with vehicle (BSA) as control, and given as mean ± SD (n = 3). Each experiment was performed in triplicate. Significantly different ($P \leq 0.05$) from Darglitazone-treated cells* and from cells treated with Darglitazone in combination with *c*9,*t*11 CLA§. B: A construct containing 1,500 bp of the 5′-flanking region of the mLXRα gene in front of a luciferase reporter [pLXRα(−1,500/+1,800)LUC] was cotransfected with 0.4 μg of an expression plasmid of RXRα (pCMV-RXRα) and 0.4 μg of an expression plasmid encoding mPPARγ (pSG5-PPARγ) into COS-1 cells. The cells were treated with 5 μM *c*9,*t*11 CLA, 5 μM *t*10,*c*12 CLA, or 1 μM Darglitazone, either alone or in addition to increasing concentrations of CLA (0, 5–25 μM) and harvested after 72 h. β-Galactosidase activity was used as an internal control. The values are presented relative to cotransfected cells stimulated with vehicle (BSA) as control, and given as mean ± SD (n = 3). Each experiment was performed in triplicate. Significantly different ($P \leq 0.05$) from Darglitazone-treated cells* and from cells treated with Darglitazone in combination with *c*9,*t*11 CLA§. C: A construct containing 1,500 bp of the 5′-flanking region of the mLXRα gene in front of a luciferase reporter [pLXRα(−1,500/+1,800)LUC] was cotransfected with 0.2 μg of an expression plasmid of RXRα (pCMV-RXRα) and 0.2 μg of an expression plasmid encoding mPPARγ (pSG5-PPARγ) into 3T3-L1 adipocytes at D11 of differentiation. The cells were treated with 25 μM *c*9,*t*11 CLA, 25 μM *t*10,*c*12 CLA, or 1 μM Darglitazone, either alone or in addition to 15 μM and 25 μM CLA, and harvested after 48 h. The luciferase activity was expressed as a function of relative light units corrected for transfection efficiency using an internal Renilla Luciferase standard. The values are presented relative to cotransfected cells stimulated with vehicle (BSA) as control (n = 1), and given as mean ± SD. This experiment was performed once, each stimulation in triplicate. * Significantly different ($P \leq 0.05$) from Darglitazone-treated cells.

cle. *c*9,*t*11 did not regulate the PPARγ targets, either alone or in combination with Darglitazone. This was different for the *t*10,*c*12 isomer, which reduced the Darglitazone-induced aP2 and LXRα mRNA expression by 50%.

## DISCUSSION

Combining the relevance of human and animal studies in vivo with the power of in vitro adipocyte models is important to better understand the role of CLA isomers in body fat accumulation. This stimulated us to study the molecular mechanisms by which CLA isomers mediate their effects in adipose cell models. The present study provides evidence that one of the mechanisms by which CLA, and in particular the *t*10,*c*12 isomer, prevents lipid accumulation in adipocytes is by acting as a PPARγ modulator.

Our data demonstrate attenuated lipid accumulation in differentiated mouse 3T3-L1 and human SGBS adipocytes, stimulated with 25 μM and 5 μM *t*10,*c*12 CLA, respectively, visualized by Oil Red O staining, and measured as mg TAG/mg protein. Moreover, at these concentrations, no significant differences in total cell number, number of living and dead cells, or protein content were ob-



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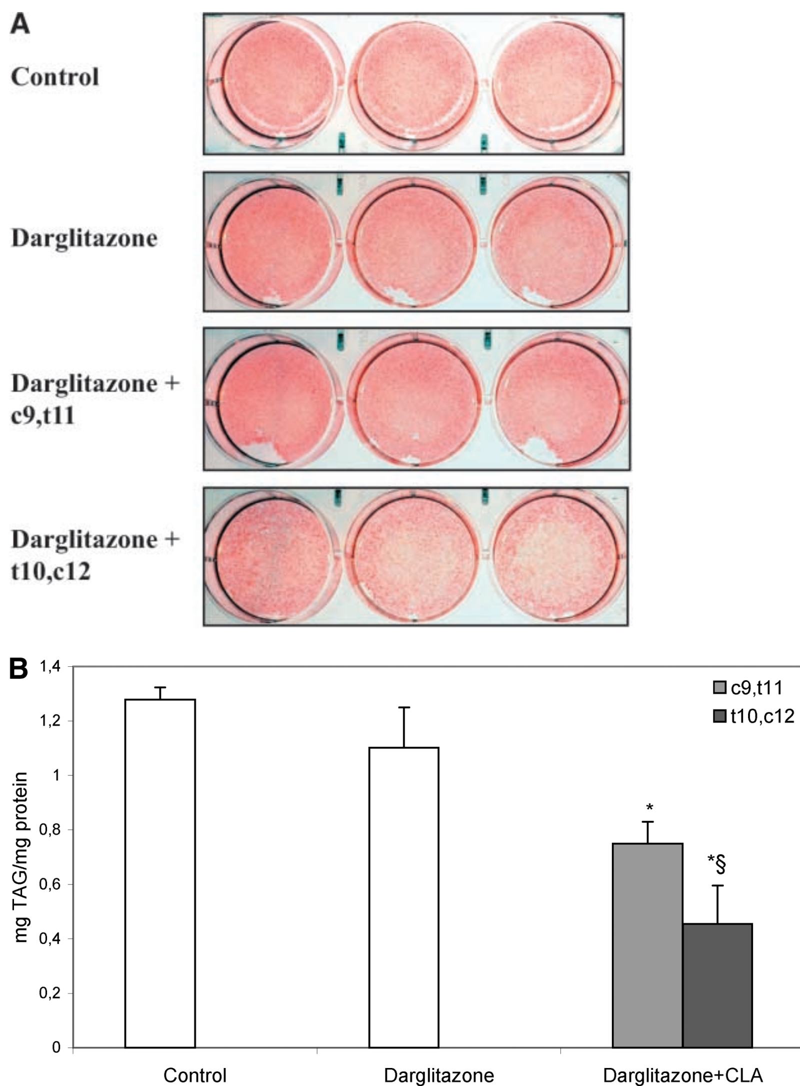


**Fig. 4.** Competition between CLA and Darglitazone on PPARγ activation in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were treated with either 1 μM Darglitazone alone or Darglitazone in addition to 25 μM of either *c*9,*t*11 or *t*10,*c*12 CLA from D0 until D11 of differentiation. Vehicle (BSA) was used as a control, (n = 3). Each experiment was performed in triplicate. A: Adipocytes stained with Oil Red O to visualize lipid content on D11 of differentiation. B: TAG levels in the adipocytes at D11 of differentiation. Results are given as mean ± SD (n = 3). Significantly different ($P \leq 0.05$) from Darglitazone-treated cells* and from cells treated with Darglitazone in combination with *c*9,*t*11 CLA§.

served between the CLA treatments for either of the cell lines. These results illustrate that at the concentrations used in our experiments, cytotoxicity is not the reason for the attenuated lipid accumulation by *t*10,*c*12 CLA treatment. Interestingly, a much lower concentration (5 μM) of *t*10,*c*12 CLA was sufficient to achieve the same reduction in lipid content in human adipocytes compared with mouse adipocytes (25 μM). Our results are in agreement with earlier studies that showed a reduced lipid accumulation when 3T3-L1 cells were stimulated with 50 μM *t*10,*c*12 CLA (29), and with a study on primary cultures of stromal vascular cells from human adipose tissue (30). *c*9,*t*11 CLA had no effect on lipid accumulation in either of the cell models used in our study. Clearly, CLA isomers have different effects on lipid accumulation, depending on the isomer type and the concentrations used.

In an effort to elucidate the molecular mechanisms by which *t*10,*c*12 CLA inhibits lipid accumulation, we have focused on the nuclear receptors PPARα and PPARγ, because they are well-known activators for specific natural and synthetic fatty acids (9, 42–45). PPARα is predominantly expressed in liver, kidney, and heart (7), and is not linked to adipocyte differentiation, as PPARγ is (3). Our data show that both CLA isomers up-regulate both full-length mouse PPARα and PPARγ [however, to a much lower extent than the well-known ligands WY 14,643 (PPARα) and Darglitazone (PPARγ)]. Supportive of our study, Moya-Camarena and coworkers demonstrated that both CLA isomers are weak activators of PPARα (31) and much lower activators of PPARγ (46). Our data show that both CLA isomers activated the LBD of PPARα slightly but failed to activate PPARγ in transactivation systems. It is obvious that the CLA isomers activate the PPAR isoforms differently. This may indicate that the CLA isomers have a different physiological role in metabolic tissues where PPARα is highly expressed compared with PPARγ, which plays a main role in adipose tissue. Along these lines, Peters and coworkers showed that the effect of a crude mixture of CLA isomers on body composition and serum TAG concentration was independent of PPARα, observing the same reduction in TAG concentration and fat mass for PPARα knockout mice as for wild-type mice (47).

Because PPARγ is adipogenic (8), we wanted to investigate whether the attenuated lipid accumulation in 3T3-L1 cells by *t*10,*c*12 CLA was due to PPARγ modulation. Indeed, *t*10,*c*12 CLA was able to modulate binding of the high-affinity ligand Darglitazone to PPARγ in the dose-



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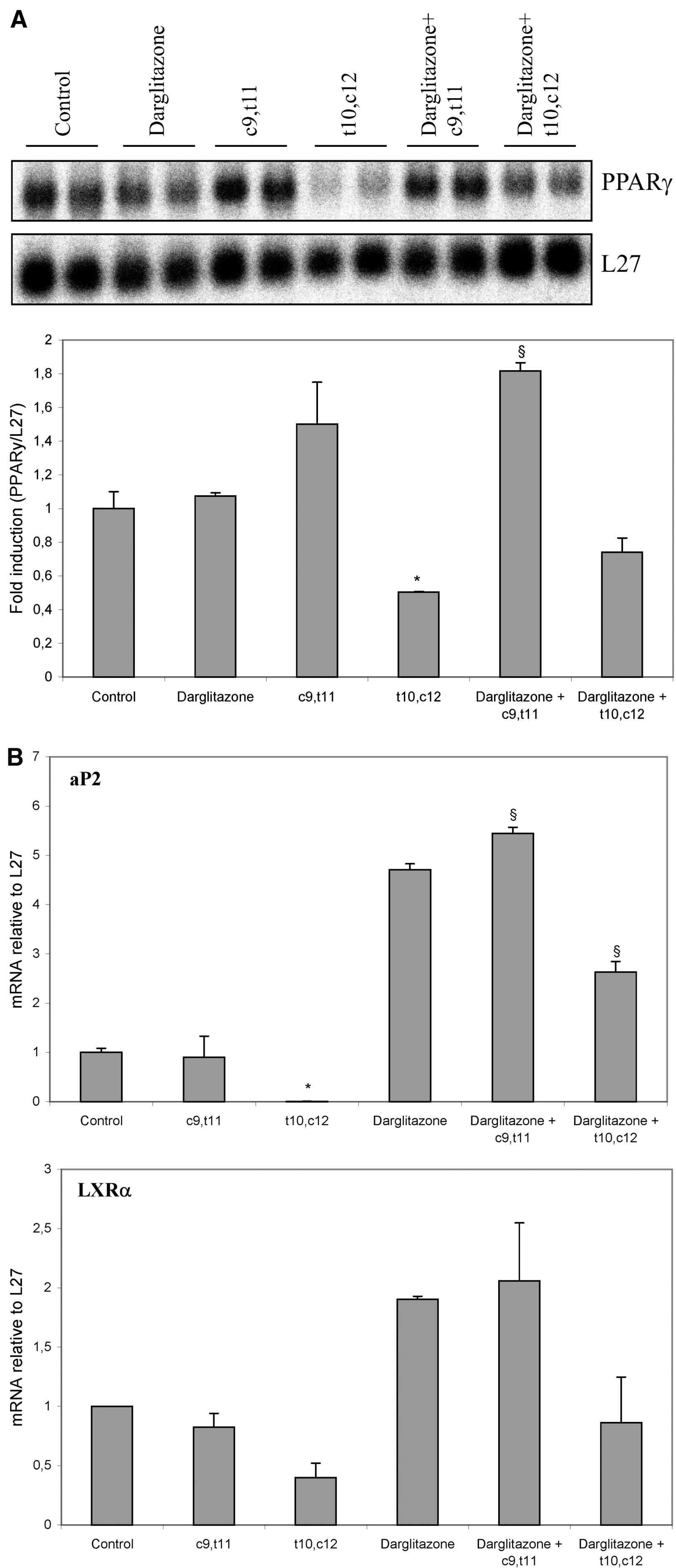


**Fig. 5.** Effect of CLA isomers on adipocyte gene expression. 3T3-L1 preadipocytes were treated with either Darglitazone (1 μM), *c*9,*t*11 CLA (25 μM), *t*10, *c*12 CLA (25 μM), or Darglitazone (1 μM) in combination with 25 μM CLA, from D0 until D11 of differentiation. Vehicle (BSA) was used as a control. Samples were subjected to Northern blot analysis. A: Northern blots (upper panel) and analysis (lower panel) for PPARγ and L27. B: Northern blot analysis of the PPARγ target genes aP2 (upper panel) and LXRα (lower panel). The intensities were measured by densitometric scanning and were normalized against L27 examined in the same samples as control. The results represent mean ± SD. Significantly different ($P \leqslant 0.05$) from control* and from Darglitazone-treated§ cells.

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dependent manner shown in transactivation studies. The effect of *t*10,*c*12 CLA on Darglitazone-induced transactivation was even more pronounced when cotransfecting with the full-length PPARγ. These results indicate that factors in addition to modulating binding to the receptor are involved in the attenuating effect of *t*10,*c*12 CLA, e.g., recruitment of cofactors and conformational changes of the receptor. In a more physiological system using 3T3-L1 cells, we were also able to demonstrate that *t*10,*c*12 CLA could inhibit Darglitazone-induced transactivation and lipid accumulation. In contrast, *c*9,*t*11 CLA failed to inhibit the effect of Darglitazone on lipid accumulation.

The modulatory effect of *t*10,*c*12 CLA was also observed on the down-regulation of PPARγ and LXRα mRNA expression. Along these lines, earlier studies have shown a down-regulation of PPARγ and aP2 mRNA expression after stimulation of 3T3-L1 cells with a mixture of CLA until D7 of differentiation (48, 49). In contrast, *t*10,*c*12 CLA stimulation until D9 of differentiation resulted in no effect on PPARγ and aP2 mRNA expression (49), whereas others found a reduced protein expression of PPARγ and aP2 after stimulation until D6 of differentiation (50).

Our results further show a reduced LXRα mRNA expression after *t*10,*c*12 CLA treatment compared with control and *c*9,*t*11 CLA treatment. Our group has shown previously that LXRα expression is increased by PPARγ activation in adipocytes, and that LXRα plays a role in lipid accumulation in adipocytes (41). The modulatory effect of *t*10,*c*12 CLA on PPARγ activation observed in this study is therefore in accordance with a subsequent down-regulation of LXRα.

Interestingly, *t*10,*c*12 reduced Darglitazone-stimulated gene expression of PPARγ and LXRα by nearly 50%. This indicates that the modulatory effect of *t*10,*c*12 CLA can also be observed in the expression of specific transcription factors involved in adipocyte differentiation and lipid accumulation.

Thus, *t*10,*c*12 CLA might affect lipid accumulation in adipocytes by acting as a PPARγ modulator. Along these lines, other compounds acting as PPARγ modulators or antagonists have been identified (14, 51–53). A novel PPARγ-specific modulator, LG100641, does not activate PPARγ, but selectively and competitively blocks TZD-induced PPARγ activation and adipocyte conversion (14). Other synthetic compounds (BADGE, PD068235, and GW0072) have also been shown to antagonize the ability of PPARγ agonists and to inhibit adipocyte differentiation in vitro (51–53). As far as we know, no natural fatty acid acting as PPARγ antagonist/modulator has been identified. However, it is known in the nuclear receptor literature that fatty acids are able to antagonize receptor activity of other nuclear receptors. The activities of LXRα and LXRβ are inhibited by polyunsaturated fatty acids that antagonize binding of their natural ligands, the cholesterol derivatives (oxysterols), and thereby inhibit transcription of specific target genes (54).

In conclusion, we demonstrate that *t*10,*c*12 CLA is able to reduce ligand-induced PPARγ activity. Furthermore, *t*10,*c*12 CLA inhibits gene expression of PPARγ and PPARγ target genes in mature adipocytes. Even though *t*10,*c*12 CLA is not a PPARγ ligand, it is a PPARγ modulator, affecting lipid accumulation in adipocytes. Because CLA improves body composition and serum lipid biochemistry, determining the mechanisms of action for CLA can provide molecular targets that may have significant impact for many related lipid-dependent diseases, including obesity, hyperlipidemia, atherosclerosis, and Type II diabetes. Along these lines, it will be of great interest to further understand the importance of *t*10,*c*12 CLA as a PPARγ modulator in relation to these diseases.

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