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Trans-10, *Cis*-12 Conjugated Linoleic Acid Antagonizes Ligand- Dependent PPARγ Activity in Primary Cultures of Human Adipocytes

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Trans-10, *Cis*-12 Conjugated Linoleic Acid Antagonizes Ligand-Dependent PPARγ Activity in Primary Cultures of Human Adipocytes,[1,2]

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Abstract

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

Introduction

Dysfunction of adipose tissue can result in insulin resistance and lipodystrophy. One major regulator in the development and function of adipose tissue is PPARγ, which induces the expression of a host of adipogenic genes such as lipoprotein lipase (LPL), insulin-stimulated glucose transporter 4 (GLUT4),[5] perilipin (PLIN), and adipocyte fatty acid-binding protein

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[5]Abbreviations used: aP2, adipocyte-specific fatty acid-binding protein; BSA, bovine serum albumin; Cip, calf intestinal phosphatase; CLA, conjugated linoleic acid; ERK, extracellular signal-related kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT4, insulin-dependent glucose transporter 4; HBSS, Hanks balanced salt solution; LPL, lipoprotein lipase; luc, luciferase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NFκB, nuclear factor kappa B; ORO, Oil Red O; PLIN, perilipin; PPRE, peroxisome proliferator response element; RXR, retinoic acid receptor; TG, triglyceride; TZD, thiazolidinedione.

(aP2). Mutations of PPARγ in humans are associated with insulin resistance and lipodystrophy (1,2). PPARγ null cells exhibit impaired adipo-genesis (3) and dominant negative mutations in PPARγ inhibit adipogenesis (4). Thus, PPARγ activity is essential in adipose tissue for glucose uptake and triglyceride (TG) accumulation.

Regulation of PPARγ occurs through a variety of proposed mechanisms, including covalent modification by phosphorylation, ligand binding, and heterodimerization with the retinoic acid receptor (RXR) (5,6). Phosphorylation of PPARγ by activation of the mitogen-activated protein kinase (MAPK) pathway has been reported to inhibit adipogenesis (7). It has been demonstrated that phosphorylation of Ser-112 of PPARγ2 results in its ubiquination and proteosome degradation (8). Activation of PPARγ by natural (i.e. PUFA) or synthetic ligands such as thiazolidinediones (TZD) initiates heterodimerization with RXR followed by their binding to peroxisome proliferator response element (PPRE) in the promoters of adipogenic target genes. The TZD are hypoglycemics that activate PPARγ, leading to upregulation of adipogenic genes, thereby enhancing insulin sensitivity. Natural ligands of PPARγ2 such as *cis*-PUFA or prostaglandins such as PGJ2 (9) have a relatively low affinity for PPARγ compared with TZD. In contrast, SFA and certain *trans* PUFA such as conjugated linoleic acid (CLA) have been reported to impair insulin sensitivity, possibly by decreasing the expression of PPARγ and many of its downstream target genes (10–13).

CLA consists of dienoic isomers of linoleic acid, including *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA. CLA decreases body fat mass in animals (14) and some humans (15). Our group has demonstrated that *trans*-10, *cis*-12 CLA decreases adipogenic gene expression and the TG content of human (pre)adipocytes (10,11). We have also demonstrated that activation of MAPK kinase (MEK)/extracellular signal-related kinase (ERK) (11) and nuclear factor κB (NFκB) (16) signaling by *trans*-10, *cis*-12 CLA were essential for its suppression of adipogenic gene expression and delipidation in human adipocytes. A number of side effects have been associated with *trans*-10, *cis*-12 CLA supplementation in humans, such as insulin resistance, hyperglycemia, and dyslipidemia (17,18). Dyslipidemia, insulin resistance, and hyperglycemia are similar characteristics found in humans with mutations in PPARγ. Two recent reports by Belury et al. (19,20) showed that the PPARγ agonist rosiglitazone prevents or attenuates inflammation, lipodystrophy, and insulin resistance in mice fed a crude mixture of CLA isomers containing equal amounts of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA. However, the isomer-specific mechanism by which CLA suppresses the expression of PPARγ and its target genes in human adipocytes remains to be elucidated. To address this issue, we examined the impact of CLA on PPARγ in the absence and presence of the PPARγ ligand rosiglitazone (BRL).

Materials and Methods

Materials

All cell culture ware were purchased from Fisher Scientific. Western Lightning chemiluminescence substrate was purchased from Perkin Elmer Life Science. The 1-step RT-PCR kit used in semiquantitative mRNA analysis was purchased from Qiagen. Immunoblotting buffers, precast gels, and gene-specific primers were purchased from Invitrogen and ribosomal 18S competimer technology internal standards and DNA-free were purchased from Ambion. Polyclonal GLUT4 antibody was a gift from Drs. S. Cushman and X. Chen (NIDDK, NIH, Bethesda, MD). aP2 antibody was a gift from Dr. D. Bernlohr (University of Minnesota). Monoclonal antibodies for PPARγ (sc7273) and polyclonal antibodies for anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sc20357) and β-actin (sc1616) were obtained from Santa Cruz Biotechnology. Anti-phospho (Thr-202/204) and total ERK antibodies were purchased from Cell Signaling Technologies. Cy3- and fluorescein-conjugated immunoglobulin G were purchased from Jackson Immunoresearch. Fetal bovine serum was

purchased from Cambrex/BioWhittaker. BRL was a gift from Glaxo Smith Kline. Isomers of CLA (+98% pure) were purchased from Matreya. The Nucleofector and Dual Glo luciferase (luc) kits were obtained from Amaxa and Promega, respectively. All other reagents and chemicals were purchased from Sigma Chemical unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue was obtained from nondiabetic females between the ages of 20 and 50 y old with a BMI ≤ 30 during abdominoplasty with consent from the Institutional Review Board at the University of North Carolina at Greensboro. Tissue was digested using collagenase and stromal vascular cells were isolated as previously described (11). Experimental treatment of cultures containing ~50% preadipocytes and ~50% adipocytes occurred on d 12 of differentiation. Each experiment was conducted in duplicate and repeated at least 3 times using a mixture of cells from 2−3 subjects unless otherwise indicated.

Preparation of fatty acids

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

Immunoblotting

Immunoblotting was conducted as previously described (11). To resolve PPARγ phospho-proteins, total cell extracts (75 μg protein) were subjected to 10% SDS-PAGE (acrylamide:bisacrylamide, 100:1, wt:wt) containing 4 mol/L urea and to electrophoresis at 80 V for 20 h as we previously described (21). Separated proteins were subsequently transferred to polyvinylidene difluoride membranes and immunoblotted with a monoclonal PPARγ antibody. For determining the phosphorylation status of PPARγ, a portion of the cell extracts from BSA vehicle and CLA treatment were incubated with 20 U of calf intestinal phosphatase (Cip) for 30 min at 37°C and for 15 min at 55°C. Subsequently, the samples were subjected to SDS-PAGE containing urea as described above.

Immunostaining of PPARγ

Cells were cultured on coverslips for immunofluorescence microscopy and stained as described previously (11) except for the permeabilization step. Fixed cells were permeabilized with 0.1% Triton X-100 for 1 min on ice. Monoclonal anti-PPARγ (1:10) were incubated overnight at 4° C. Fluorescent images were captured with a SPOT digital camera mounted on an Olympus BX60 fluorescence microscope.

Transient transfections of human adipocytes

For measuring PPARγ activity, primary human adipocytes were transiently transfected with the multimerized PPAR-responsive (luc) reporter construct pTK-PPRE33-luc (22) using the Amaxa Nucleofector as previously described (21). On d 6 of differentiation, 1×10^6 cells from a 60-mm plate were trypsinized and resuspended in 100 μL of nucleofector solution (Amaxa) and mixed with 2 μg of pTK-PPRE3×-luc and 25 ng pRL-CMV for each sample. Electroporation was performed using the V-33 nucleofector program (Amaxa). Cells were replated in 96-well plates after 10 min of recovery in calcium-free RPMI media. Firefly luc activity was measured using the Dual-Glo luc kit and normalized to *Renilla* luc activity from the cotransfected control pRL-CMV vector. All luc data are presented as a ratio of firefly luc to Renilla luc activity. We consistently obtained ~75% transfection efficiency revealed by parallel transfections with a green fluorescent protein reporter construct. Both adipocytes and nonadipocytes were transfectable using this protocol based on aP2 immunostaining and 4′,6-diamidino-phenylindole nuclear staining.

RNA analysis

Following treatment, cultures were harvested for total RNA using Tri-Reagent according to the manufacturer's protocol. Contaminating DNA was removed with DNAase (DNA-free, Ambion). One microgram of RNA from each sample was used for semiquantitative RT-PCR using the One-Step RT-PCR kit (Qiagen) as previously described in Brown et al. (10). The gene-specific primer pairs used were previously described (10).

Lipid staining

Lipid staining of cultures of human adipocytes was conducted as previously described (10) using Oil Red O (ORO).

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

Newly differentiated cultures of adipocytes were incubated with BSA vehicle, 30 μmol/L *cis*-9, *trans*-11 CLA, 30 μmol/L *trans*-10, *cis*-12 CLA, 30 μmol/L *trans*-10, *cis*-12 CLA + 1 μmol/L BRL, or 1 μmol/L BRL in adipocyte media for 2 d. Then, for an additional 2 d, cultures were incubated in 1 mL of serum-free basal DMEM containing 1000 mg/L D-(+)-glucose with or without 20 pmol/L of human insulin with BSA vehicle, 30 μmol/L *cis*-9, *trans*-11 CLA, 30 μmol/L *trans*-10, *cis*-12 CLA, 30 μmol/L *trans*-10, *cis*-12 CLA + 1 μmol/L BRL, or 1 μmol/L BRL in adipocyte media for another 2 d. Following the experimental treatments, insulin-stimulated uptake of [3H]-2-deoxy-glucose was measured following a 90-min incubation in the presence of 100 nmol/L human insulin as described previously (16).

Statistical analysis

Statistical analyses were performed for data in Figure 1 testing the main effects of BRL and CLA and the interaction of the 2 (BRL × CLA) using 2-way ANOVA (JMP version 6.03, SAS Institute). Analyses for significant differences for data in Figure 4C were conducted using 1-way ANOVA. Student's *t* tests were used to compute individual pairwise comparisons of least square means ($P < 0.05$). Data are expressed as the means ± SE.

Results

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

To determine the extent to which CLA decreased PPARγ activity, basal- and ligand-induced activation of PPARγ activity were examined. There were no significant differences in basal levels of PPARγ activity due to CLA treatment in the absence of BRL (Fig. 1). However, PPARγ activity in BRL-stimulated cultures (+BRL) was lower in cultures treated with 30 μmol/L *trans*-10, *cis*-12 CLA compared with control and 30 μmol/L *cis*-9, *trans*-11 CLA-treated cultures. The extent to which *trans*-10, *cis*-12 CLA decreased PPARγ activity (~40%) was comparable to that of PPARγ antagonist GW9662, which inhibited ligand-induced PPARγ activity without affecting basal activity (data not shown).

Given the inverse relationship between PPARγ activity and its phosphorylation status (7), we wanted to determine the kinetics of PPARγ phosphorylation during treatment with *trans*-10, *cis*-12 CLA. *Trans*-10, *cis*-12 CLA caused a band shift in PPARγ1/2 after 24 h of treatment (Fig. 2A). Intriguingly, robust ERK1/2 phosphorylation at 24 h accompanied the PPARγ1/2 band shift, consistent with ERK1/2's role as a donor of phosphate groups to nuclear PPARγ1/2 and with our published data demonstrating that ERK1/2 is required for CLA's suppression of adipogenic gene expression and glucose uptake (11). However, because a PPARγ band shift could be due to processes other than phosphorylation (e.g. by acetylation, methylation, or sumylation), Cip was added to the cell extracts to remove phosphorylated groups. *Trans*-10, *cis*-12 CLA-induced band shifts of PPARγ1/2 were either lowered or attenuated by phosphatase

treatment (Fig. 2*B*). Taken together, these data suggest that *trans*-10, *cis*-12 CLA promotes PPAR*γ* and ERK phosphorylation, which contributes at least in part to CLA's isomer-specific reduction of PPAR*γ* activity.

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

We previously demonstrated that a physiological level (e.g. 30 *μ*mol/L) of *trans*-10, *cis*-12 CLA decreased the mRNA levels of PPAR*γ* and several of its target genes in differentiating cultures of human stromal vascular cells (10) and in the newly differentiated cultures of human adipocytes (11). However, the isomer-specific impact of CLA on PPAR*γ* protein levels in human adipocytes is unknown. PPAR*γ*2 protein levels were decreased after 4 d and undetectable after 6 d of treatment with *trans*-10, *cis*-12 CLA compared with the BSA vehicle or *cis*-9, *trans*-11 CLA-treated cultures (Fig. 3*A*). Consistent with these data, newly differentiated cultures treated with 30 *μ*mol/L *trans*-10, *cis*-12 CLA for 4 d had dramatically less nuclear PPAR*γ* staining compared with BSA vehicle-treated cultures (Fig. 3*B*). These data demonstrate that *trans*-10, *cis*-12 CLA decreases PPAR*γ* protein levels in an isomer-specific manner in newly differentiated human adipocytes.

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

To further evaluate the antagonistic effects of *trans*-10, *cis*-12 CLA on PPAR*γ* activity, we examined the extent to which cosupplementation with the PPAR*γ* agonist BRL could prevent *trans*-10, *cis*-12 CLA suppression of adipogenic genes and proteins, glucose uptake, and TG accumulation. *Trans*-10, *cis*-12 CLA decreased the mRNA (Fig. 4*A*) and protein (Fig. 4*B*) levels of PPAR*γ*, aP2, LPL, and GLUT4 compared with BSA vehicle controls or *cis*-9, *trans*-11 CLA. Although BRL cosupplementation prevented CLA-mediated reductions in aP2, LPL, and PLIN gene expression, it did not prevent CLA suppression of PPAR*γ*2 or GLUT4 mRNA levels (Fig. 4*A*). Consistent with these data, BRL cosupplementation prevented CLA suppression of aP2 protein expression but did not prevent CLA suppression of PPAR*γ* or GLUT4 protein levels (Fig. 4*B*).

CLA isomer-specific reduction of insulin-stimulated glucose uptake (Fig. 4*C*) or TG accumulation (Fig. 4*D*) was not prevented by cosupplementation with BRL. Collectively, these data demonstrate that *trans*-10, *cis*-12 CLA chronically suppresses adipogenic gene and protein expression, glucose uptake, and TG content, which are only partially prevented by a PPAR*γ* ligand.

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

Next, we wanted to determine whether the delipidating effects of CLA could be rescued by CLA withdrawal in the absence or presence of a PPAR*γ* ligand. Surprisingly, withdrawal of *trans*-10, *cis*-12 CLA treatment for 2 wk did not restore the mRNA levels of LPL, PLIN, or GLUT4 gene (group 1, Fig. 5*A*) or the protein levels of PPAR*γ* or GLUT4 (group 1, Fig. 5*B*). Interestingly, the pattern of gene and protein expression in group 1 was almost identical to that of the cultures treated for 1 wk with *trans*-10, *cis*-12 CLA (Fig. 4), indicating the effects of CLA were sustained over 2 wk. Consistent with these gene and protein data, cultures treated with *trans*-10, *cis*-12 CLA had less stainable TG 2 wk after withdrawal (group 1, Fig. 5*C*) compared with controls.

BRL supplementation for 2 wk following CLA withdrawal rescued PPAR*γ*, aP2, and LPL gene expression compared with BSA vehicle- or *cis*-9, *trans*-11 CLA-treated cultures, whereas PLIN and GLUT4 were partially rescued (Group 2, Fig. 5*A*). BRL supplementation for 2 wk following CLA withdrawal reversed or attenuated *trans*-10, *cis*-12 CLA suppression of aP2 and GLUT4 protein levels, respectively, compared with cultures not receiving BRL for 2 wk (group 1). Although CLA-treated cultures supplemented for 2 wk with BRL (group 2) had

more PPAR*γ* protein compared with those not receiving BRL for 2 wk (group 1), PPAR*γ* protein levels did not return to the levels of the BSA vehicle- or *cis*-9, *trans*-11 CLA-treated cultures. Similarly, supplementation of cultures with BRL for 2 wk during CLA withdrawal (group 2) increased the TG content of cultures treated with *trans*-10, *cis*-12 CLA compared with cultures not receiving BRL during withdrawal (group 1). Interestingly, BRL was only effective in preventing delipidation when it was cosupplemented with CLA and then supplemented for another 2 wk following CLA withdrawal (group 2, Fig. *5C*). Taken together, these data demonstrate that *trans*-10, *cis*-12 CLA-mediated delipidation persists after CLA withdrawal and is relatively refractory to supplementation with a PPAR*γ* ligand unless the ligand is supplemented during and after CLA treatment.

Discussion

The PPAR*γ* agonist rosiglitazone has been demonstrated to prevent or attenuate inflammation, lipodystrophy, and insulin resistance in mice fed a crude mixture of CLA isomers (e.g. primarily *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA) (19,20). These data suggest an antagonism between one or both CLA isomers and PPAR*γ*. However, the isomer-specific mechanism by which CLA suppresses the activity of PPAR*γ* in human adipocytes remains unknown. We demonstrate in this article that *trans*-10, *cis*-12, but not *cis*-9, *trans*-11, CLA attenuates ligand-induced activation of PPAR*γ* (Fig. 1), possibly via phosphorylation of PPAR*γ* by ERK1/2 (Fig. 2). Inactivation of PPAR*γ* leads to suppression of protein and mRNA levels of PPAR*γ* and several of its target genes in newly differentiated human adipocytes (Figs. 3, 4, 5). BRL cosupplementation did not prevent insulin resistance caused by *trans*-10, *cis*-12 CLA (Fig. 4). Furthermore, we show that *trans*-10, *cis*-12 CLA-mediated suppression of TG accumulation does not return to control levels following CLA withdrawal or by supplementation with a PPAR*γ* agonist following CLA treatment (Fig. 5). Only BRL cosupplementation followed by 2 wk of BRL supplementation restored the TG content of *trans*-10, *cis*-12 CLA-treated cultures to control levels. Taken together, these data provide further support for the concept that CLA's antiadipogenic effects in humans are due to the *trans*-10, *cis*-12 isomer and not the *cis*-9, *trans*-11 isomer and are directly linked to the suppression of PPAR*γ* activity, adipogenic gene and protein expression, insulin-stimulated glucose uptake, and TG content, which appears to be due in part to an antagonism of ligand-mediated activation of PPAR*γ*.

Potential mechanisms explaining the isomer-specific attenuation of PPAR*γ* activity by CLA are shown in our working model (Fig. 6). We propose that *trans*-10, *cis*-12 CLA, a metabolite, or a signal activated by CLA suppresses PPAR*γ* activity by: *1*) phosphorylating PPAR*γ* via activation ERK1/2; 2) inhibiting ligand activation and heterodimer formation with RXR; or *3*) impairing DNA binding of the PPRE to target genes, thereby decreasing adipogenic gene transcription, insulin-stimulated glucose uptake, and TG synthesis.

PPARγ phosphorylation

Support for the first 2 mechanisms comes from our discovery that *trans*-10, *cis*-12 CLA suppresses adipogenic gene expression and metabolism through activation of ERK1/2 (11) and NFκB (16). Reports demonstrating that NFκB (23–27) and MAPK (28–30) activation hinders PPAR*γ* DNA-binding affinity or transcriptional activation provides a potential mechanism by which *trans*-10, *cis*-12 CLA suppresses the expression of PPAR*γ* target genes, leading to delipidation. ERK1/2 activates NFκB (31) and inactivates PPAR*γ* (32), resulting in its ubiquination and proteosome degradation (33). Further support comes from studies showing that PPAR*γ* agonists attenuate cytokine-mediated inflammation by suppressing NFκB and/or MAPK signaling (34–37). Clearly, the activity of PPAR*γ* is regulated by its phosphorylation status via phosphatases and kinases (33). Indeed, the phosphorylation of PPAR*γ* at a consensus

MAPK site within its A/B domain (e.g. Ser-112) by ERK1/ 2 or c-Jun-NH2-terminal kinase reduces its transcriptional activation potential, leading to insulin resistance and/or decreased adipogenesis (8,28–30,32,37,38). Interestingly, we previously reported that the MEK/ERK inhibitor U0126 blocked *trans*-10, *cis*-12 CLA suppression of adipogenic genes and glucose and fatty acid uptake (11). Consistent with these data, we found that *trans*-10, *cis*-12 CLA simultaneously increased the phosphorylation of ERK1/2 and PPARγ (Fig. 2*A*). Based on these data, our working hypothesis is that *trans*-10, *cis*-12 CLA antagonizes PPARγ's activity acutely and PPARγ expression chronically in adipocytes via NFκB and ERK1/2 activation, leading to decreased glucose and fatty acid uptake and TG synthesis.

Ligand binding

CLA may also compete with endogenous (i.e. unsaturated fatty acids) or exogenous (i.e. rosiglitazone-BRL) ligands for activation of PPARγ. Low affinity PPARγ ligands such as PUFA increase PPARγ activity and target gene expression (39). Several CLA isomers, including *cis*-9, *trans*-11 CLA, have been shown to be ligands for PPARγ (13,40) or its partner RXR (39). Consistent with the reported antagonism between PPARγ and inflammation, *cis*-9, *trans*-11 CLA has been shown to suppress NFκB activation and inflammatory cytokine production by lipopolysaccharide in dendritic cells (41) and in white adipose tissue of obese mice (42). However, we found that cosupplementation of *trans*-10, *cis*-12 CLA-treated cultures with up to 30 μmol/L *cis*-9, *trans*-11 CLA did not reverse insulin resistance or adipogenic gene expression (data not shown). In contrast, Granlund et al. (13) demonstrated that both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA decreased the activity of a darglitazone-stimulated, LXRα-PPRE-LUC reporter in a dose-dependent manner up to 25 μmol/L in COS-1 cells and 3T3-L1 cells. We have also demonstrated in 3T3-L1 that both CLA isomers antagonize ligand-induced activation of PPARγ (10). Alternatively, CLA phosphorylation of PPARγ in the A/B domain could reduce PPARγ affinity for ligand and/or cofactor recruitment (33).

Transcriptional activation

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

One possible explanation for the long-term effects of CLA following withdrawal could be that CLA accumulates within the phospholipid and neutral lipid fractions of the cell, as we have previously shown for both isomers (10). Thus, CLA could continue to antagonize PPARγ/RXR activity following withdrawal, thereby impacting endogenous ligand production, phosphorylation, and/or directly interfering with their transcriptional activation.

In summary, although cosupplementation with BRL, a high affinity ligand for PPARγ, generally prevented or attenuated *trans*-10, *cis*-12 CLA suppression of adipogenic gene and protein expression and TG content, it did not prevent CLA's suppression of a PPARγ reporter construct or insulin-stimulated glucose up-take. Furthermore, BRL supplementation for 2 wk after CLA withdrawal did not completely rescue its antiadipogenic and TG-lowering effects. Taken together, these data suggest that *trans*-10, *cis*-12 CLA may decrease PPARγ activity acutely by increasing PPARγ phosphorylation via ERK1/2 and chronically by decreasing PPARγ transcription, thereby decreasing the amount PPARγ available for ligand binding, leading to the suppression of insulin-stimulated glucose uptake and TG accumulation.

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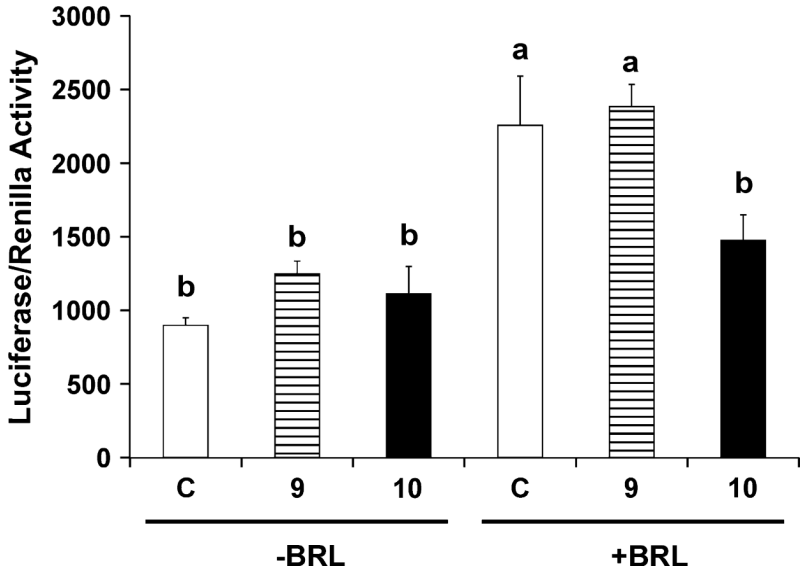

FIGURE 1.

Trans-10, *cis*-12 CLA blocks ligand-induced activation of PPARγ in human adipocytes. Cultures of newly differentiated human adipocytes were transfected on d 6 with pTK-PPRE33-luc and pRL-CMV. Twenty four hours later, transfected cells were treated with dimethyl sulfoxide vehicle control (C), 30 μmol/L *trans*-10, *cis*-12 CLA, or 30 μmol/L *cis*-9, *trans*-11 CLA in the absence or presence of 0.1 μmol/L BRL for 24 h. Values are means ± SEM, $n = 3$. Means without a common letter differ, $P < 0.05$.

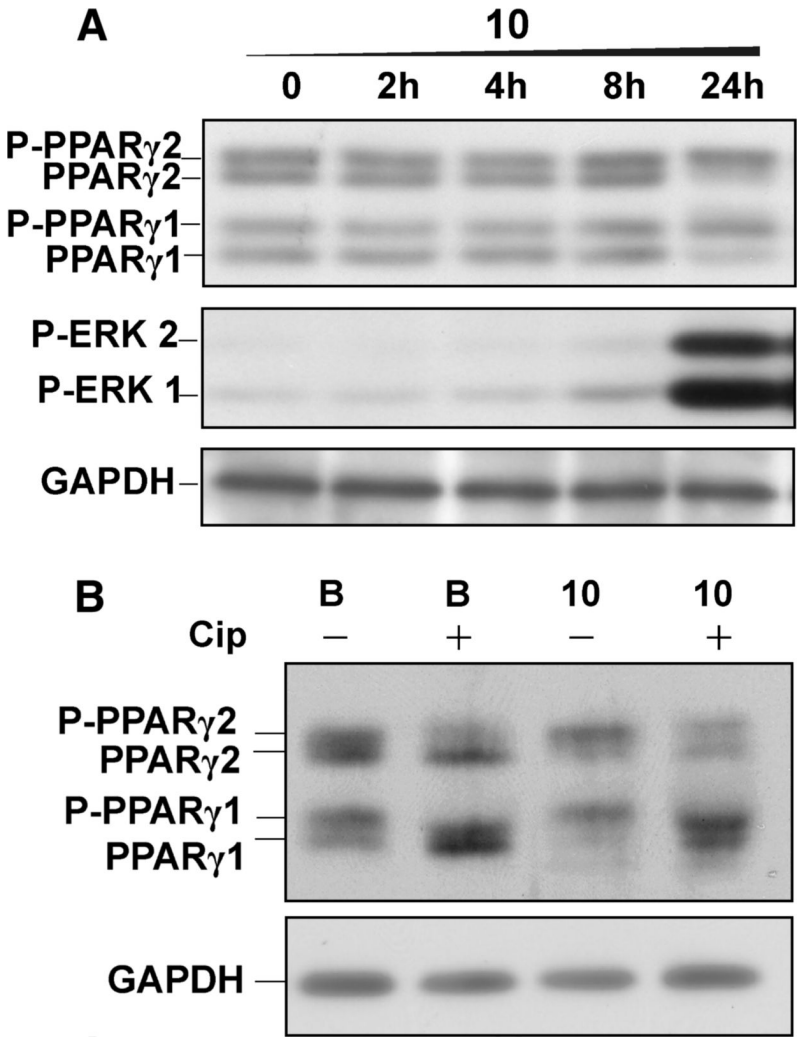

FIGURE 2.

Trans-10, *cis*-CLA increases PPARγ phosphorylation in human adipocytes. (*A*) Cultures of newly differentiated human adipocytes were serum starved for 24 h and then treated without (0) or with 30 μmol/L *trans*-10, *cis*-12 CLA (10) for 2, 4, 8, or 24 h. Subsequently, cell extracts were harvested, proteins separated by SDS-PAGE urea, and immunoblotted for the phosphorylated and unphosphorylated forms of PPARγ1/2, ERK1/2, and GAPDH (load control). (*B*) Cultures were treated for 24 h with BSA vehicle or 30 μmol/L *trans*-10, *cis*-12 CLA (10). A portion of the cell extracts from BSA vehicle and CLA treatment were incubated with Cip. Proteins were separated with SDS-PAGE urea and probed with antibodies targeting PPARγ1/2 and GAPDH. Data in *A* and *B* are representative of 2 independent experiments.

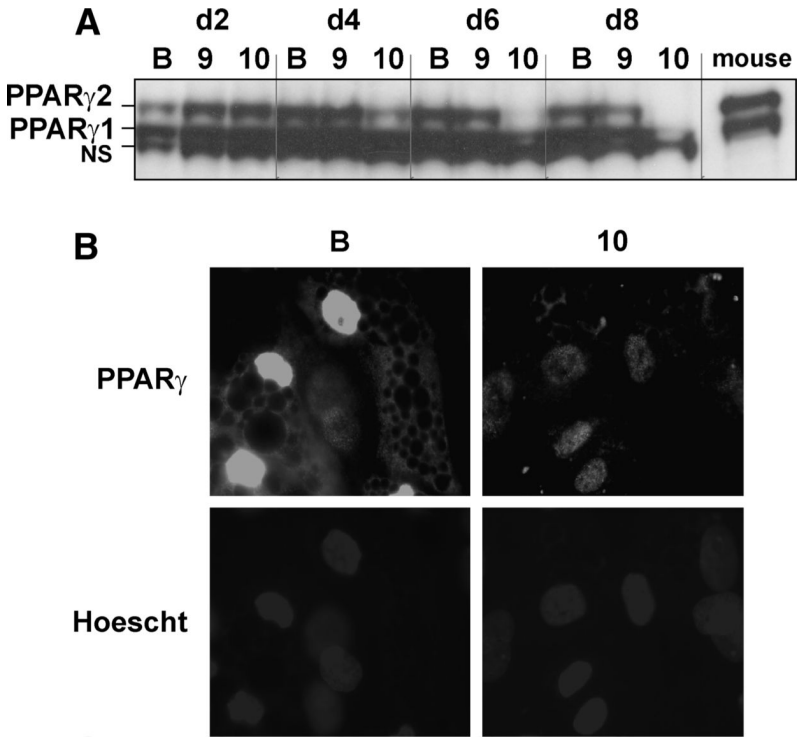

FIGURE 3.

Trans-10, *cis*-12 CLA decreases PPAR*γ* protein levels in human adipocytes. (*A*) Cultures of newly differentiated human adipocytes were treated with BSA vehicle (B), 30 *μ*mol/L *cis*-9, *trans*-11 CLA (9), or 30 *μ*mol/L *trans*-10, *cis*-12 CLA (10) for 2, 4, 6, or 8 d. Cells extracts were immunoblotted for PPAR*γ*. To identify PPAR*γ*1/2 in cultures of human adipocytes, cell extracts from 3T3-L1 adipocytes (mouse) were isolated and immunoblotted for PPAR*γ*. A 3rd band was identified in human adipocytes and labeled as nonspecific (NS). (*B*) Cultures were treated with BSA vehicle (B) or 30 *μ*mmol/L *trans*-10, *cis*-12 CLA (10) for 4 d. PPAR*γ* was detected using immunofluorescence microscopy. Data are representative of 2 independent experiments.

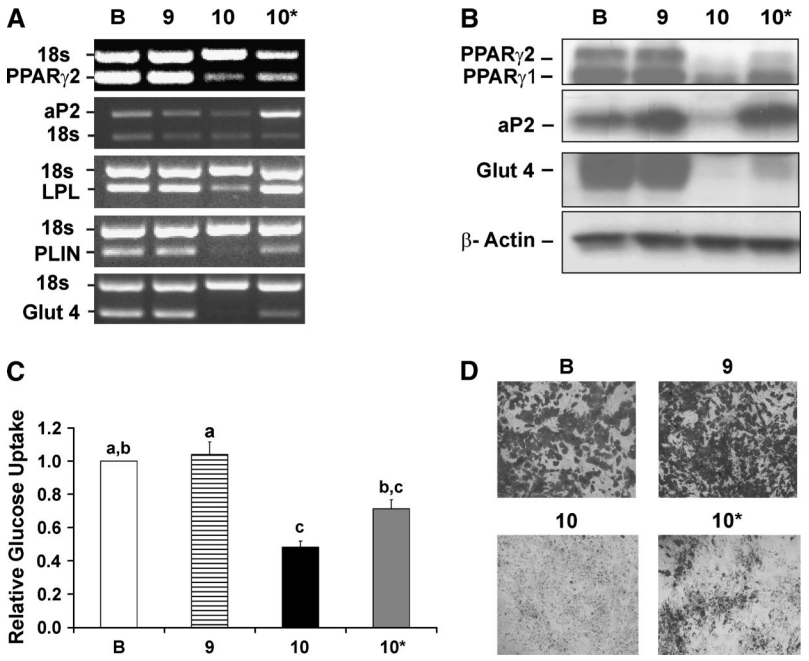

FIGURE 4.

Trans-10, *cis*-12 CLA antagonizes ligand-activated PPARγ expression, glucose uptake, and TG accumulation in human adipocytes. (*A*) Cultures of newly differentiated human adipocytes were treated for 1 wk with either BSA vehicle (B), 30 μmol/L *cis*-9, *trans*-11 CLA (9), 30 μmol/L *trans*-10, *cis*-12 CLA (10), or 30 μmol/L *trans*-10, *cis*-12 CLA + 1 μmol/L BRL (10*) and then harvested. RNA was isolated and the mRNA levels of PPARγ2, aP2, LPL, PLIN, and Glut 4 were measured using semiquantitative RT-PCR. 18S ribosomal RNA was used as an internal control. (*B*) Cultures were treated as in *A* for 1 wk and then cell extracts were isolated and immunoblotted for PPARγ, aP2, Glut4, and β-actin. (*C*) Cultures were treated as in *A* for 4 d and then insulin-stimulated uptake of [^{3}H]-2-deoxyglucose was measured. Values are means ± SEM; $n = 6$. Means without a common letter differ, $P < 0.05$. (*D*) Cultures were treated as in *A* for 1 wk and then stained with ORO and phase-contrast photo-micrographs were taken using an Olympus inverted microscope with a 10× objective. Data in *A, B,*,and *D* are representative of 2−3 independent experiments.

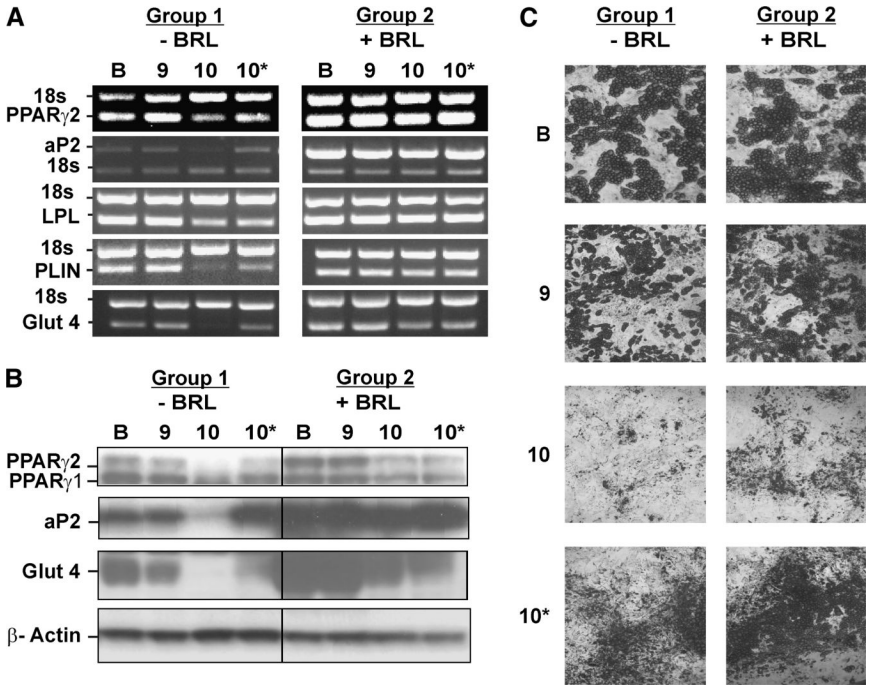

FIGURE 5.

Effects of withdrawal of *trans*-10, *cis*-12 CLA in the presence of a PPARγ ligand in human adipocytes. Cultures of newly differentiated human adipocytes were treated for 1 wk with either BSA vehicle (B), 30 μmol/L *cis*-9, *trans*-11 CLA (9), 30 μmol/L *trans*-10, *cis*-12 CLA (10), or 30 μmol/L *trans*-10, *cis*-12 CLA + 1 umol/L BRL (10*) and then had their treatments withdrawn for 2 wk (group 1, -BRL), or were treated with 1 μmol/L BRL for 2 wk during CLA withdrawal (group 2, +BRL). (*A*) RNA was isolated and the mRNA levels of PPARγ2, aP2, LPL, PLIN, and Glut4 were measured using semiquantitative RT-PCR. 18S ribosomal RNA was used as an internal control. (*B*) Cell extracts were isolated and immuno-blotted for PPARγ, aP2, Glut4, and β-actin. (*C*) Cultures were stained with ORO and phase-contrast photomicrographs were taken using an Olympus inverted microscope with a 10× objective. Data are representative of 2−3 independent experiments.

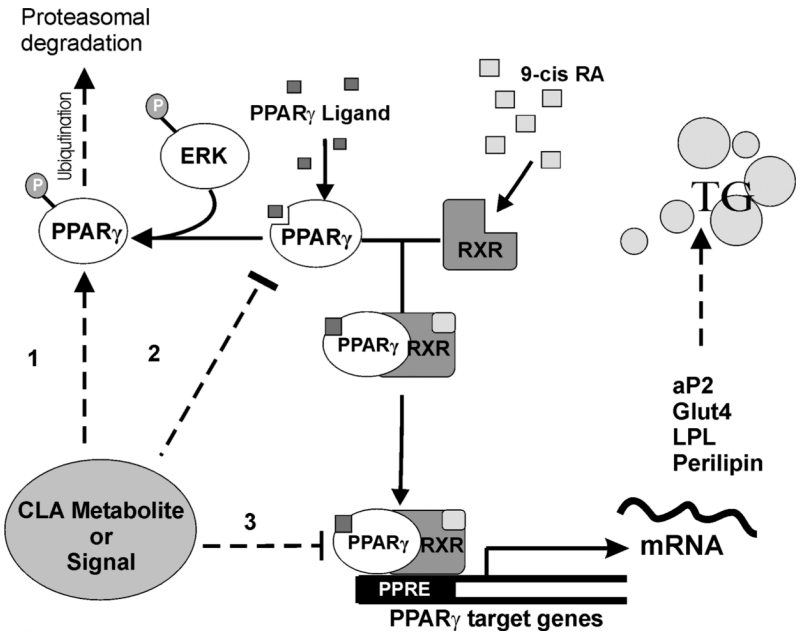

FIGURE 6.

Working model CLA, metabolites, or signals suppress PPAR*γ* activity by: *1*) phosphorylating PPAR*γ* via activation of NFκB and ERK1/2; *2*) inhibiting ligand activation and/or heterodimer formation with RXR; or *3*) impairing transcriptional activation of target genes, thereby decreasing TG synthesis.