

## **Trans-10, Cis-12, But Not Cis-9, Trans-11, Conjugated Linoleic Acid Attenuates Lipogenesis in Primary Cultures of Stromal Vascular Cells from Human Adipose Tissue<sup>1</sup>**

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**ABSTRACT** We have previously shown that both a commercially available mixture of conjugated linoleic acid (CLA) isomers and the *trans*-10, *cis*-12 isomer of CLA reduced the triglyceride (TG) content and induced apoptosis in differentiating cultures of murine 3T3-L1 preadipocytes. However, the influence of CLA isomers on differentiating human (pre)adipocytes is unknown. Therefore, we conducted a series of studies using primary cultures of stromal vascular cells isolated from human adipose tissue to determine: 1) the influence of seeding density and thiazolidinedione (TZD) concentration on TG content; 2) the chronic dose response of *cis*-9, *trans*-11 CLA vs. *trans*-10, *cis*-12 CLA on TG content; 3) whether chronic linoleic acid supplementation could rescue the TG content of CLA-treated cultures; and 4) whether *trans*-10, *cis*-12-mediated reduction in cellular TG was due to decreased lipogenesis and/or increased lipolysis. In expt. 1, the TG content [ $\mu\text{mol}/(\text{L} \cdot 10^6 \text{ cells})$ ] increased as both seeding density and TZD concentration increased. For example, cultures seeded at  $4 \times 10^4 \text{ cells}/\text{cm}^2$  and supplemented with  $10 \mu\text{mol}/\text{L}$  BRL 49653 had 10-fold more TG than similarly seeded cultures without BRL 49653. In expt. 2, TG content decreased as the level of *trans*-10, *cis*-12 CLA increased from 1 to  $10 \mu\text{mol}/\text{L}$ , whereas the TG content increased with increasing concentrations of either linoleic acid or *cis*-9, *trans*-11 CLA. In expt. 3, linoleic acid supplementation restored the TG content of cultures treated with *trans*-10, *cis*-12 CLA compared with cultures treated with CLA alone, suggesting that attenuation of TG content by CLA is reversible. In expt. 4, glucose incorporation into total lipid decreased with increasing levels of *trans*-10, *cis*-12 CLA, whereas neither CLA isomer acutely affected lipolysis. These data suggest that the reported antiobesity actions of a supplement containing a crude mixture of CLA isomers given to humans may be due to inhibition of lipogenesis by the *trans*-10, *cis*-12 isomer. J. Nutr. 131: 2316–2321, 2001.

**KEY WORDS:** • conjugated linoleic acid • human (pre)adipocytes • differentiation • triglyceride • lipogenesis • lipolysis

Conjugated linoleic acid (CLA)<sup>3</sup> consists of a group of positional and geometric fatty acid isomers that are derived from linoleic acid [18:2(n-6)]. CLA is found in ruminant meats, pasteurized cheeses and dairy products and, therefore, is a natural part of the diet. Several research groups have demonstrated the antiobesity properties of a crude mixture of CLA isomers (1–11). For example, mice, pigs and hamsters fed low levels of CLA (<1.5 g/100 g) had less body fat and more lean body mass than did controls (7–11).

Several in vitro studies have shown that treatment with a crude mixture of 20–200  $\mu\text{mol}/\text{L}$  CLA isomers decreases the

proliferation (12,13) and lowers the lipid content (3–5,12–14) of murine (pre)adipocytes. Brodie et al. (12) and Choi et al. (14) demonstrated that 25–100  $\mu\text{mol}/\text{L}$  of mixed CLA isomers reduced mRNA levels of adipocyte-specific genes PPAR $\gamma$ 2 and adipocyte fatty acid binding protein in cultures of 3T3-L1 preadipocytes. Moreover, the *trans*-10, *cis*-12 isomer of CLA was determined to be the bioactive isomer that reduced lipoprotein lipase activity (5), increased lipolysis (5), induced apoptosis (13), reduced stearoyl-CoA desaturase activity and expression (14) and decreased the triglyceride (TG) content of cultures of murine 3T3-L1 preadipocytes (5,13,14). In contrast, Satory and Smith (15) found that a crude mixture of CLA isomers increased de novo lipogenesis in cultures 3T3-L1 preadipocytes. Furthermore, both *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA increased markers of differentiation in porcine adipocytes in vitro (16). Therefore, differences within and between species in response to CLA treatment of (pre)adipocytes have been found.

In humans, the influence of CLA treatment is less clear. For example, CLA treatment (3.4–6.8 g/d) for 3 mo reduced body fat mass of obese and overweight adult men and women (17).

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<sup>3</sup> Abbreviations used: BSA, bovine serum albumin; CLA, conjugated linoleic acid; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HEPES, *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid]; HBSS, Hank's balanced salt solution; SV, stromal vascular; TG, triglyceride; TZD, thiazolidinedione.

In contrast, Zambell et al. (18) found that CLA consumption (3 g/d, mixed isomers) over 3 mo did not affect fat mass, fat-free mass, percent body fat or body weight in humans. This discrepancy may be due to the type and amount of CLA isomers used along with the body weights and energy intakes of the subjects.

Whereas CLA attenuates body fat in animals and reduces the TG content of several murine preadipocyte models, potential antiobesity properties in humans are disputable and require additional examination. Thus, examining the impact of the predominant isomers of CLA found in CLA supplements (e.g., *cis*-9, *trans*-11 and *trans*-10, *cis*-12) on the differentiation of stromal vascular (SV) cells isolated from human adipose tissue could show whether CLA has direct effects on adipose tissue, which may play a role in altering adiposity *in vivo*. Therefore, the purpose of this study was to: 1) establish optimal culturing conditions for differentiating primary cultures of human SV cells; 2) determine which isomer(s) of CLA attenuate TG content; 3) examine whether the proposed attenuation of CLA of TG content is reversible; and 4) determine whether CLA decreases TG content by decreasing lipogenesis and/or increasing lipolysis in primary cultures of human adipocytes.

## MATERIALS AND METHODS

### Cell isolation and culture conditions

**Isolation and culture of SV cells from human adipose tissue.** SV cells and all media were provided by Zen-Bio (Research Triangle Park, NC). Briefly, abdominal adipose tissue (expt. 1–4a) and thigh adipose tissue (expt. 4b) were obtained from middle-aged women with body mass indexes <30.0 kg/m<sup>2</sup> during liposuction or elective surgery with consent. Subsequently, tissue was minced and enzymatically digested for 45 min in a Krebs-Ringer buffer containing 1 g/L collagenase (CLS-1; Worthington Biochemical Corp., Lakewood, NJ), 15 g/L bovine serum albumin (BSA), and 5 mmol/L glucose. Digestion was carried out at a 5 mL/1 g ratio (digestion solution:tissue mass). The digest was then filtered through 200- and 60- $\mu$ m mesh and pelleted by centrifuging at 600  $\times$  g for 5 min. The SV cells were resuspended in an RBC lysis buffer for 10 min and recentrifuged to remove most of the contaminating endothelial cells. Cultures of SV cells were grown in proliferation medium containing 90% Dulbecco's modified Eagle's medium (DMEM)/Ham's Nutrient Broth F-10 (1:1, v/v), 100 mL/L fetal bovine serum (FBS), 15 mmol/L HEPES (pH 7.4), 60 U/mL penicillin, 6  $\times$  10<sup>4</sup> U/L streptomycin and 25 mg/L amphotericin B. Cultures were incubated at 37°C in a humidified O<sub>2</sub>:CO<sub>2</sub> (95:5%) atmosphere. SV cells were grown to 80% confluency and then cryopreserved in liquid nitrogen in aliquots (2  $\times$  10<sup>6</sup> cells/mL).

**Induction of cell differentiation.** Cryopreserved aliquots were subsequently thawed, seeded in T-150 flasks at 1  $\times$  10<sup>4</sup>/cm<sup>2</sup> and grown in proliferation medium until 80% confluent. At this time the cells were removed via trypsinization, seeded (3  $\times$  10<sup>4</sup>/cm<sup>2</sup>, except for expt. 1) in 24-well or 96-well (exp. 4b) Falcon dishes and allowed to attach for 24 h in proliferation medium. After attachment, cultures were grown for the next 3 d in differentiation medium containing DMEM/Ham's F-10 (1:1, v/v), 30 mL/L FBS, 15 mmol/L HEPES (pH 7.4), 33  $\mu$ mol/L biotin, 17  $\mu$ mol/L pantothenate, 100 nmol/L human insulin, 1  $\mu$ mol/L dexamethasone (DEX), 6  $\times$  10<sup>4</sup> U/L penicillin, 6  $\times$  10<sup>4</sup> U/L streptomycin, 25 mg/L fungizone, 0.25 mmol/L isobutylmethylxanthine and TZD (expt. 1 = BRL 49653; expt. 2–4 = Zen Bio's proprietary reagent). Thereafter, cultures were exposed to adipocyte medium consisting of 97% DMEM/Ham's F-10 (1:1, v/v), 15 mmol/L HEPES (pH 7.4), 30 mL/L FBS, 33  $\mu$ mol/L biotin, 17  $\mu$ mol/L pantothenate, 100 nmol/L human insulin, 1  $\mu$ mol/L DEX, 6  $\times$  10<sup>4</sup> U/L penicillin, 6  $\times$  10<sup>4</sup> U/L streptomycin and 25 mg/L fungizone. Adipocyte media was replaced every 3 d. After 10–12 d under these culturing conditions, ~35% of the cells exhibited mor-

phology of mature adipocytes. After 18 d in culture, at least 50% of the cells contained visual lipid droplets.

**Experimental designs.** Experiment 1 was designed to determine optimal culturing conditions during differentiation of primary cultures of SV cells isolated from human adipose tissue. Specifically, we wanted to determine how seeding density and TZD (PPAR $\gamma$  agonist BRL 49653) concentration influenced TG content [ $\mu$ mol/(L  $\times$  10<sup>6</sup> cells)]. SV cells were seeded at increasing densities (2, 3 or 4  $\times$  10<sup>4</sup>/cm<sup>2</sup>) in differentiation media containing 0, 1 or 10  $\mu$ mol/L TZD (BRL 49653 during the first 3 d of differentiation) in this 3  $\times$  3 factorial design. All cultures received the same adipocyte media after 3 d of differentiation. The cultures were harvested on d 11–12 of differentiation and TG content and cell number were measured. Another set of cultures was stained with Oil Red O and counterstained with Mayer's hematoxylin to assess cellular differentiation potential (n = 3 per treatment combination).

The objective of expt. 2 was to evaluate the dose response of *trans*-10, *cis*-12 CLA, *cis*-9, *trans*-11 CLA and linoleic acid on the TG content of the cultures. SV cells were seeded at a density of 3  $\times$  10<sup>4</sup>/cm<sup>2</sup> and continuously treated with increasing concentrations (1, 3, 10 or 30  $\mu$ mol/L) of linoleic acid, *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA. All cultures contained TZD (Zen Bio's proprietary agent that stimulated adipogenesis to the same degree as BRL 49653) for the first 3 d of differentiation. A set of control cultures contained only the vehicle (BSA) plus TZD (Zen Bio's proprietary agent added during the first 3 d of differentiation). TG content and cell number were evaluated on d 11 of differentiation.

Experiment 3 was designed to determine whether supplementing the cultures with linoleic acid could reverse the *trans*-10, *cis*-12 CLA-mediated reduction in TG content. SV cells were seeded at a density of 3  $\times$  10<sup>4</sup>/cm<sup>2</sup> and continuously treated with 10  $\mu$ mol/L *trans*-10, *cis*-12 CLA alone, 10  $\mu$ mol/L *trans*-10, *cis*-12 CLA plus linoleic acid at 10, 30 or 100  $\mu$ mol/L or linoleic acid alone at 10, 30 or 100  $\mu$ mol/L. All cultures contained TZD (1  $\mu$ mol/L BRL 49653) for the first 3 d of differentiation. TG and cell number were assessed on d 11 of differentiation.

Experiment 4, a and b were designed to determine whether the *trans*-10, *cis*-12 CLA-mediated reduction in TG content was due to decreased lipogenesis and/or increased lipolysis. In expt. 4a (lipogenesis), SV cells were seeded at a density of 3  $\times$  10<sup>4</sup>/cm<sup>2</sup> and continuously treated with increasing concentrations (3, 10 or 30  $\mu$ mol/L) of linoleic acid, *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA. A set of control cultures received vehicle (BSA). All cultures contained TZD (Zen Bio's proprietary agent) for the first 3 d of differentiation, received differentiation media (d 1–3), adipocyte media (d 4–9) and low-glucose (~5 mmol/L) adipocyte media (d 10–12) before measuring glucose incorporation into total lipid. On d 12, cultures of adipocytes were incubated for 2 h with <sup>14</sup>C-labeled glucose and the radioactivity in the lipid fraction was determined by scintillation counting. Time course data (not shown) indicated a linear increase in radio-labeled glucose incorporation into lipid over 2 h.

In expt. 4b, basal lipolysis was measured on d 18 of differentiation after the cultures had been treated with fatty acids for 5 h. Cultures were grown in basal media (e.g., adipocyte media lacking FBS, DEX and insulin) for 24 h before the measurement of lipolysis. Lipolysis was determined by measuring free glycerol release into the media after acute (5-h) treatment. A preliminary study (data not shown) demonstrated that glycerol release into the media was linear between 0, 1, 3 and 5 h. A set of vehicle control cultures was treated with 1  $\mu$ mol/L isoproterenol to determine the lipolytic sensitivity of the cultures to a  $\beta$ -adrenergic agent known to activate adenylate cyclase. All cultures contained TZD (Zen Bio's proprietary agent) for the first 3 d of differentiation.

**Treatment specifications.** Linoleic acid (Nu Check Prep, Elysian, MN; 99% pure), *cis*-9, *trans*-11 CLA (Matreya, Pleasant Garden, PA; 98% pure) and *trans*-10, *cis*-12 CLA (Matreya; 98% pure) were complexed to fatty acid free albumin (1 mmol/L BSA:4 mmol/L fatty acid), and added to postconfluent SV cultures at various concentrations, except expt. 4b in which all fatty acids were dissolved in dimethyl sulfoxide (DMSO). All cultures contained the same amount of vehicle (BSA in expt. 1–4a, DMSO in expt. 4b). All cultures received differentiation media for d 1–3 and adipocyte media from d

4 onward unless otherwise indicated. Fresh fatty acids were added with each media change until the day of harvest. With the exception of expt. 4b (lipolysis), all cultures were chronically treated with fatty acids (e.g., beginning on d 1 of the differentiation program) until their time of harvest during late stages of differentiation (d 10–18). All of the treatment combinations had a sample size of  $n = 6$  unless otherwise indicated.

**Determination of cell number.** Adherent cells were harvested in 500  $\mu\text{L}$  cell counting solution containing 0.01 mol/L monobasic  $\text{NaPO}_4$ , 0.154 mol/L NaCl, 25 mmol/L EDTA and 20 g/L BSA. After gentle trituration to deter cell clumping, cell number was determined using the Coulter Multi-Sizer IIE Counter (Coulter Electronics, Hi-aleah, FL).

**Quantification of TG content.** Cells were harvested in 500  $\mu\text{L}$  cell counting solution and sonicated. Triton X-100 (5 mL/100 mL) was added to all lysates to ensure homogenous lipid distribution in all samples. Intracellular TG content was measured using a colorimetric assay that quantifies the glycerol content of the samples (Infinity TG reagent 343–25P; Sigma, St. Louis, MO). This assay involves the enzymatic hydrolysis of TG by lipases to free fatty acid and glycerol. The glycerol moiety, through a series of oxidation-reduction reactions, then associates with 3,5-dichloro-2-hydroxybenzene sulfonate and 4-aminoantipyrine to produce a red dye. The absorbency of this dye is directly proportional to the concentration of TG present in each lysate. Each sample was transferred to a 96-well plate, and the absorbency quantified at 520 nm on a microtiter plate reader (Tecan-SLM, Research Triangle Park, NC). TG concentration was determined based on a standard curve using Sigma's glycerol standard (G-1394) and data are expressed as  $\mu\text{mol}/(\text{L} \cdot 10^6 \text{ cells})$ .

**Lipid staining.** The presence of intracellular lipid was visualized by staining cultures with Oil Red O. Briefly, cell monolayers were washed twice with 1 mL Hank's Balanced Salt Solution (HBSS) and then fixed for 1 h at 4°C in a solution containing 100 mL/L formalin and 0.36 mol/L calcium chloride. After fixation, cells were washed twice with deionized water and stained using 3 mL/L Oil Red O in isopropanol for 15 min at room temperature. The cells were rinsed again with deionized water. The nuclei then were counterstained with Mayer's Hematoxylin (1 g/L) for 3 min, then rinsed a final time with deionized water for 3 min. Counterstaining allows for quantifying the percentage of cells that have undergone differentiation (e.g., total cell number per field/number of cells having appreciable amounts of Oil Red O stain). Photomicrographs were taken of the Oil Red O-stained cells to provide visual indication of the degree of lipid accumulation in relation to nuclei.

**Glucose incorporation into total lipid.** Incorporation of  $^{14}\text{C}$ -glucose into cellular lipid was determined on d 12 of differentiation in cultures chronically treated with fatty acids or vehicle. After the addition of fatty acids and low glucose ( $\sim 5 \text{ mmol/L}$ ) medium to the cultures on d 12, 1.0  $\mu\text{Ci}$  [ $^{14}\text{C}$ ]-D-glucose ([ $^{14}\text{C}$ ]-D-glucose; SA  $\sim 250 \text{ mCi/mmol}$ ; ICN, Costa Mesa, CA)/mL medium was added to the cultures for 2 h. Our time course study indicated a linear increase in radio-labeled glucose incorporation into lipid over a 2-h period (data not shown). After 2 h, media containing unincorporated  $^{14}\text{C}$ -glucose was immediately removed and the cultures were washed with 1 mL HBSS to remove unincorporated  $^{14}\text{C}$ -glucose. An additional 1 mL of HBSS was added and, after vigorous trituration, cells were transferred to glass vials. Five milliliters of a chloroform:methanol (2:1) solution was added to each vial and they were vortexed for 1 min. All samples were then centrifuged for 5 min at  $1000 \times g$  to further separate the hydrophobic and hydrophilic phases. The lower hydrophobic phase was removed from the tubes and dried under nitrogen at 40°C. Five milliliters of scintillation cocktail (Scinti Verse; Fisher Scientific, Norcross, GA) was added to each sample, and the  $^{14}\text{C}$  content was determined by liquid scintillation counting on a Beckman LS 6000 Scintillation Counter (Beckman Instruments, Palo Alto, CA). To control for unincorporated  $^{14}\text{C}$ -glucose that may have accompanied the cultures into the lipid extraction vials, a set of cultures were exposed to  $^{14}\text{C}$ -glucose for 5 s and subsequently washed, harvested and fractionated. The radioactivity in the lipid fraction from these cultures was subtracted from the total counts. Cell numbers were determined from parallel experiments in separate culture

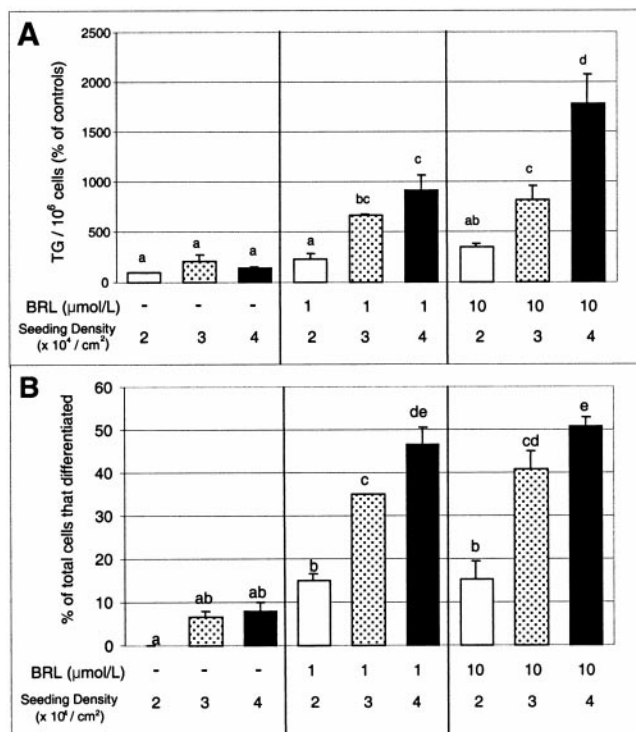
dishes at the time of radioisotope addition to the medium. Mean  $^{14}\text{C}$ -glucose incorporation is expressed as  $\text{pmol}/(\text{L} \cdot 10^6 \text{ cells})$ .

**Lipolysis assay.** On d 17 of differentiation, cultures were grown in basal adipocyte media (adipocyte media minus FBS, DEX and insulin). On d 18 of differentiation, cultures of mature adipocytes were washed and incubated in Krebs-Ringer buffer supplemented with  $\sim 5 \text{ mmol/L}$  glucose and incubated for 5 h at 37°C with the fatty acid treatments or 1.0  $\mu\text{mol/L}$  isoproterenol (positive control for lipolysis). All fatty acids were dissolved in DMSO (final concentration = 1 mL/L). A set of vehicle controls contained 1 mL/L DMSO. Conditioned media (100  $\mu\text{L}$ ) was removed from each well, and the lipolytic rate was determined by quantifying the amount of free glycerol in each sample using Sigma's TG kit (GPO-Trinder; Sigma) (19).

**Statistics.** Analyses of statistically significant differences between treatment means (e.g., main effects and their interactions) were conducted using two-way (e.g., expt. 1 = seeding density  $\times$  BRL concentration; expt. 2–4 = treatment  $\times$  dose) least squares ANOVA General Linear Models procedures and a commercially available software program (SUPERANOVA; Abacus Concepts, Berkeley, CA). Differences between treatment means were identified by Student's  $t$  test and considered significant at  $P < 0.05$ .

## RESULTS

**Experiment 1.** Increasing seeding density and TZD concentration increased the TG content [ $\mu\text{mol}/(\text{L} \cdot 10^6 \text{ cells})$ ] of the cultures (Fig. 1A). The influence of increasing seeding density on TG content was greatest in the cultures containing either 1 or 10  $\mu\text{mol/L}$  TZD. This effect was greatest in cultures



**FIGURE 1** Effects of TZD BRL 49653 and seeding density on the TG content (A) and differentiation potential (B) in cultures of SV cells isolated from human adipose tissue. Cultures were treated with BRL 49653 during the first 3 d of differentiation and received adipocyte media thereafter. In expt. 1a ( $n = 6$ ), cultures were harvested on d 13 and TG content [ $\mu\text{mol}/(\text{L} \cdot 10^6 \text{ cells})$ ] was measured. In expt. 1b ( $n = 3$ ), cultures were stained with Oil Red O to identify mature adipocytes and immediately counterstained with the nuclear stain hematoxylin to identify nondifferentiated cells. Means ( $\pm$  SEM) not sharing a letter differ,  $P < 0.05$ .

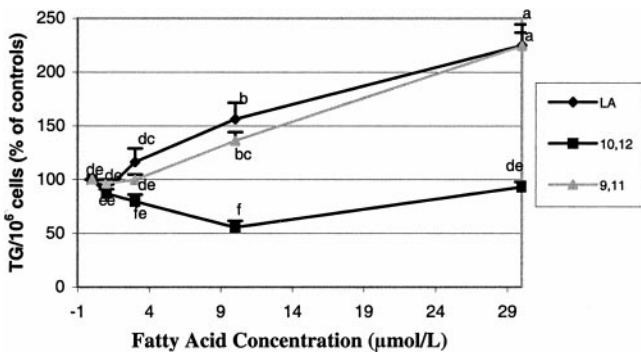
supplemented with 10  $\mu\text{mol/L}$  TZD, where doubling the seeding density increased the TG content approximately fivefold. The data in Figure 1B provide insight into how seeding density and TZD concentration influenced the number of cells that phenotypically differentiate into adipocytes (e.g., accumulate visually detectable lipid droplets). The data in Figure 1B closely parallel the TG content data in Figure 1A, suggesting that the increase in TG content was due to an increase in the number of cells that have differentiated into adipocytes. The exception to this observation was the cultures seeded at the highest seeding density and TZD concentration. This treatment group had nearly twice as much TG compared with the group seeded at the same density ( $4 \times 10^4$ ) and supplemented with 1  $\mu\text{mol/L}$  TZD but had nearly the same percentage of cells that differentiated (47% vs. 50%). This suggests that the increase in TZD concentration from 1 to 10  $\mu\text{mol/L}$  increased adipocyte size or lipid filling rather than adipocyte number.

**Experiment 2.** The TG content of the cultures increased in a dose-dependent manner as the level of linoleic acid and *cis*-9, *trans*-11 CLA increased (Fig. 2). In contrast, as the level of *trans*-10, *cis*-12 increased from 1 to 10  $\mu\text{mol/L}$ , the TG content decreased. However, the TG content of cultures treated with 30  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA was not significantly different than that of vehicle controls.

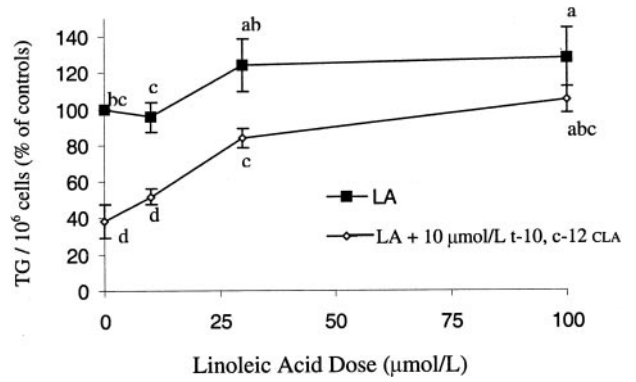
**Experiment 3.** Cultures treated with 10  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA alone had ~60% less TG than the vehicle controls (Fig. 3). Interestingly, when 10  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA-treated cultures were supplemented with 10, 30 or 100  $\mu\text{mol/L}$  linoleic acid, they had 26%, 55% and 64% more TG, respectively, than those cultures treated with 10  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA alone. In fact, the *trans*-10, *cis*-12 CLA-treated cultures supplemented with 100  $\mu\text{mol/L}$  linoleic acid had a TG content similar to the BSA controls, suggesting that linoleic acid supplementation reverses the TG-lowering effect of CLA.

**Experiment 4a.** Incorporation of  $^{14}\text{C}$ -glucose into total cellular lipid per  $10^6$  cells decreased as the level of *trans*-10, *cis*-12 CLA increased in the cultures (Fig. 4). Cultures treated with 30  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA had 80% less  $^{14}\text{C}$ -glucose incorporated into cellular lipid compared with the vehicle controls. In contrast, neither linoleic acid nor *cis*-9, *trans*-11 CLA influenced glucose incorporation into total lipid.

**Experiment 4b.** Isoproterenol-stimulated lipolysis in cultures of abdominal and thigh adipocytes was ~2.5- and 1.5-fold greater, respectively, than control cultures (Fig. 5). In



**FIGURE 2** Effects of increasing levels of linoleic acid, *trans*-10, *cis*-12 CLA, and *cis*-9, *trans*-11 CLA on the TG content [ $\mu\text{mol/L} \cdot 10^6$  cells] in differentiating cultures of SV cells isolated from human adipose tissue. Cultures were treated with 0–30  $\mu\text{mol/L}$  of each fatty acid continuously and harvested on d 13. Means ( $\pm$  SEM,  $n = 6$ ) not sharing a letter differ,  $P < 0.05$ .

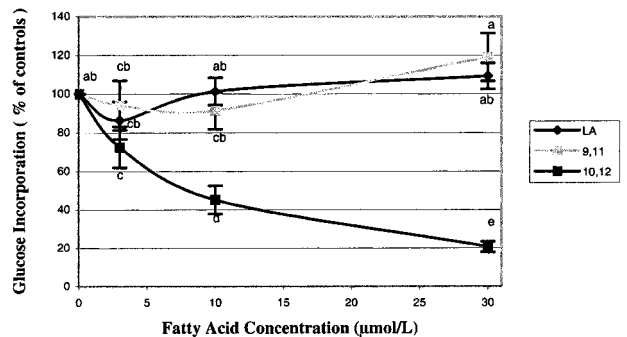


**FIGURE 3** Linoleic acid reverses the suppression of the TG content by CLA [ $\mu\text{mol/L} \cdot 10^6$  cells] in human preadipocytes. Means ( $\pm$  SEM,  $n = 6$ ) not sharing a letter differ,  $P < 0.05$ .

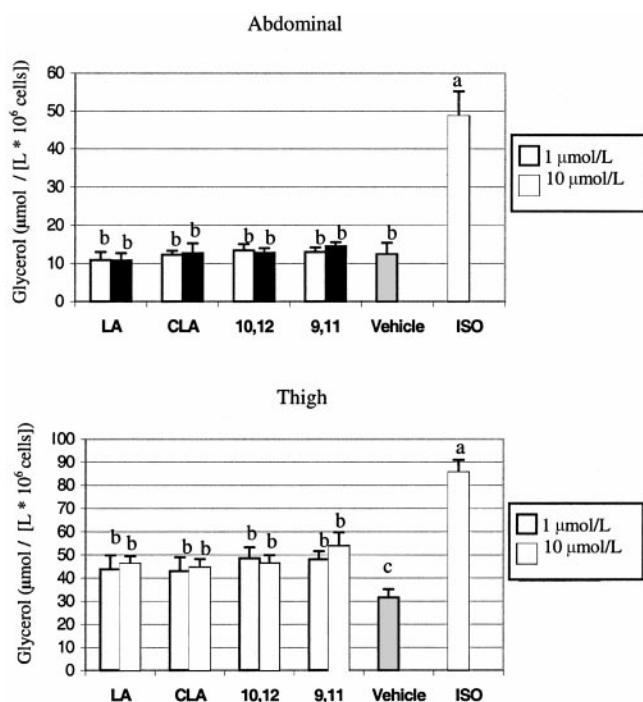
contrast, lipolysis was not altered by any of the acute fatty acid treatments in cultures of abdominal adipocytes compared with the DMSO controls. In cultures of thigh adipocytes, although all fatty acid treatments stimulated lipolysis, there were no significant differences among the types or doses of fatty acids.

DISCUSSION

The present study provides direct evidence that chronic treatment with *trans*-10, *cis*-12 CLA, but not with *cis*-9, *trans*-11 CLA, decreases the TG content and glucose incorporation into total lipid in human primary SV cultures. Furthermore, linoleic acid supplementation of the cultures reversed the TG-lowering actions of *trans*-10, *cis*-12 CLA, suggesting that CLA is not toxic to the cultures and that it may lower TG content by interfering with TG esterification or cellular differentiation. To our knowledge, this is the first time the lipid-lowering actions of *trans*-10, *cis*-12 CLA have been demonstrated in primary cultures of SV cells isolated from human adipose tissue. These data suggest that the antioesity effects of a crude mixture of CLA isomers observed in rodents (2–7,20), hamsters (19), pigs (8,9,21) and humans (15,22) may indeed be due to the direct actions of the *trans*-10, *cis*-12 CLA isomer on adipose tissue mass and cellularity. In additional support of this concept, our group (13,23,24) and the group of Pariza et al. (5,14) have clearly shown that the *trans*-10, *cis*-12 isomer of CLA is the TG-lowering isomer



**FIGURE 4** Effects of linoleic acid, *cis*-9, *trans*-11 CLA, and *trans*-10, *cis*-12 CLA on  $^{14}\text{C}$ -glucose incorporation into total cellular lipid [ $\text{pmol/L} \cdot 10^6$ ] in human preadipocytes. Data are expressed as percentage of vehicle (BSA) controls. Means ( $\pm$  SEM,  $n = 6$ ) not sharing a letter differ,  $P < 0.05$ .



**FIGURE 5** The lipolytic effects of 5 h of treatment of SV cells isolated from human abdominal (A) and thigh (B) adipose tissue with linoleic acid, a crude mixture of CLA isomers, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA and isotroterol. Means ( $\pm$  SEM,  $n = 6$ ) not sharing a letter differ,  $P < 0.05$ .

found in the commercially available crude mixture of CLA isomers using 3T3-L1 preadipocytes as the cell model.

Our results are further substantiated by research demonstrating that *trans*-10, *cis*-12 CLA is the antiadipogenic isomer of CLA in some animals and cultures of preadipocytes. In vivo, ICR mice consuming 2.5 g/kg diet *trans*-10, *cis*-12-enriched CLA had lower body fat percentages than controls or mice fed 2.5 g/kg diet *cis*-9, *trans*-11-enriched CLA (5). Furthermore, Baumgard et al. (25) found that only the *trans*-10, *cis*-12 isomer of CLA reduced milk fat percentage and yield in Holstein cows. In vitro, Park et al. (5) showed that 3T3-L1 preadipocytes treated for 48 h with *trans*-10, *cis*-12 CLA beginning on d 4 of differentiation contained less intracellular TG and glycerol than *cis*-9, *trans*-11 CLA-treated cultures. More recently, Choi et al. (14) found that *trans*-10, *cis*-12 CLA inhibited the expression of stearoyl-CoA desaturase-1 without reducing PPAR $\gamma$ 2 or adipocyte fatty acid binding protein mRNA levels in 3T3-L1 preadipocytes.

Studies by Blankson et al. (17) and Vessby and Smedman (22) have demonstrated the antiobesity actions of CLA in humans. Blankson et al. (17) showed that overweight and obese subjects consuming supplements containing either 3.4 or 6.8 g/d of a crude mixture of CLA isomers (equal amounts of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers) for 3 mo had less body fat than did placebo-treated controls. Vessby and Smedman (22) also found that subjects consuming a CLA supplement (4.2 g/d of a crude mixture of CLA isomers) for 3 mo had significantly less body fat than did placebo-treated controls. Taken together, our data suggest that the antiobesity effects of CLA reported in these two human studies may be due to the direct actions of the *trans*-10, *cis*-12 isomer on adipocyte size and TG content, because CLA treatment did not reduce cell number compared with control cultures (data not shown). Furthermore, we hypothesize that the *cis*-9, *trans*-11 CLA

isomer in the crude CLA mixture does not reduce adiposity, because it increased the TG content of our human preadipocyte cultures in a dose-dependent manner (Fig. 2). However, the precise mechanism(s) by which *trans*-10, *cis*-12 CLA reduces the TG content is not yet known.

Several proposed mechanisms for the TG-lowering actions of CLA include: 1) decreasing fatty acid esterification into TG; 2) interfering with the preadipocyte differentiation program; 3) decreasing lipogenesis; or 4) increasing lipolysis. The present study suggests that chronic *trans*-10, *cis*-12 CLA treatment decreases the TG content of cultures of human adipocytes, at least in part, by decreasing glucose incorporation into total lipid in a dose-dependent manner (Fig. 4).

In contrast to the attenuation of glucose incorporation into total lipid by CLA, acute treatment (5 h) of mature human adipocytes with either CLA isomer did not affect lipolysis (Fig. 5). Because we did not measure the impact of linoleic acid supplementation to CLA-treated cultures on lipogenesis, we do not know how linoleic acid reversed the TG-lowering actions of CLA. We speculate, however, that the supplemental linoleic acid was esterified, thereby increasing the TG content of the CLA-treated cultures. Support for this hypothesis is based on the fact that increasing levels of linoleic acid to the cultures increased the TG content (Fig. 2) without affecting glucose incorporation into total lipid (Fig. 4). We have duplicated the TG-rescuing effect of linoleic acid in CLA-treated cultures in 3T3-L1 preadipocytes as well (24). In the present study, the linoleic acid-mediated increase in TG content was much more robust in expt. 2 (Fig. 2) compared with expt. 3 (Fig. 3). We speculate that this was due to the different type of TZD used in these experiments. Future studies examining the effects of CLA and linoleic acid on TG esterification and on regulators of preadipocyte differentiation, such as PPAR $\gamma$ 2 and C/EBP $\alpha$ , would provide additional insight into how CLA decreases TG content of human adipocytes, i.e., by affecting differentiation per se or only by decreasing lipogenesis.

In an attempt to determine the mechanism by which *trans*-10, *cis*-12 CLA inhibits TG accumulation, we have assessed the expression of PPAR $\gamma$ 2 protein in cultures of 3T3-L1 preadipocytes (23). Acute *trans*-10, *cis*-12 CLA treatment (2 d) of the murine cultures increased—and chronic treatment (6 d) decreased—the expression of PPAR $\gamma$ 2 protein. Similar to our results in 3T3-L1 cells, Brodie et al. (12) found that both linoleic acid and 50  $\mu$ mol/L of a crude mix of CLA isomers reduced PPAR $\gamma$  mRNA levels on d 7 of differentiation. In contrast to the results of Brodie et al., Choi et al. (14) reported that although a crude mixture of CLA isomers reduced PPAR $\gamma$ 2 mRNA expression, 45  $\mu$ mol/L *trans*-10, *cis*-12 CLA did not affect PPAR $\gamma$ 2 mRNA levels. Furthermore, Houseknecht et al. (1) found that 100–200  $\mu$ mol/L of a crude mixture of CLA isomers activated the expression of PPAR $\gamma$  in CV-1 cells transiently transfected with a human PPAR $\gamma$  reporter gene construct. Therefore, the influence of CLA on PPAR $\gamma$ 2 expression seems to be dependent on the genetic background of the cell or animal model, duration and timing of treatment, type of CLA isomer used, PPAR $\gamma$  isoform examined and differences in expression of PPAR $\gamma$  mRNA vs. protein.

Concerning the potential inhibition of TG esterification by CLA, Choi et al. (14) found that *trans*-10, *cis*-12 CLA reduced stearoyl-CoA desaturase activity, suggesting that CLA may be interfering with the desaturation of long-chain fatty acids and their subsequent esterification into TG. They also demonstrated that 3T3-L1 preadipocytes treated with 45  $\mu$ mol/L *trans*-10, *cis*-12 CLA had lower levels of both 16:1 and 18:1 in

their cellular lipids. Azain et al. (20) found that Sprague-Dawley rats fed 5.0 g/kg diet mixed isomers of CLA for 7 or 49 d had lower levels of 16:1 and 18:1, along with higher levels of 18:2 in their adipose tissue. In support of these findings, we discovered that 50  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA-treated 3T3-L1 cultures had lower amounts of 16:1 (in the neutral lipid fraction) and 18:1, *cis*-11 (in both the neutral and polar lipid fractions) and higher amounts of 18:2, *cis*-9, *cis*-12 (in both the neutral and polar lipid fractions) compared with BSA controls (23). Finally,  $\Delta$ -6 desaturation of linoleic acid in rat hepatic microsomes was decreased in the presence of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (26). However, only the *trans*-10, *cis*-12 isomer of CLA inhibited  $\Delta$ -9 desaturation of stearic acid. Taken together, these data suggest that CLA treatment could decrease TG content by reducing the synthesis of monounsaturated fatty acids, such as 16:1 and 18:1, because these fatty acids are the major monounsaturated fatty acids of membrane phospholipids and TG found in murine adipocytes (27).

Previously, we have shown that treatment with mixed CLA isomers or *trans*-10, *cis*-12 CLA induced biochemical (i.e., nuclear condensation and increased percentage of cells in the sub-G<sub>1</sub> phase) and morphological (i.e., rounding and membrane blebbing) changes that are characteristic of apoptosis (13). A number of studies also have shown that CLA is capable of inducing apoptosis. For example, cells in the adipose tissue of C57BL/6J mice fed 10.0 g/kg diet of mixed isomers of CLA underwent apoptosis (6). Additional studies in primary rat mammary cells (28) as well as N-nitroso-N-methyl urea mammary cells (29) have also demonstrated that CLA induces apoptosis. However, in the present study using the human SV cultures, there were no obvious visual signs of apoptosis (i.e., a large number of nonadherent cells) and cell number was not decreased by CLA treatment. Furthermore, treatment of cultures with >30  $\mu\text{mol/L}$  CLA actually increased TG content above control levels (data not shown). In 3T3-L1 cultures in our laboratory, high levels of CLA (>50  $\mu\text{mol/L}$  *trans*-10, *cis*-12 or >200  $\mu\text{mol/L}$  mixed CLA isomers) are apoptotic/cytotoxic, whereas in our human cultures these levels do not cause cell death, suggesting that the TG-lowering actions of CLA are influenced by differences in culturing conditions or in the source of adipocytes (e.g., animal vs. human, primary vs. cell line and embryonic vs. adult). Taken together, these data suggest that although CLA treatment decreases the TG content of both murine and human adipocytes, it may not be by the same mechanism(s).

In conclusion, we have found that *trans*-10, *cis*-12 CLA is the TG-lowering isomer of CLA in primary cultures of SV cells isolated from human adipose tissue. In contrast, *cis*-9, *trans*-11 CLA increased the TG content of the cultures. Supplementation with linoleic acid was able to reverse the TG-lowering effects of *trans*-10, *cis*-12 CLA. Furthermore, chronic treatment of the cultures decreases the TG content in part by decreasing glucose incorporation into total lipid, whereas acute CLA treatment did not affect lipolysis. Future research is needed to discover the precise mechanism through which *trans*-10, *cis*-12 decreases TG content.

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