

## Trans-10, Cis-12 Conjugated Linoleic Acid Increases Fatty Acid Oxidation in 3T3-L1 Preadipocytes<sup>1</sup>

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**ABSTRACT** The purpose of this study was to examine the effect of 0–50  $\mu\text{mol/L}$  *trans*-10, *cis*-12 conjugated linoleic acid (CLA) and *cis*-9, *trans*-11 CLA isomers on lipid and glucose metabolism in cultures of differentiating 3T3-L1 preadipocytes. Specifically, we investigated the effects of 6 d of CLA treatment on the following: 1) <sup>14</sup>C-glucose and <sup>14</sup>C-oleic acid incorporation and esterification into lipid; 2) <sup>14</sup>C-glucose and <sup>14</sup>C-fatty acid oxidation; and 3) basal and isoproterenol-stimulated lipolysis. *Trans*-10, *cis*-12 CLA supplementation (25 and 50  $\mu\text{mol/L}$ ) increased both <sup>14</sup>C-glucose and <sup>14</sup>C-oleic acid incorporation into the cellular lipid fraction, which was primarily triglyceride (TG), compared with bovine serum albumin (BSA) controls. Although glucose oxidation (<sup>14</sup>C-glucose to <sup>14</sup>C-CO<sub>2</sub>) was unaffected by CLA supplementation, oleic acid oxidation (<sup>14</sup>C-oleic acid to <sup>14</sup>C-CO<sub>2</sub>) was increased by ~55% in the presence of 50  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA compared with BSA controls. In contrast, 50  $\mu\text{mol/L}$  linoleic acid (LA) and *cis*-9, *trans*-11 CLA-treated cultures had ~50% lower CO<sub>2</sub> production from <sup>14</sup>C-oleic acid compared with control cultures after 6 d of fatty acid exposure. Finally, 50  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA modestly increased basal, but not isoproterenol-stimulated lipolysis compared with control cultures. Thus, the TG-lowering actions of *trans*-10, *cis*-12 CLA in cultures of 3T3-L1 preadipocytes may be via increased fatty acid oxidation, which exceeded its stimulatory effects on glucose and oleic acid incorporation into lipid. J. Nutr. 132: 450–455, 2002.

**KEY WORDS:** • conjugated linoleic acid • preadipocytes • glucose and fatty acid oxidation  
• glucose and fatty acid incorporation • lipolysis

Conjugated linoleic acid (CLA)<sup>3</sup> consists of a group of positional and geometric fatty acid isomers derived from linoleic acid (LA) [18:2(n-6)]. CLA is found as a mixture of isomers (predominantly *cis*-9, *trans*-11 CLA) in pasteurized cheeses, dairy products and the meat of ruminant animals. Commercial sources of CLA used in most research studies contain predominantly *cis*-9, *trans*-11 (~40%) and *trans*-10, *cis*-12 (~40%) CLA. Feeding a crude mixture of CLA isomers reduces body fat (1–14), while increasing lean body mass in hamsters (11), rodents (2–6) and pigs (7,8). In humans, CLA consumption (3.4–6.8 g/d) for 3 mo reduced body fat mass of obese and overweight adult men and women (15). In contrast, Zambell et al. (16) found that CLA consumption (3 g/d mixed isomers) over 3 mo did not affect fat mass, fat-free mass, percentage of body fat or body weight in normal-weight human subjects, whereas Medina et al. (17) and Berven et al. (18) found no change in body fat or body weight when overweight or obese subjects consumed mixed CLA isomers.

Thus, the effects of CLA on obesity and lean body mass in humans remain controversial.

In vitro, several studies have shown that treatment with a mixture of CLA isomers lowers the lipid content of murine (pre)adipocytes (2,5,19). Moreover, the *trans*-10, *cis*-12 isomer of CLA is the isomer that is believed to reduce triglyceride (TG) content (5,19,20). More recently, we have shown that although *trans*-10, *cis*-12 CLA lowers TG content, the isomer differentially affects peroxisome proliferator-activated receptor  $\gamma$ -2 (PPAR  $\gamma$ -2) protein expression without significantly altering the level of adipogenic fatty acids such as arachidonic acid in cultures of 3T3-L1 preadipocytes (21). In contrast, Choi et al. (20) found that *trans*-10, *cis*-12 CLA reduced stearoyl-CoA desaturase activity and mRNA levels without affecting PPAR- $\gamma$  or adipocyte fatty acid binding protein mRNA, suggesting that CLA may be interfering with the desaturation of long-chain fatty acids and their subsequent esterification into TG.

The aforementioned studies suggest that the antiobesity actions of a crude mixture of CLA isomers may be due to the direct influence of *trans*-10, *cis*-12 CLA on lipid metabolism rather than adipocyte differentiation per se. However, the specific mechanism by which CLA reduces the TG content of (pre)adipocyte cultures remains to be determined. Several possible mechanisms include the following: 1) decreased incorporation of glucose and/or fatty acids into TG (de novo lipogenesis); 2) increased oxidation of glucose and/or fatty acids; or 3) increased lipolysis. Therefore, the purpose of this

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<sup>3</sup> Abbreviations used: BSA, bovine serum albumin; CE, cholesterol ester, CLA, conjugated linoleic acid; CPT-1, carnitine palmitoyltransferase-1; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FFA, free fatty acid; LA, linoleic acid; PPAR  $\gamma$ -2, peroxisome proliferator activated receptor  $\gamma$ -2; TG, triglyceride; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

study was to examine in differentiating cultures of 3T3-L1 preadipocytes the effects of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers on  $^{14}\text{C}$  glucose and  $^{14}\text{C}$ -oleic acid incorporation and esterification into cellular lipids (Exp. 1),  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -oleic acid oxidation to  $^{14}\text{C}$ -CO<sub>2</sub> (Exp. 2) and basal and isoproterenol-stimulated lipolysis (Exp. 3).

## MATERIALS AND METHODS

**Cell model.** Postconfluent monolayers of 3T3-L1 preadipocytes were used as the cellular model for these studies. 3T3-L1 preadipocytes are a nontransformed cell line, which is a continuous substrain of Swiss albino 3T3 murine cells developed through clonal expansion (22). These cells can be converted from a preadipocyte to adipose-like phenotype when appropriately stimulated. In addition, these cells are capable of differentiating in culture in response to agents that induce adipose tissue differentiation *in vivo*.

**Experimental designs and culture conditions.** As outlined in Figure 1, the objective of Exp. 1 was to determine whether increasing doses of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers of CLA decreased the incorporation of  $^{14}\text{C}$ -glucose (Exp. 1a) and  $^{14}\text{C}$ -oleic acid (Exp. 1b) into the organic cellular fraction in cultures of 3T3-L1 preadipocytes. In Exp. 1c, the effect of CLA supplementation on  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -oleic acid incorporation into specific neutral and polar lipid classes was examined. In Exp. 2, the ability of *trans*-10, *cis*-12 CLA to increase the oxidation of  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -oleic acid (thereby producing increased amounts of  $^{14}\text{C}$ -CO<sub>2</sub>) compared with *cis*-9, *trans*-11 CLA- or LA-treated cultures was assessed. Finally, Exp. 3 determined whether *trans*-10, *cis*-12 CLA increased basal and/or isoproterenol-stimulated lipolysis. All reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Both isomers of CLA and LA were complexed to fatty acid-free bovine serum albumin (1 mmol/L BSA:4 mmol/L fatty acid) and added to postconfluent, differentiating cultures on either d 1 of differentiation (Exps. 1 and 2) or d 5 (Exp. 3) of differentiation (Fig. 1) as previously described (19). Pure LA (99% pure according to the manufacturers) was obtained from Nu-Chek-Prep, Elysian, MN. The *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA isomers (98%+ pure according to the manufacturers) were obtained from Matreya, Pleasant Gap, PA. All treatments contained 0.2 mmol/L  $\alpha$ -tocopherol to prevent lipid peroxidation as previously described (19). BSA controls were without fatty acids and had a BSA concentration similar to the fatty acid treatment within each experiment. Media were changed at 2-d intervals and fresh fatty acids added at each media change until the day of harvest.

Cells were seeded at a density of  $3.3 \times 10^3/\text{cm}^2$  in 12-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and cultured in Dulbecco's modified Eagle's medium (DMEM), 10% bovine calf serum and antibiotics until confluent. Two days postconfluency, the cells were stimulated to differentiate with DMEM containing 10%

fetal bovine serum (FBS) (charcoal stripped to remove endogenous fatty acids), 10 mg/L insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 0.1  $\mu\text{mol/L}$  dexamethasone, 0.2 mmol/L  $\alpha$ -tocopherol and 1% antibiotics. On d 3 of differentiation, the above media was replaced with DMEM, 10% stripped FBS, 2.5 mg/L insulin, 0.2 mmol/L  $\alpha$ -tocopherol and 1% antibiotics. From d 5 onward, media containing DMEM, 10% stripped FBS, 0.2 mmol/L  $\alpha$ -tocopherol and 1% antibiotics were used. For the  $^{14}\text{C}$ -glucose incorporation and oxidation experiments, the media on d 5 of differentiation was replaced with low-glucose DMEM (~5 mmol/L).

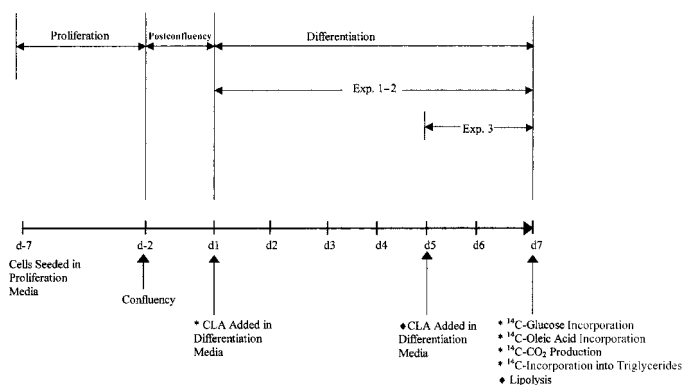
**Cell number (Exps. 1–3).** Adherent cells were harvested in a cell counting solution (25 mmol/L glucose, 0.154 mmol/L NaCl, 0.01 mol/L NaPO<sub>4</sub> [monobasic], 5 mmol/L EDTA, 2% albumin, pH 7.4) and counted on a Coulter Multisizer IIE (Coulter, Miami, FL).

**Glucose incorporation (Exp. 1a).**  $^{14}\text{C}$ -glucose incorporation was determined on d 7 of differentiation after chronic treatment with 0 (BSA control), 25 or 50  $\mu\text{mol/L}$  *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA. On d 7, the media were replaced with low glucose DMEM supplemented with 37 KBq [U- $^{14}\text{C}$ ]-D-glucose/L medium [specific activity: 9.2 GBq/mmol (ICN, Costa Mesa, CA)] for 2 h at 37°C. A time-course study indicated a linear increase in radiolabeled glucose incorporation into lipid over a 4-h period (data not shown). After 2 h, media were removed, the cell monolayers were washed twice, harvested in PBS with vigorous trituration and added to glass vials containing 3.75 mL of a chloroform/methanol solution (2:1, v/v). To separate the aqueous and organic fractions, the cellular mixture was capped and mixed vigorously for 1 min. Chloroform (1.25 mL) was added and the tube mixed vigorously for 1 min. Water (1.25 mL) was added and the tube mixed vigorously for 1 min. The mixture was then centrifuged at  $200 \times g$  for 5 min and the bottom layer (organic) was transferred to a scintillation vial. The top layer (aqueous) was poured into another scintillation vial and 1 mL of this mixture was transferred to a scintillation vial. The organic fraction was then allowed to evaporate under N<sub>2</sub> gas. Scintillation fluid (5 mL; Scintisafe, Fisher Scientific, Norcross, GA) was added to the vials containing the aqueous and organic fractions and allowed to stand for 1 h. The  $^{14}\text{C}$  content was determined on a Beckman LS 6000 Scintillation Counter (Beckman Instruments, Palo Alto, CA). Mean glucose incorporation is expressed as nmol/(h  $\cdot$  10<sup>6</sup> cells). To control for unincorporated residual  $^{14}\text{C}$ -glucose, a set of cultures was exposed to  $^{14}\text{C}$ -glucose and immediately washed, harvested and fractionated. The radioactivity in the lipid and aqueous fractions was subtracted from the total counts in the respective treated fractions.

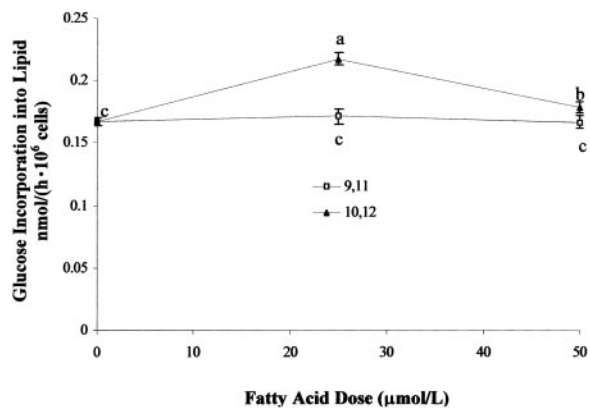
**Oleic acid incorporation (Exp. 1b).**  $^{14}\text{C}$ -oleic acid incorporation was determined on d 7 of differentiation after chronic treatment of the cultures with 0, 25 or 50  $\mu\text{mol/L}$  *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA or LA. On d 7, the media were supplemented with 18.5 KBq [1- $^{14}\text{C}$ ]-oleic acid/L media [specific activity: 1.9 GBq/mmol (NEN-Perkin Elmer Life Sciences, Boston, MA)] for 2 h at 37°C. A time-course study indicated a linear increase in radiolabeled oleic acid incorporation into lipid over a 3-h period (data not shown). Unincorporated  $^{14}\text{C}$ -oleic acid was subtracted from each assay using the procedure described above. Mean oleic acid incorporation is expressed as nmol/(h  $\cdot$  10<sup>6</sup> cells).

**Glucose and oleic acid incorporation into lipid classes (Exp. 1c).**  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -oleic acid incorporation into polar and neutral lipids was determined on d 7 of differentiation after chronic treatment with 50  $\mu\text{mol/L}$  LA, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA or vehicle (BSA). On d 7, the media were replaced with DMEM (low-glucose DMEM for the  $^{14}\text{C}$ -glucose experiment) supplemented with 18.5 KBq [U- $^{14}\text{C}$ ]-D-glucose [specific activity: 9.2 GBq/mmol (ICN)] or 18.5 KBq [1- $^{14}\text{C}$ ]-oleic acid/L medium [specific activity: 1.9 GBq/mmol (NEN-Perkin Elmer Life Sciences)] for 2 h at 37°C.  $^{14}\text{C}$  incorporation into the organic fraction was then isolated as described above for  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -oleic acid incorporation. The organic fraction was dried under N<sub>2</sub> gas and frozen in scintillation vials at -20°C until analysis.

Quantification of  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -oleic acid incorporation into the neutral lipid fraction was determined using radio-HPLC. HPLC was performed using a Waters 600E multisolute delivery system with 717 plus autosampler and 996 photodiode detector (Waters, Milford, MA) connected to an in-line  $\beta$ -radiochromatography detector (Radiomatics Flo-one/Beta, Flow Scintillation Analyzer 500 TR Series, Packard In-



**FIGURE 1** Design of experiments. Cultures were seeded and allowed to proliferate until they reached confluency. Two days postconfluency, cultures were induced to differentiate. Fatty acids and 0.2 mmol/L  $\alpha$ -tocopherol were added fresh at each change of media. Experiments were conducted as shown above.



**FIGURE 2** Effect of treatment with increasing doses of *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (9,11), or *trans*-10, *cis*-12 CLA (10,12) for 6 d on <sup>14</sup>C-glucose incorporation into the lipid fraction after 2 h of incubation with 37 KBq <sup>14</sup>C-glucose. Means ( $\pm$ SEM;  $n = 6$ ) not sharing a superscript are different,  $P < 0.05$ .

strument, Meriden, CT). The column was a Hibar Pre-Packed Column RT 250-4 (Lichrospher silica 5  $\mu$ m) purchased from Alltech Associates (Deerfield IL). Chromatographic conditions followed the procedure of Patton et al. (23) with slight modification. Mobile phase was composed of hexane/tetrahydro-furan/acetic acid (500:20:0.1). The flow rate was 1 mL/min for the first 5 min at which point it was increased to 1.5 mL/min. Liquid scintillation cocktail (Biosafe II, Research Products International, Mount Prospect, IL) was mixed with HPLC eluate at a ratio of 3:1 (v/v) before entering the 0.5 counting loop of the detector. TG, cholesterol ester (CE), monoglycerol and free fatty acid (FFA) peaks were identified at 205 nm based on the retention times compared with injection of each individual standard. The retention times of TG, CE and FFA were confirmed by injection of <sup>14</sup>C-labeled triolein (2 GBq/mmol, American Radiolabeled Chemicals, St. Louis, MO). Signals from the photodiode detector were processed by computer using Millennium 2.1 HPLC software. <sup>14</sup>C-TG content was determined by collecting the TG peak from the  $\beta$  flow monitor in scintillation fluid (Biosafe II) followed by counting on a Beckman LS 6500 scintillation counter. To control for unincorporated residual <sup>14</sup>C-glucose and <sup>14</sup>C-oleic acid, a set of cultures was exposed to <sup>14</sup>C-glucose or <sup>14</sup>C-oleic acid, immediately washed, harvested and fractionated. The basal radioactivity in the lipid fraction was then subtracted from the total counts in the treated samples. Data were expressed as nmol/(h · 10<sup>6</sup> cells).

**<sup>14</sup>C-CO<sub>2</sub> production from <sup>14</sup>C-glucose. (Exp. 2a)** <sup>14</sup>C-CO<sub>2</sub> production from <sup>14</sup>C-glucose was determined on d 7 of differentiation after chronic treatment with 50  $\mu$ mol/L LA, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, or 50  $\mu$ mol/L *trans*-10, *cis*-12 CLA plus 50  $\mu$ mol/L LA. Experiments were conducted in 25 cm<sup>2</sup> flasks (T25 Falcon flasks) that were supplemented with 18.5 KBq [U-<sup>14</sup>C]-D-glucose/L medium [specific activity: 9.2 GBq/mmol (ICN)] and sealed with a rubber stopper fitted with a plastic center well (Kontes, Vineland, NJ) containing filter paper. After a 90-min incubation, the filter paper was injected with 100  $\mu$ L ethidium hydroxide (Sigma). After 30 min, 0.5 mL of 1 mol/L H<sub>2</sub>SO<sub>4</sub><sup>2-</sup> was injected into the monolayers to terminate the reaction and liberate the <sup>14</sup>C-CO<sub>2</sub>. The cultures were allowed to sit for 30 min for the <sup>14</sup>C-CO<sub>2</sub> to collect on the filter paper. Wells containing the filter paper were clipped from the rubber stopper, placed in 5 mL of scintillation fluid and counted using a Beckman LS 6000 Scintillation counter. <sup>14</sup>C-CO<sub>2</sub> production was calculated as dpm/(h · 10<sup>6</sup> cells) and expressed as a percentage of vehicle control (BSA).

**<sup>14</sup>C-CO<sub>2</sub> production from <sup>14</sup>C-oleic acid. (Exp. 2b.)** <sup>14</sup>C-CO<sub>2</sub> production from <sup>14</sup>C-oleic acid was determined on d 7 of differentiation after chronic treatment with 50  $\mu$ mol/L LA, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA or 50  $\mu$ mol/L *trans*-10, *cis*-12 CLA plus 50  $\mu$ mol/L LA. Experiments were conducted in 25 cm<sup>2</sup> flasks (T25 Falcon flasks) that were supplemented with 18.5 KBq [1-<sup>14</sup>C]-oleic acid/L medium [specific activity: 1.9 GBq/mmol (NEN-Perkin Elmer Life Sciences)] and sealed with a rubber stopper fitted with a plastic center well. <sup>14</sup>C-CO<sub>2</sub> produc-

tion was then determined as above, calculated as dpm/(h · 10<sup>6</sup> cells), and expressed as a percentage of control (BSA).

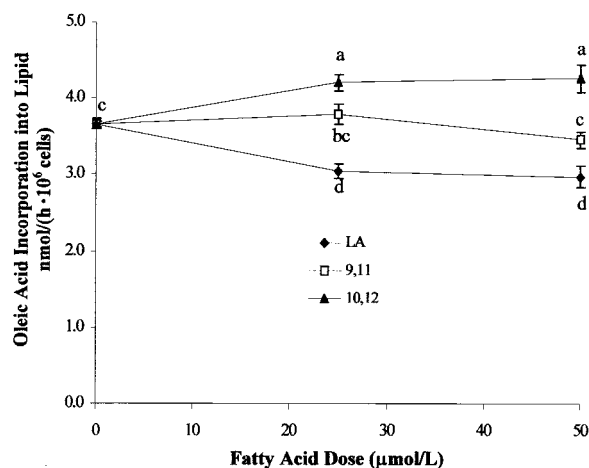
**Lipolysis (Exp. 3).** On d 5 of differentiation, the media were supplemented with 50  $\mu$ mol/L LA, *cis*-9, *trans*-11 CLA, *trans*-10, *cis*-12 CLA, *trans*-10, *cis*-12 CLA plus 50  $\mu$ mol/L LA or vehicle (BSA) control. After 48 h of treatment, conditioned media were removed for glycerol determination and fresh differentiation media were added containing 1  $\mu$ mol/L isoproterenol to stimulate lipolysis and 10 U adenosine deaminase to prevent adenosine-induced inhibition of lipolysis at 37°C for 2 h. Conditioned media were then removed for glycerol determination. Cells were harvested using cell counting solution to determine cell number. Glycerol content was measured using a commercially available colorimetric kit (Sigma #339-10), modified for cell culture as previously described (24). This procedure measures glycerol by enzyme-coupled reduction of a dye that absorbs light at 520 nm and can be quantified spectrophotometrically. Free glycerol in the media was expressed as  $\mu$ mol/(h · 10<sup>6</sup> cells).

**Statistical analyses.** Data were analyzed using a commercially available software package (SuperANOVA, Abacus Concepts, Berkeley CA). For Exp. 1 a and b, a three-way least-squares ANOVA (fatty acid  $\times$  dose  $\times$  replicate) was conducted and the fatty acid  $\times$  dose interactions were compared for significance at the  $P < 0.05$  level. For Exps. 1c, 2 and 3, a two-way ANOVA (fatty acid  $\times$  replicate) was performed, and the effect of fatty acid treatment was compared for significance at the  $P < 0.05$  level. Each treatment combination per experiment was conducted in triplicate and repeated at least once (e.g.,  $n = 6$ ) unless otherwise indicated.

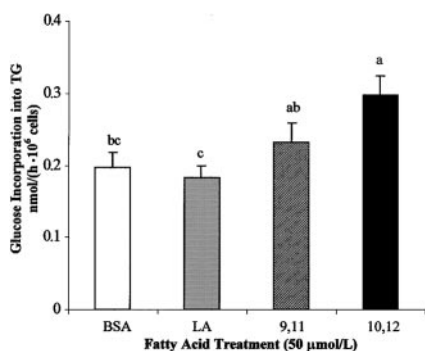
## RESULTS

**Exp. 1.** <sup>14</sup>C-glucose incorporation (Exp. 1a) into the lipid fraction is shown in Figure 2. Cultures treated with 25 and 50  $\mu$ mol/L *trans*-10, *cis*-12 CLA had greater glucose incorporation into the lipid fraction compared with vehicle controls. *Cis*-9, *trans*-11 CLA, on the other hand, had no effect ( $P > 0.05$ ) on glucose incorporation into the lipid fraction. Approximately 40% of the initial <sup>14</sup>C-glucose remained in the media after incubation (data not shown). There were no treatment differences ( $P > 0.05$ ) in unincorporated <sup>14</sup>C-glucose.

Similar to the glucose incorporation data, cultures treated with 25 and 50  $\mu$ mol/L *trans*-10, *cis*-12 CLA in Exp. 1b had higher rates of <sup>14</sup>C-oleic acid incorporation into lipid compared with all other treatments (Fig. 3). In contrast, there was no effect ( $P > 0.05$ ) of *cis*-9, *trans*-11 CLA, whereas cultures treated with 25



**FIGURE 3** Effect of treatment with increasing doses of linoleic acid (LA), *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (9,11) or *trans*-10, *cis*-12 (10,12) CLA for 6 d on <sup>14</sup>C-oleic acid incorporation into the lipid fraction after 2 h of incubation with 18.5 KBq <sup>14</sup>C-oleic acid. Means ( $\pm$ SEM;  $n = 6$ ) not sharing a superscript are different,  $P < 0.05$ .

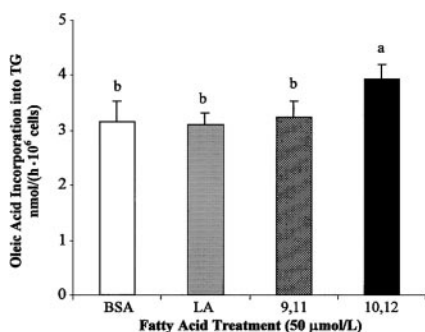


**FIGURE 4** Effect of treatment with 50 μmol/L linoleic acid (LA), *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (9,11), *trans*-10, *cis*-12 CLA (10,12) or bovine serum albumin (BSA; vehicle control) for 6 d on <sup>14</sup>C-glucose incorporation into triglycerides (TG) after 2 h of incubation with 37 KBq <sup>14</sup>C-glucose. Means (+SEM; *n* = 5) not sharing a superscript are different, *P* < 0.05.

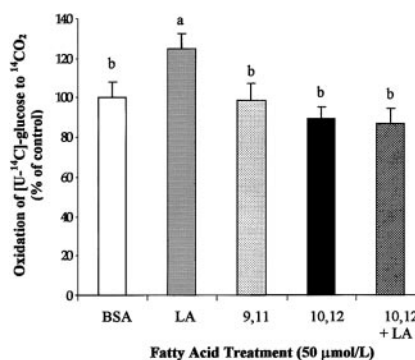
and 50 μmol/L LA had lower rates of <sup>14</sup>C-oleic acid incorporation into lipid compared with BSA controls. Approximately 45% of the initial <sup>14</sup>C-oleic acid remained in the media after incubation (data not shown). There were no treatment differences (*P* > 0.05) in unincorporated <sup>14</sup>C-oleic acid.

The organic lipid extract of the cultures was fractionated into lipid classes using HPLC coupled to a radiometric detector (Exp. 1c). <sup>14</sup>C-TG was the only detectable peak for cultures receiving either <sup>14</sup>C-glucose or <sup>14</sup>C-oleic acid. Approximately 90% of the recovered radioactivity was found in the TG peak. <sup>14</sup>C-glucose incorporation into HPLC-isolated TG was higher in cultures supplemented with *trans*-10, *cis*-12 CLA compared with LA-treated cultures and BSA controls (Fig. 4). However, there was no difference (*P* > 0.05) in <sup>14</sup>C-glucose incorporation into TG between the two CLA isomer treatments. <sup>14</sup>C-oleic acid incorporation into TG was greater in cultures treated with 50 μmol/L *trans*-10, *cis*-12 CLA compared with all other treatments (Fig. 5). There was no difference in <sup>14</sup>C-oleic acid incorporation into TG in cultures supplemented with LA or *cis*-9, *trans*-11 CLA compared with BSA controls.

**Exp. 2.** To determine the effect of *trans*-10, *cis*-12 CLA on glucose (Exp. 2a) and fatty acid (Exp. 2b) oxidation, cultures were treated with either <sup>14</sup>C-glucose or <sup>14</sup>C-oleic acid for 2 h and <sup>14</sup>C-CO<sub>2</sub> production was measured. Cultures treated with 50 μmol/L LA produced more <sup>14</sup>C-CO<sub>2</sub> after incubation with



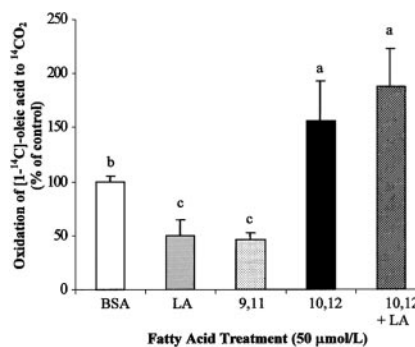
**FIGURE 5** Effect of treatment with 50 μmol/L linoleic acid (LA), *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (9,11), *trans*-10, *cis*-12 CLA (10,12) or bovine serum albumin (BSA; vehicle control) for 6 d on <sup>14</sup>C-oleic acid incorporation into triglycerides (TG) after 2 h of incubation with 37 KBq <sup>14</sup>C-oleic acid. Means (+SEM; *n* = 5) not sharing a superscript are different, *P* < 0.05.



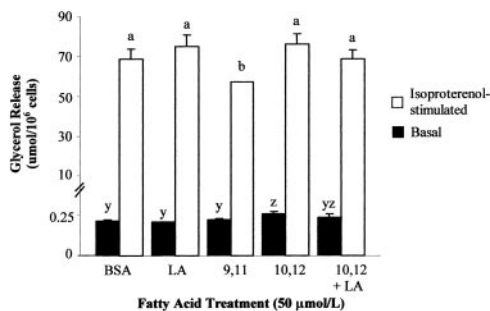
**FIGURE 6** Effect of treatment with 50 μmol/L linoleic acid (LA), *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (9,11), *trans*-10, *cis*-12 CLA (10,12), *trans*-10, *cis*-12 CLA plus 50 μmol/L linoleic acid (10,12 + LA) or bovine serum albumin (BSA; vehicle control) for 6 d on <sup>14</sup>C-CO<sub>2</sub> production after incubation with <sup>14</sup>C-glucose for 2 h. <sup>14</sup>C-CO<sub>2</sub> production [dpm/(h · 10<sup>6</sup> cells)] is expressed as percentage <sup>14</sup>C-CO<sub>2</sub> production of BSA controls. Means (+SEM; *n* = 6) not sharing a superscript are different, *P* < 0.05.

<sup>14</sup>C-glucose compared with BSA controls (Fig. 6). There was no difference in <sup>14</sup>C-CO<sub>2</sub> production from <sup>14</sup>C-glucose after any of the other fatty acid treatments. In contrast, cultures treated with 50 μmol/L *trans*-10, *cis*-12 CLA and *trans*-10, *cis*-12 CLA plus 50 μmol/L LA had ~55 and ~85% higher rates of <sup>14</sup>CO<sub>2</sub> production, respectively, after incubation with <sup>14</sup>C-oleic acid compared with controls (Exp. 2b, Fig. 7). Cultures treated with LA or *cis*-9, *trans*-11 CLA, on the other hand, produced ~50% less <sup>14</sup>C-CO<sub>2</sub> from <sup>14</sup>C-oleic acid compared with BSA controls.

**Exp. 3.** As shown in Figure 8, basal glycerol release was ~18% higher in *trans*-10, *cis*-12 CLA and *trans*-10, *cis*-12 CLA plus LA-treated cultures compared with BSA controls. After a 2-h incubation period with 1 μmol/L isoproterenol plus 1 μmol/L adenosine deaminase, glycerol release increased by ~300-fold. However, there was no difference (*P* > 0.05) in glycerol release among cultures treated with *trans*-10, *cis*-12 CLA, LA or *trans*-10, *cis*-12 CLA plus LA compared with controls. In contrast, cultures treated with 50 μmol/L *cis*-9, *trans*-11 CLA had 11% lower isoproterenol-stimulated release of glycerol into the medium compared with BSA controls.



**FIGURE 7** Effect of treatment with 50 μmol/L linoleic acid (LA), *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (9,11), *trans*-10, *cis*-12 CLA (10,12), *trans*-10, *cis*-12 CLA plus 50 μmol/L linoleic acid (10,12 + LA) or bovine serum albumin (BSA; vehicle control) for 6 d on <sup>14</sup>C-CO<sub>2</sub> production after incubation with <sup>14</sup>C-oleic acid for 2 h. <sup>14</sup>C-CO<sub>2</sub> production [dpm/(h · 10<sup>6</sup> cells)] is expressed as percentage <sup>14</sup>C-CO<sub>2</sub> production of BSA controls. Means (+SEM; *n* = 6) not sharing a superscript are different, *P* < 0.05.



**FIGURE 8** Effect of treatment with 50  $\mu\text{mol/L}$  linoleic acid (LA), *cis*-9, *trans*-11 conjugated linoleic acid (CLA) (9,11), *trans*-10, *cis*-12 CLA (10,12), *trans*-10, *cis*-12 CLA plus 50  $\mu\text{mol/L}$  linoleic acid (10,12 + LA) or bovine serum albumin (BSA; vehicle control) for 48 h on glycerol release before and after 2 h of stimulation with isoproterenol in the presence of adenosine deaminase. Means ( $\pm$ SEM;  $n = 6$ ) not sharing a superscript are different,  $P < 0.05$ .

## DISCUSSION

To our knowledge, this is the first study to demonstrate that *trans*-10, *cis*-12 CLA increases fatty acid (specifically oleic acid) oxidation in cultures of 3T3-L1 preadipocytes. In contrast, LA and *cis*-9, *trans*-11 CLA-treated cultures had lower oleic acid oxidation rates compared with all other treatments. Moya-Camarena et al. (25) found an increase in the activity of acyl-CoA oxidase, a peroxisomal enzyme involved in fatty acid oxidation, in rat hepatoma cells after treatment with 0.14–0.4  $\mu\text{mol/L}$  mixed isomers of CLA for 1–24 h. Similarly, in vivo, Sakano et al. (26) observed an increase in  $\beta$ -oxidation (assessed by an increase in ketone body formation) in rats fed 1 g/kg diet mixed isomers of CLA for 2 wk. Furthermore, Martin et al. (27) found that the *trans*-10, *cis*-12 isomer of CLA increased the activity of acyl-CoA oxidase, as well as hepatic and adipose carnitine palmitoyltransferase-1 (CPT-1)-the rate-limiting enzyme in  $\beta$ -oxidation, in rats consuming 10 g/kg diet *trans*-10, *cis*-12 CLA for 6 wk. A similar increase in CPT-1 activity was observed in mice fed 5 g/kg diet mixed isomers of CLA for 32 d (2). Martin et al. (27) also suggested that *trans*-10, *cis*-12 CLA, as a result of its geometric and positional structure, may be more efficiently oxidized by the enzymes of the  $\beta$ -oxidation pathway than either *cis*-9, *trans*-11 CLA or other polyunsaturated fatty acids (PUFA). Taken together, these data suggest that *trans*-10, *cis*-12 CLA may decrease cellular TG by increasing fatty acid oxidation.

In another study, West et al. (13) found a decrease in nighttime respiratory quotient in mice consuming 1.0–1.2% (wt/wt) mixed isomers of CLA for 6 wk, indicating an increase in fat oxidation after CLA consumption. Furthermore, fatty acid analyses of various tissues have consistently found higher levels of *cis*-9, *trans*-11 CLA than *trans*-10, *cis*-12 CLA (28–33), although this is not true of the serum fatty acid profile of rats fed 1% (wt/wt) mixed CLA isomers for 2 wk (34). However, the degree to which this stimulation in fatty acid oxidation may be responsible for the TG-lowering actions of CLA remains to be determined.

Contrary to our hypothesis, 50  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA increased  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -oleic acid incorporation into lipid. We initially hypothesized that *trans*-10, *cis*-12 CLA would decrease glucose and/or fatty acid incorporation into lipid, thereby decreasing TG synthesis. However, Satory and Smith (35) also reported an increase in glucose incorporation into lipid using 3T3-L1 preadipocytes, although these researchers used a crude mixture of CLA isomers (~40% *cis*-9,

*trans*-11 and 40% *trans*-10, *cis*-12). Furthermore, they assessed glucose incorporation into the lipid fraction without determining incorporation into TG specifically. To our knowledge, this is the first time that the effects of individual isomers of CLA, vis-à-vis *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA, on lipogenesis in preadipocytes have been examined. In keratinocyte cultures, Jun et al. (36) observed an increase in  $^3\text{H}$ -acetate incorporation into cholesterol esters and  $^3\text{H}$ -glycerol into TG in cultures treated with 50–250  $\mu\text{mol/L}$  mixed isomers of CLA. Thus, our observed increase in glucose and oleic acid incorporation is consistent with several CLA studies and argues against the hypothesis that CLA reduces TG in adipocytes via a reduction in lipogenesis.

However, data from radiolabeled substrate studies are subject to interpretation, given their limitations. For example, one limitation of radiolabel studies is that the unlabeled endogenous pool size is unknown; therefore it is not possible to assess the actual flux of isotopes within the cultures. Therefore, the exogenous radiolabeled oleic acid may have been a larger portion of the endogenous fatty acid pool available for esterification in the *trans*-10, *cis*-12 CLA-treated cultures (because more of the available fatty acids were either released to the media through lipolysis or oxidized by  $\beta$ -oxidation). Thus, an increased proportion of  $^{14}\text{C}$ -oleic acid may have been incorporated into the lipid fraction, although total TG was reduced. The same may hold true for our  $\beta$ -oxidation results in that  $^{14}\text{C}$ -oleic acid may represent a larger proportion of the fatty acids available for oxidation in the *trans*-10, *cis*-12 CLA-treated cultures. This would result in relatively more  $^{14}\text{CO}_2$  produced from  $^{14}\text{C}$ -oleic acid, whereas *cis*-9, *trans*-11 CLA and LA-treated cultures would have a decreased amount of labeled fatty acid in proportion to the total endogenous fatty acid pool. However, the addition of LA to *trans*-10, *cis*-12 CLA-treated cultures did not reduce  $^{14}\text{CO}_2$  production as expected, and the reason for this finding is unclear.

*Trans*-10, *cis*-12 CLA treatment of differentiated 3T3-L1 adipocytes also increased basal, but not isoproterenol-stimulated lipolysis. An increase in basal lipolysis was also observed in 3T3-L1 adipocytes treated with 100  $\mu\text{mol/L}$  mixed CLA isomers for 48 h (2). However, a similar experiment with *trans*-10, *cis*-12 CLA by Park et al. (5) showed a 30-fold increase in basal glycerol release, whereas we observed an 18% increase in basal glycerol release. The reasons for these CLA-mediated differences in basal lipolysis are unclear.

The *trans*-10, *cis*-12 CLA-dependent increase in glucose and oleic acid incorporation, fatty acid oxidation and basal lipolysis suggests that *trans*-10, *cis*-12 CLA may increase fatty acid metabolism in adipocytes. More specifically, these results suggest that *trans*-10, *cis*-12 CLA may lower TG by increasing fatty acid turnover. In support of this concept, mice fed 1 g/kg diet mixed CLA isomers had increased adipose uncoupling protein-2 mRNA, a key regulator of the oxidative phosphorylation pathway (12), suggesting that CLA may increase energy expenditure by reducing the efficiency of energy metabolism. Furthermore, West et al. (13) found an increase in energy expenditure, but no change in de novo fatty acid synthesis, in mice consuming 1 g/kg diet mixed CLA in the presence of a high fat diet.

CLA has also been shown to increase the release of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in mice fed 1% mixed CLA isomers for 8 wk (12). In addition to inhibiting insulin-dependent stimulation of lipogenesis, TNF- $\alpha$  decreased the expression of acyl-CoA carboxylase, lipoprotein lipase and fatty acid synthase mRNA as well as increased delipidation of adipocytes [for review, see (37)]. This TNF- $\alpha$ -mediated impairment of insulin signaling, down-regulation of lipogenic enzymes and

delipidation may explain the observed reduction in TG content seen in CLA-treated adipocytes [for review, see (38)]. However, the mechanism by which CLA induces TNF- $\alpha$  production remains to be determined.

It is also important to note that we assessed the effects of chronic CLA treatment on lipid synthesis and oxidation after 6 d of fatty acid supplementation. Thus, it is possible that the effects of acute CLA supplementation on lipid metabolism during the first few days of differentiation may differ from our chronic treatment data. Recent research suggests that adipocytes reach a limit in their ability to synthesize and store lipid [for review, see (39)]. Thus, in vivo, once adipocytes attain a certain level of lipid storage, it is believed that lipid-synthesizing enzymes are down-regulated and lipid metabolism is shifted to the liver. Thus, it is possible that in our experiments, cultures treated with BSA alone, LA or *cis*-9, *trans*-11 CLA reached maximal lipid storing capacity and subsequently down-regulated the enzymes necessary for lipid synthesis and storage, whereas *trans*-10, *cis*-12 CLA-treated cultures were still actively synthesizing lipid at d 6 of differentiation. This would explain the increased  $^{14}\text{C}$ -oleic acid incorporation into lipid and TG seen with *trans*-10, *cis*-12 CLA treatment compared with LA and BSA control cultures. Therefore, future studies examining the acute effects of CLA on lipid metabolism during early differentiation are warranted.

In conclusion, we have shown that *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA have differential effects on lipid metabolism in 3T3-L1 preadipocytes. Specifically, 50  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA increased oleic acid incorporation into TG by  $\sim 25\%$  compared with controls. In addition, cultures treated with 50  $\mu\text{mol/L}$  *trans*-10, *cis*-12 CLA had a  $\sim 55\%$  increase in oleic acid, but not glucose oxidation, whereas basal lipolysis was increased by  $\sim 18\%$  compared with controls. The observed mild increase in TG synthesis from  $^{14}\text{C}$ -oleic acid as well as the dramatic increase in fatty acid oxidation suggests that *trans*-10, *cis*-12 CLA treatment may increase metabolism and fatty acid turnover in preadipocytes. Increased fatty acid turnover may explain how *trans*-10, *cis*-12 CLA reduces TG content in 3T3-L1 preadipocytes; however, this possibility remains to be examined further.

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