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# Linoleic acid partially restores the triglyceride content of conjugated linoleic acid-treated cultures of 3T3-L1 preadipocytes

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**Abstract**

We have previously demonstrated that a crude mixture of commercially available conjugated linoleic acid (CLA) isomers suppressed triglyceride (TG) content and induced apoptosis in post-confluent cultures of murine 3T3-L1 preadipocytes. Furthermore, we found that 100 $\mu$M of trans-10, cis-12 isomer of CLA had a greater TG-lowering and apoptotic effect than the crude mixture of CLA isomers. Therefore, the purpose of this study was to: 1) compare the potencies of the two main isomers found in the crude mixture of CLA isomers, e.g. cis-9, trans-11 (41%) and trans-10, cis-12 (44%); and 2) determine if the TG-reducing actions of CLA could be attenuated by the addition of increasing levels of linoleic acid to the cultures. Preadipocyte differentiation was assessed on day 7 of the differentiation protocol by measuring TG content (per $10^6$ cells), cell size, and lipid staining. In experiment 1, post-confluent cultures of 3T3-L1 preadipocytes treated for the first 6 d of differentiation with 100 $\mu$M of a crude mixture of CLA isomers or 44 $\mu$M of trans-10, cis-12 CLA had less TG content than all other cultures. In contrast, cultures supplemented with 41 $\mu$M of the cis-9, trans-11 CLA isomer had the same amount of TG as the BSA controls. In experiment 2, post-confluent cultures of 3T3-L1 preadipocytes treated for the first 6 d of differentiation with 50 $\mu$M trans-10, cis-12 CLA had less TG content and a greater number of smaller cells (10–12.5 microns) compared to all other treatments. CLA-treated cultures supplemented with increasing levels of linoleic acid (50–200 $\mu$M) had greater TG contents and greater numbers of larger cells (15–20 microns) than cultures treated with 50 $\mu$M of the trans-10, cis-12 CLA isomer alone. These data demonstrate that: 1) the TG-lowering effects of the crude mixture of CLA isomers is due almost exclusively to the trans-10, cis-12 isomer; and 2) linoleic acid partially reverses CLA's attenuation of TG content, suggesting that these unsaturated fatty acids may compete for incorporation into TG or phospholipid-derived eicosanoids that regulate preadipocyte differentiation. 

*Keywords:* Conjugated linoleic acid; Linoleic acid; Differentiation; 3T3-L1 preadipocytes; Triglyceride content

## 1. Introduction

Conjugated linoleic acid (CLA) refers to a group of isomers of linoleic acid (cis-9, cis-12 octadecadienoic acid). These isomers can either be positional (shifting of double bonds to 9–11 or 10–12 positions), geometric (cis/trans variations), or a combination of both. CLA is found naturally in foods, predominantly in dairy products (4.7–5.5 g/kg of fat) and ruminant meats (4.3–5.6 g/kg of fat) [1]. CLA is formed via a biohydrogenation process within the rumen by colonic bacteria as well as via the mechanical processing of dairy products [2–4]. Recent research suggests that humans are capable of producing small amounts of CLA via this isomerization process [5]. Serum levels of CLA isomers in non-vegetarians have been reported to be in the 20–70 $\mu$M range, with the cis-9, trans-11 and the trans-10, cis-12 isoforms representing ~80% and ~10%, respectively [6].

A crude mixture of CLA isomers has been shown to have a variety of potential health benefits in both animal and cell culture models. CLA was first introduced as a possible anticarcinogenic agent in rodents by Pariza and Hargraves [7], with several other studies having since confirmed these findings [8,9]. CLA has also been shown to possess antiatherogenic [10,11], antidiabetic [12], and immune enhancing properties [13,14].

More recently, a crude mixture of CLA isomers has been shown to reduce body fat and enhance fat-free mass in rodents [15–17] and in pigs [18,19]. Rats fed a crude mixture of CLA isomers had less adipose tissue mass due to having smaller, but not fewer, adipocytes [20]. In mice, these antiobesity actions of a crude mixture of CLA isomers

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have been attributed to the trans-10, cis-12 isomer [21]. Studies by our group [22] and Pariza's group [23] examining potential mechanisms of actions of CLA using the murine 3T3-L1 cell line further support the suggestion that trans-10, cis-12 CLA is the antiadipogenic isomer of CLA. However, the mechanism by which CLA attenuates adipogenesis is not well understood.

Although it has been clearly shown that CLA inhibits 3T3-L1 preadipocyte proliferation [22,24,25], there is disagreement about CLA's effects on cellular differentiation. One study reported a stimulatory effect [24] and others demonstrated an inhibitory effect of CLA on preadipocyte differentiation [22,23,25]. Two of these studies [24,25] used non-physiological fatty acid vehicles such as DMSO or ethanol, and three of these studies did not protect their fatty acids against lipid peroxidation [23–25]. In contrast, we complex our fatty acids to albumin instead of ethanol since *in vivo* free fatty acids are normally bound to albumin. Furthermore, we supplement our fatty acid-containing cultures with $\alpha$-tocopherol to protect them from oxidation. In support of these culturing procedures, we have shown that ethanol enhances the TG content of 3T3-L1 cultures compared to BSA-treated cultures and that supplementing with $\alpha$-tocopherol can prevent some, but not all, of the TG-lowering effects of CLA [22]. Whether this difference in TG content is due to peroxidation of unsaturated fatty acids cultured without supplemental vitamin E is unknown.

Therefore, the objective of this study was to determine: 1) which of the two major isomers (cis-9, trans-11 or trans-10, cis-12) in the commercially-available crude mixture of CLA isomers is responsible for CLA's TG-lowering actions; and 2) if CLA's antiadipogenic actions could be reversed by supplementing CLA-treated cultures with linoleic acid, an unconjugated geometric isomer of CLA.

## 2. Materials and methods

### *2.1. Cell culture conditions*

Freshly split 3T3-L1 preadipocytes were seeded at a density of $3.3 \times 10^3/cm^2$ in 12-well Falcon plates (Becton Dickinson, Franklin Lakes, NJ) and cultured in proliferation media containing Dulbecco's Modified Eagle's Medium (DMEM; GIBCO BRL, Grand Island, NY), 10 mM N-2-hydroxymethylpiperazine-N′-2-ethanesulfonic acid (Hepes), 10% bovine calf serum (BCS; Hyclone, Logan, UT), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cultures were incubated at 37°C in a humidified $O_2$:$CO_2$ (90:10%) atmosphere. After approximately 5 days under these conditions, cultures reached confluence. Two days post-confluence, cells were grown in differentiation medium containing DMEM, 10% fetal bovine serum (FBS; charcoal-dextran stripped to remove endogenous fatty acids), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ug/ml insulin, 0.5 mM 3-isobutylmethylxanthine, and 0.1 $\mu$M dexamethasone. On day 3 of differentiation the medium was replaced with DMEM containing 10% FBS, 2.5 $\mu$g/ml insulin, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The medium from day 5 onward contained DMEM, 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. All cell culture reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. Dr. Howard Green (Harvard University) generously provided the 3T3-L1 preadipocytes.

### *2.2. Fatty acid treatments*

A crude mixture of CLA isomers (Nu-check-Prep, Elysian, MN, mainly 41% cis-or trans-9, trans-11 isomers; 44% trans-10, cis-12 isomers; and 10% cis-10, cis-12 isomers according to the manufacturers), cis-9, trans-11 CLA (Matreya, Inc., Pleasant Garden, PA; 98% pure), trans-10, cis-12 CLA (Matreya, Inc., 98% pure), and linoleic acid (Nu-check-Prep, 99% pure) were complexed to fatty acid-free albumin (BSA; 1 mM BSA: 4 mM fatty acid), and added to post-confluent cultures of 3T3-L1 preadipocytes at various concentrations ($n = 6–9$ per treatment combination). Media was changed at two-day intervals, and fresh fatty acids were added each media change until the day of harvest. In experiment 1, post-confluent cultures were treated continuously during the first 6 d of the differentiation protocol with either BSA alone (vehicle control), 44 or 100 $\mu$M linoleic acid (fatty acid control), 44 or 100 $\mu$M mixed CLA isomers (approximately 41% cis-9, trans-11 and 44% trans-10, cis-12 CLA isomers), or the concentration of each specific isomer found in 100 $\mu$M of the crude mixture of CLA isomers (e.g., 41 $\mu$M cis-9, trans-11 CLA vs. 44 $\mu$M trans-10, cis-12 CLA). All cultures contained 0.2 mM $\alpha$-tocopherol (Sigma) to protect fatty acids from peroxidation.

In experiment 2, cultures were treated for the first 6 d of differentiation with either 50 $\mu$M trans-10, cis-12 CLA isomer alone, 50 $\mu$M trans-10, cis-12 CLA, CLA plus linoleic acid at 50, 100, or 200 $\mu$M, or linoleic acid alone at 50, 100, or 200 $\mu$M. A set of control cultures contained only the vehicle (7.5% BSA). All cultures contained 0.2 mM $\alpha$-tocopherol (Sigma) to protect fatty acids against lipid peroxidation.

### *2.3. Determining cell number and cell size*

Cells were harvested on day 7 of differentiation in 500 $\mu$l cell counting solution (0.01 M monobasic $NaPO_4$, 0.154 M NaCl, 25 mM glucose, 5 mM EDTA, and 2% BSA) according to a procedure developed by Dr. Phil Pekala, Department of Biochemistry, East Carolina University (personal communication). After 1 h incubation in cell counting solution at 37°C, any remaining adherent cells were gently triturated off the bottom of the wells. This procedure minimizes cell lysis and clumping compared to trypsinization. Cell number and cell size distribution were determined using a Coulter Multi-Sizer Cell Counter (Coulter Electron-

ics, Hialeah, FL) [22]. Adipocytes with diameters from 10 to 60 $\mu$m were counted and sized.

### 2.4. Quantifying TG content

Cells harvested in cell counting solution were sonicated prior to the addition of 5% (v/v) Triton X-100 to ensure homogenous lipid distribution in all samples [22]. TG content was measured using a colorimetric assay that quantifies the glycerol content of the samples (GPO-Trinder, Sigma; St. Louis, MO). This assay involves the enzymatic hydrolysis of TG by lipases to free fatty acids and glycerol. The glycerol moiety, through a series oxidation-reduction reactions, then associates with 3,5 dichloro-2-hydroxybenzene sulfonate and 4-aminoantipyrine to produce a red colored dye. The absorbence of this dye is proportional to the concentration of TG present in each sample. Following these reactions, an aliquot of each sample was transferred to a 96 well plate, and the absorbence quantified at 520 nm on a microtiter plate reader (Tecan-SLM, Research Triangle Park, NC). TG data are expressed as ug of TG per $10^6$ cells.

### 2.5. Lipid staining

The presence of intracellular lipid was visualized by staining the cultures with Oil Red O [22]. Cell monolayers were washed twice with 1 ml Hank's Balanced Salt Solution (HBSS), and then fixed for 1 hour in a 10% formalin solution (10% formalin, 4% calcium chloride, and deionized water) at 4°C. After fixation, cells were washed twice with deionized water and stained using a 0.3% Oil Red O in isopropanol for 15 minutes at room temperature. Following staining, cells were rinsed several times using 20% ethanol and deionized water to remove any undissolved stain. The stained adipocytes were then viewed under our Olympus IMT-2 inverted microscope, and photographs were taken of representative fields in each well.

### 2.6. Statistics

Analyses of statistical differences between treatment means were conducted using two-way (treatment × replication) analysis of variance (ANOVA) procedures with a commercially available software program (SUPER-ANOVA; Abacus Concepts, Berkeley, CA). Treatment mean differences were considered significant at $P < 0.05$.

## 3. Results

In experiment 1, cultures treated with 100 $\mu$M mixed CLA isomers and 44 $\mu$M trans-10, cis-12 CLA had less TG content than all other cultures (Fig. 1). Interestingly, cultures treated with the cis-9, trans-11 CLA isomer had more TG content than linoleic acid-treated cultures. In experiment 2, cultures treated with 50 $\mu$M trans-10, cis-12 CLA

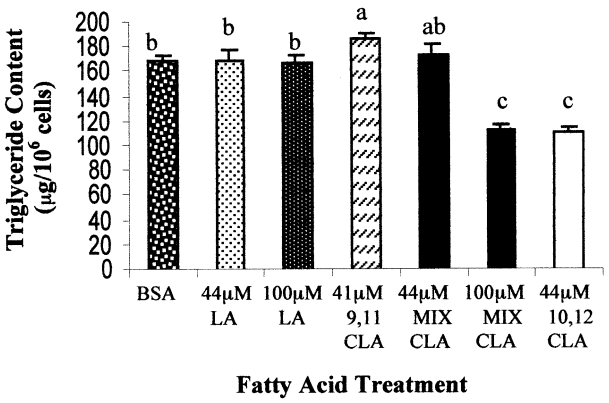


Fig. 1. Cultures were treated throughout the first 6 d of differentiation with either vehicle alone (BSA), linoleic acid (LA), a crude mixture of CLA isomers (MIX) or pure CLA isomers (cis-9, trans-11 and trans-10, cis-12). Cultures were treated continuously and harvested on day 6 for the determination of triglyceride levels and cell number. Means (±SEM; n = 6–9) not sharing a common superscript are significantly different ($P < 0.05$).

alone had 54% less TG content ($\mu$g/$10^6$ cells) compared to the BSA controls (Fig. 2). Cultures treated with 50 $\mu$M trans-10, cis-12 CLA plus 50, 100, or 200 $\mu$M linoleic acid had 47, 49, and 62% more TG content, respectively, than those cultures treated with 50 $\mu$M trans-10, cis-12 CLA alone. CLA-treated cultures supplemented with 200 $\mu$M linoleic acid had 25% less TG content than the BSA controls. In contrast, cultures supplemented with either 50, 100, or 200 $\mu$M linoleic acid alone had more TG content compared to respective cultures treated with CLA plus linoleic acid.

Cell size (Fig. 3) and morphology (Fig. 4) were also altered by CLA and linoleic acid treatments. Cultures treated with 50 $\mu$M trans-10, cis-12 CLA alone had a greater number of smaller cells (10–12.5 micron range) compared to the BSA controls and the linoleic acid supplemented cultures (Fig. 3). In contrast, cultures treated with trans-10, cis-12 CLA alone had significantly fewer cells in the 15–20 micron range compared to control and linoleic acid supplemented cultures. Interestingly, CLA-treated cultures supplemented with 50 $\mu$M linoleic acid had fewer smaller cells (10–12.5 micron range) and more cells in the larger 15–20 micron range than cultures treated with trans-10, cis-12 CLA alone (Fig. 3). These differences in cell size and TG between cultures treated with CLA alone and those treated with CLA plus linoleic acid can be seen in Figure 4. Linoleic acid treatment increased both cellular content of TG and the number of cells containing TG. Lipid locules in cells treated with the trans-10, cis-12 isomer of CLA alone were much smaller in size than all other cultures (Fig. 4). As the level of linoleic acid in CLA-treated cultures increased, cells became less multilocular and more unilocular (Fig. 4).

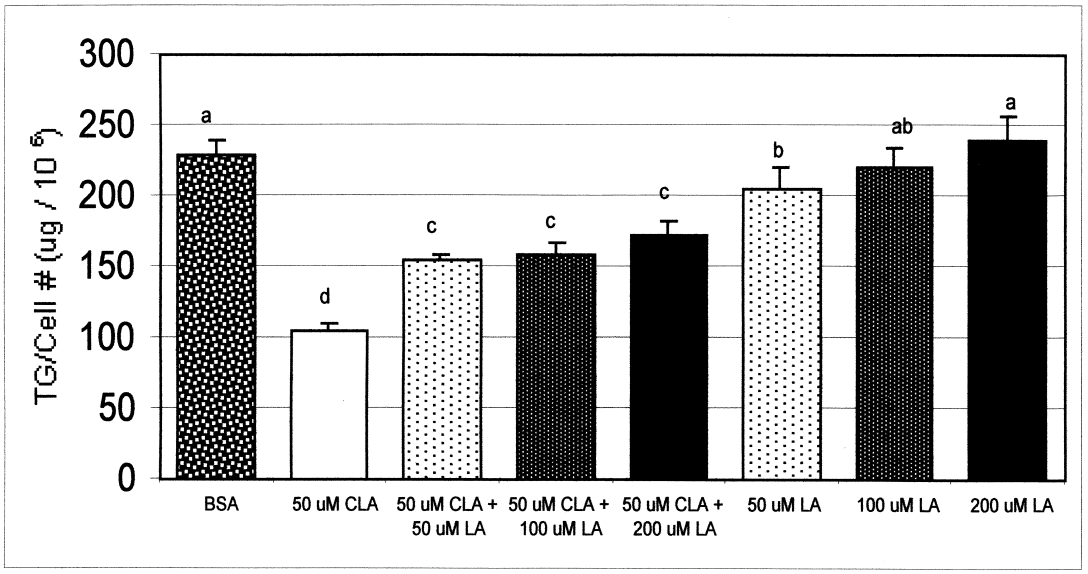


Fig. 2. Effect of trans-10, cis-12 conjugated linoleic acid (CLA) and linoleic acid (LA) on triglyceride content ($\mu g/10^6$ cells) in cultures of 3T3-L1 preadipocytes. Reported values are mean ± SE (n = 6–9, collected from 2–3 separate experiments). Data were analyzed by two-way analysis of variance, (fatty acid treatment × experimental repetition). Means not sharing a common superscript are significantly different ($P < 0.05$).

## 4. Discussion

The present study provides direct evidence that the trans-10, cis-12, not the cis-9, trans-11, isomer of CLA lowers the TG content and reduces cell size compared to control and linoleic acid-treated cultures of 3T3-L1 (pre)adipocytes. These data on the antiadipogenic actions of trans-10, cis-12 CLA are in agreement with *in vivo* data using a mouse model [21] and data obtained using 3T3-L1 preadipocytes [21–23] and adipocytes [21]. Furthermore, this study provides the first evidence that linoleic acid partially restores the TG content and adipocyte size of CLA-treated cultures.

The precise mechanism by which CLA reduces TG content is unclear. Trans-10, cis-12 CLA may reduce TG con-

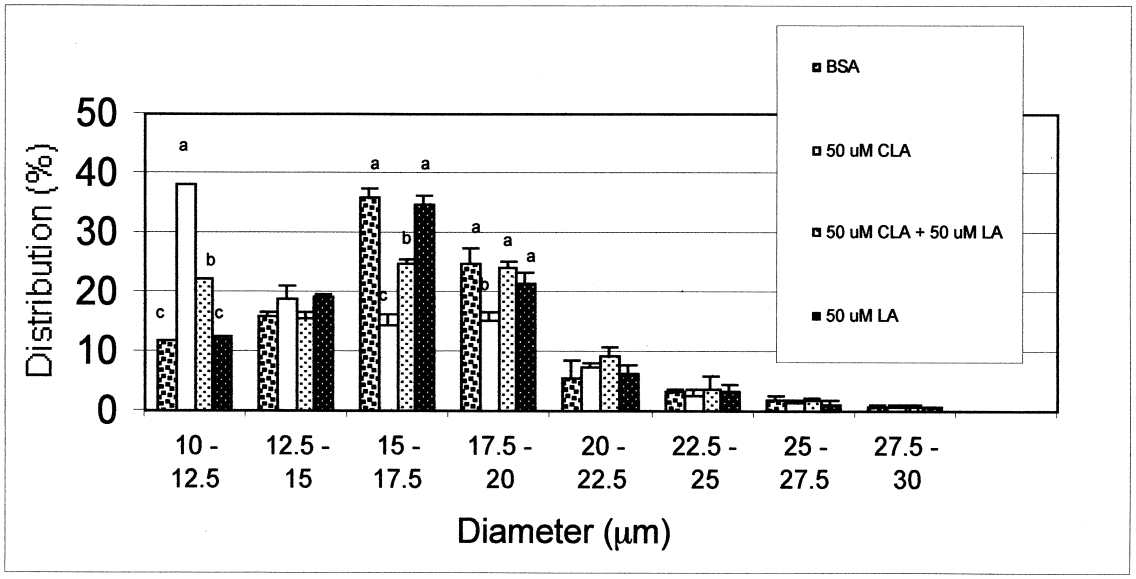


Fig. 3. Effect of trans-10, cis-12 conjugated linoleic acid (CLA) and linoleic acid (LA) on 3T3-L1 adipocyte size distribution. Reported values are the mean ± SE of cell size distributions (n = 6, collected from 2 separate experiments). Data are presented as a percent of total cells for each treatment combination. Data were analyzed by two-way analysis of variance (fatty acid treatment × experimental repetition). Means within a cell size range (i.e., 10–12.5 $\mu$M) not sharing a common superscript are significantly different ($P < 0.05$). The treatments shown in this figure were selected based on the treatment differences in triglyceride content shown in Fig. 2.

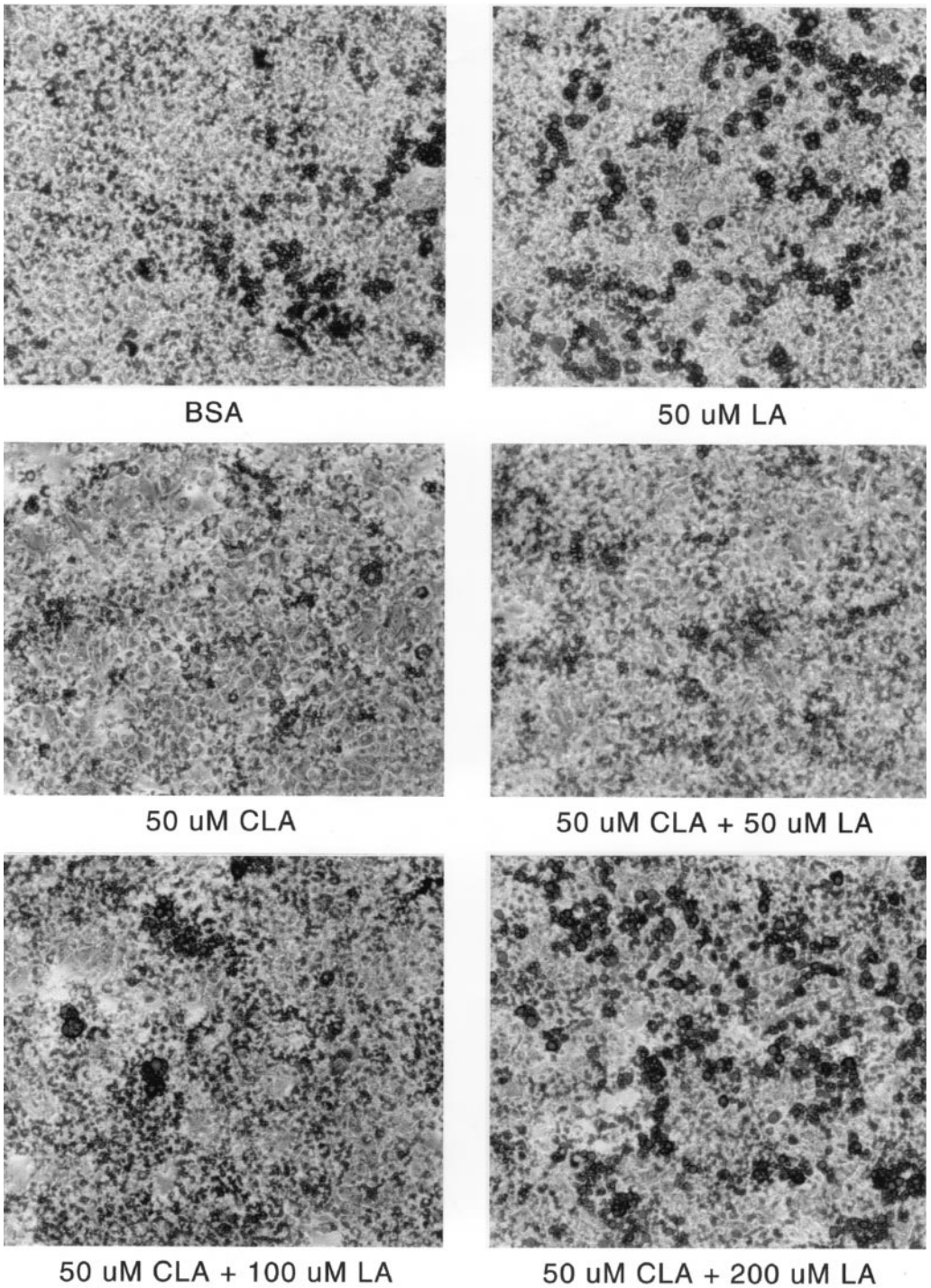


Fig. 4. Photographs (10×) of cultures of 3T3-L1 adipocytes, taken on day 6 of differentiation. All cultures were fixed in formalin and stained with Oil Red O to improve visibility of lipid droplets. Oil red O-stained cells appear black in micrographs.

tent by attenuating adipogenesis (i.e., decreased expression of differentiation markers and rates of lipogenesis), increasing lipolysis, or simply decreasing fatty acid esterification into TG. Furthermore, since 50 $\mu$M linoleic acid partially reversed CLA's suppression of cellular TG, these two fatty acids may compete for synthesis of lipids critical for differentiation. However, linoleic acid supplementation above 50 $\mu$M did not further increase TG content. One class of lipids that CLA and linoleic acid may compete for is membrane phospholipids that contain arachidonic acid (AA) at the sn-2 position such as phosphatidylcholine. Therefore, CLA may compete with linoleic acid for the delta-6 desaturase-elongase-delta-5 desaturase pathway thereby altering AA and phospholipid synthesis [26]. Furthermore, prostaglandin

metabolites of AA such as prostaglandins $D_2$ and $J_2$ are known to regulate cell maturation, including activation of $PPAR_\gamma$ [27] which controls the adipogenic genes aP2 and lipoprotein lipase [28]. Support for this concept comes from animal and cell culture studies demonstrating that: 1) CLA is detectable in the cellular lipids of animal tissue and cultures of cells when treated with CLA [3,8,13,24,26,29–32]; 2) CLA treatment is associated with a decrease in AA in cultures of 3T3-L1 preadipocytes and keratinocytes [24, 26]; 3) levels of AA-derived series-2 prostaglandins ($PGE_2$) are decreased by CLA treatment in animals [29,30] and cell cultures [26]; 4) mRNA of the transcription factors PPAR$\gamma$ and C/EBP$\alpha$ and fatty acid binding protein (aP2) were decreased in CLA-treated cultures of murine preadipocytes [25]; and 5) trans-10, cis-12, but not cis-9, trans-11, CLA treatment reduces levels of murine hepatic [33,34] and adipose tissue SCD [23], an essential enzyme for the delta-9 desaturation of palmitic and stearic acid.

It is not clear from the literature whether signaling molecules that activate PPAR$\gamma$ are first synthesized into membrane phospholipids or are directly metabolized into lipid second messengers. Therefore, CLA might compete with unsaturated fatty acids such as linoleic acid for metabolism into lipid signals such as AA and its prostanoid metabolites that directly regulate PPAR$\gamma$ and adipogenesis without first going through the phospholipid pool. However, Choi et al. [23] recently demonstrated that 45 $\mu$M trans-10, cis-12 CLA had no impact on PPAR$\gamma$ gene expression in post-confluent 3T3-L1 (pre)adipocytes. Instead, these authors suggest that the trans-10, cis-12 isomer of CLA may influence TG and phospholipid synthesis and subsequent adiposity by inhibiting SCD activity, thereby reducing the ratio of monounsatured fatty acids to saturated fatty acids. These CLA-mediated changes in fatty acid profiles of phospholipids could alter membrane fluidity and subsequent cellular metabolism.

In another study using monkey kidney CV-1 cells, Houseknecht et al. [12] demonstrated that a crude mixture of CLA isomers increased the activation of a PPAR$\gamma$ reporter gene construct. They suggest that CLA's insulin-sensitizing effects are due to its activation of PPAR$\gamma$ and are similar to the mechanism by which thiazolidinediones lower blood glucose. Therefore, there is disagreement concerning CLA's influence on PPAR$\gamma$ expression based on the specific type and amount of isomer used and the tissue site of action. Future studies measuring trans-10, cis-12 CLA's impact on PPAR$\gamma$ protein expression in preadipocytes and PPAR$\gamma$ activity in fibroblasts and preadipocytes with PPAR$\gamma$ expression vectors and reporter gene constructs will provide greater insight about CLA's potential mechanism of action.

Finally, CLA may also reduce adipocyte TG content and cell size by increasing lipolysis. In support of this idea, the trans-10, cis-12 isomer of CLA reduced intracellular TG and enhanced glycerol release into the medium in cultured 3T3-L1 preadipocytes [21]. The lipolytic effect of CLA was accompanied by a decrease in lipoprotein lipase activity. Alternatively, Choi et al. [23] suggest that the trans-10, cis-12 isomer of CLA may reduce TG content by inhibiting monounsaturated fatty acid synthesis via down regulating SCD activity. This would limit the two major monounsaturated fatty acids found in membrane phospholipids and TG in cultures of 3T3-L1 (pre)adipocytes.

In conclusion, the results of this study demonstrate that the TG-lowering effect of a commercially available mixture of CLA isomers is due primarily to the trans-10, cis-12, not the cis-9, trans-11, isomer of CLA and that these antiadipogenic actions can be partially reversed by supplementing the cultures with linoleic acid. Thus, there may be a competitive interaction between trans-10, cis-12 CLA and linoleic acid. However, it is not known if this competition occurs via a common metabolic pathway (e.g., lipogenesis or lipolysis) or an alteration in regulatory genes important in preadipocyte differentiation (e.g., PPAR$\gamma$ or C/EBP$\alpha$). Future studies examining these potential mechanism(s) by which CLA lowers TG, especially in primary cultures of human preadipocytes, are needed.

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