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Trans-10, *cis*-12 conjugated linoleic acid decreases *de novo* lipid synthesis in human adipocytes☆,☆☆,★

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Abstract

Conjugated linoleic acid (CLA) reduces adiposity *in vivo*. However, mechanisms mediating these changes are unclear. Therefore, we treated cultures of human adipocytes with *trans*-10, *cis*-12 (10,12) CLA, *cis*-9, *trans*-11 (9,11) CLA or other trans fatty acids (FA), and measured indices of lipid metabolism. The lipid-lowering effects of 10,12 CLA were unique, as other trans FA did not reduce TG content to the same extent. Using low levels of [^{14}C]-CLA isomers, it was shown that both isomers were readily incorporated into acylglycerols and phospholipids, albeit at lower levels than [^{14}C]-oleic or [^{14}C]-linoleic acids. When using [^{14}C]-acetic acid and [^{14}C]-pyruvic acid as substrates, 30 μM 10,12 CLA, but not 9,11 CLA, decreased *de novo* synthesis of triglyceride, free FA, diacylglycerol, cholesterol esters, cardiolipin, phospholipids and ceramides within 3–24 h. Treatment with 30 μM 10,12 CLA, but not 9,11 CLA, decreased total cellular lipids within 3 days and the ratio of monounsaturated FA (MUFA) to saturated FA, and increased C18:0 acyl-CoA levels within 24 h. Consistent with these data, stearoyl-CoA desaturase (SCD)-1 mRNA and protein levels were down-regulated by 10,12 CLA within 7–12 h, respectively. The mRNA levels of liver X receptor (LXR)α and sterol regulatory element binding protein (SREBP)-1c, transcription factors that regulate SCD-1, were decreased by 10,12 CLA within 5 h. These data suggest that the isomer-specific decrease in *de novo* lipid synthesis by 10,12 CLA is due, in part, to the rapid repression of lipogenic transcription factors that regulate MUFA synthesis, suggesting an anti-obesity mechanism unique to this trans FA.

Keywords: Conjugated linoleic acid; Adipocytes; Lipid synthesis; Stearoyl-CoA desaturase

Abbreviations: aP2/FABP4, adipocyte fatty acid binding protein 4; BSA, bovine serum albumin; CLA, conjugated linoleic acid; DGAT2, diacylglycerol transferase-2; ER, endoplasmic reticulum; EA, elaidic acid; EOS, ceramide-containing sphingosine; FA, fatty acids; FAME, fatty acid methyl esters; FBS, fetal bovine serum; GC, gas chromatography; GPDH, glycerol-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin layer chromatography; LXR, liver X receptor; MUFA, monounsaturated fatty acid; NS, nonhydroxy ceramide; NFκB, nuclear factor kappa B; OA, oleic acid; ORO, oil red O; PBS, phosphate buffered saline; PPAR, peroxisome proliferator-activated receptor; q-PCR, real-time polymerase reaction; SCD-1, stearoyl-CoA desaturase-1; SFA, saturated fatty acid; SGBS, Simpson–Golabi–Behmel Syndrome; SREBP-1c, sterol regulatory element binding protein; SV, stromal vascular; TBP, TATA binding protein; TLC, thin layer chromatography; TG, triglyceride; TVA, trans vaccenic acid; VA, vaccenic acid; WAT, white adipose tissue.

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★ The authors' responsibilities were as follows: TO, conducted studies in Figs. 1–6 and 8–9 and prepared the first draft of this manuscript; NF, daily direction of the research conducted by TO; SC, conducted the cell experiments in shown in Fig. 7, which were sent to TO for subsequent analyses; KM, prepared and conducted statistical analyses of data shown in Figs. 1–2 and 5–9, and conducted experiment with ceramide inhibitors (data not shown); SG, conducted experiments with ceramide inhibitors (data not shown); OL, synthesized and provided the [^{14}C]-CLA isomers; MW, provided the SGBS cells and culturing methods; SM, co-mentor for TO and CO-I on NIH CLA grant; and MM, PI of NIH CLA grant and revised the manuscript with input from TO and co-authors.

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1. Introduction

Conjugated linoleic acid (CLA) consists of a group of positional and geometric fatty acid (FA) isomers of linoleic acid (C18:2; *cis*-9, *cis*-12 octadecadienoic acid). Isomers of CLA are found naturally in ruminant meats and dairy products, and in relatively larger quantities in dietary supplements or fortified foods. The two major isomers of CLA, *trans*-10, *cis*-12 (10,12) and *cis*-9, *trans*-11 (9,11), have been reported to prevent obesity, diabetes, atherosclerosis or cancer depending on the specific doses, isomers and models used. There is a great deal of interest in CLA as a weight loss treatment, because of its ability to decrease body weight and body fat mass in many animal studies, and in some human studies (reviewed in Refs. [1,2]). Of the two major isomers, 10,12 CLA is responsible for its anti-obesity properties [3–7].

Potential mechanisms by which 10,12 CLA prevents obesity include (1) increasing energy expenditure and lipolysis, (2) inducing adipocyte apoptosis and (3) deceasing adipogenesis or lipogenesis (reviewed in Ref. [2]). For example, CLA suppresses preadipocyte differentiation in animal [8–14] and human preadipocytes [15], in part, via attenuating the expression or activity of peroxisome proliferator activated receptor (PPAR) γ, a transcription factor essential for adipogenesis. Indeed, rodents supplemented with 10,12 CLA had decreased expression of PPARγ and its target genes [14,16–20]. In mature, primary human adipocytes or murine 3T3-L1

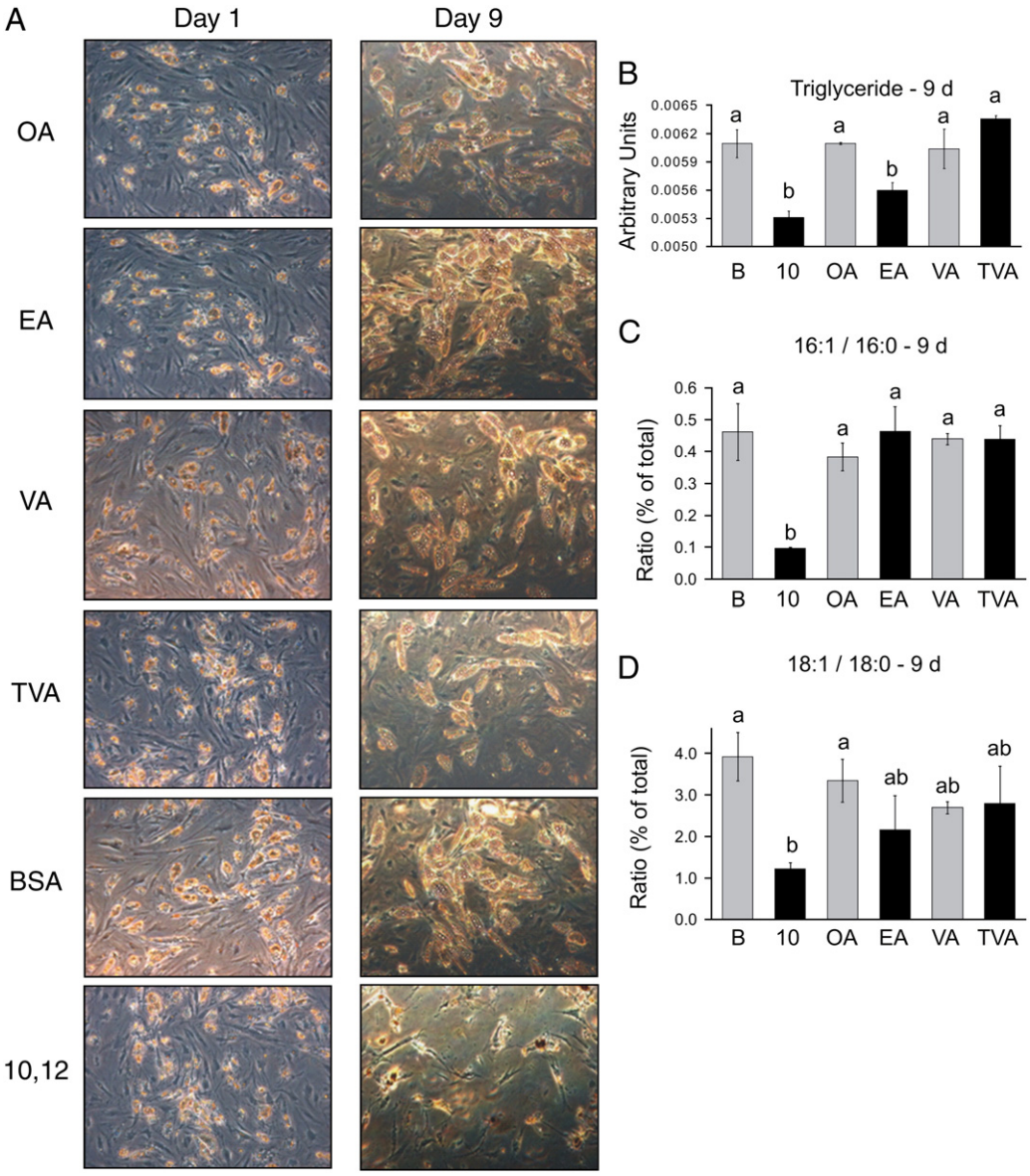

Fig. 1. Chronic treatment with *trans*-10, *cis*-12 CLA, but not with other trans FA, decreases TG content and the MUFA/SFA ratio. Cultures of newly differentiated SGBS adipocytes were treated on Day 6 of differentiation for 9 days with 30 μM oleic acid (OA; cis 9, C18:1), elaidic acid (trans 9, C18:1), vaccenic acid (VA; cis 11, C18:1), trans vaccenic acid (TVA; trans 11, C18:1), BSA vehicle or 10,12 CLA. (A) On Days 0 and 9, cultures were visualized using a Leica DM IRBE inverted light microscope with a 10× objective. The pictures are representative of two independent experiments. Left panel, Day 0; right panel, Day 9. (B) Cultures were harvested and the lipids extracted [25] and spotted onto HPTLC plates and separated in hexane/diethylether/acetic acid (70:30:1 v/v). The separated spots were visualized by spraying the plate with a 3% copper-acetate/15% phosphoric acid solution and charred in a heating cabinet at 100°C for 10–20 min, and the quantification of the lipids was based on the intensities of the charred lipids (arbitrary units) using version 4.0.3 of Scion Image. Means (±S.E.M., $n=2$) not sharing a common superscript are significantly different ($P<.05$). (C and D) The FA composition of the total lipids was determined (% of total), from which the ratios of 16:1/16:0 and 18:1/18:0 were calculated. Means (±S.E.M., $n=2$) not sharing a common superscript are significantly different ($P<.05$). Data in all panels are representative of two independent experiments.

adipocytes, 10,12 CLA decreases the expression or activity of PPARγ [8,21] and PPARγ target genes and lipid content [15,22]. Other transcription factors involved in adipogenesis (e.g., CCAT/enhancer binding protein α, C/EBPα) or lipogenesis (e.g., sterol regulatory element binding protein 1c [SREBP-1c], liver X receptor α [LXRα]) may also be impaired by CLA [5,14,15,18,19,22]. We previously showed that chronic 10,12 CLA treatment of differentiating primary human preadipocytes decreased the mRNA levels of the delta-9 desaturase stearoyl-CoA desaturase (SCD)-1, a SREBP-1c target gene, after 6 days and the monounsaturated fatty acid/saturated fatty acid (MUFA/SFA) ratio after 12 days of treatment, suggesting that down-regulation of SCD-1 precedes reductions in the MUFA/SFA ratio [22]. However, the effects and kinetics of CLA on regulators of lipid metabolism and *de novo* lipid metabolism in mature primary human adipocytes have not been reported.

Thus, we wanted to understand the specificity and kinetics of these anti-lipogenic actions of CLA isomers in human adipocytes; i.e., Does CLA directly attenuate lipogenic transcription factors, which in turn alter lipid metabolism? Or does CLA initially alter lipid metabolism, which in turn affects the expression or activity of these transcription factors? To address these questions, we compared the delipidating effects of 10,12 CLA to other trans FA. Furthermore, we examined the kinetics of CLA's incorporation into lipid classes and its impact on *de novo* lipid synthesis and the expression of LXR, SREBP-1c and their target SCD-1 in newly differentiated human adipocytes.

2. Materials and methods

2.1. Materials

All cell culture ware were purchased from Fisher Scientific (Norcross, GA, USA). Methanolic-HCl (3N) kit was purchased from Supelco (Belafonte, PA, USA), anhydrous methanol 99.8% from Aldrich (Steinheim, Germany) and the GC standard 68A from NU-CHEK-PREP, Inc. (Elysian, MN, USA). Antibodies for SCD-1 (N-20), donkey-anti goat (SC-2020) and glycerol-3-phosphate dehydrogenase (GAPDH) (FL-35) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), and the anti-rabbit antibody from Promega (Madison, WI, USA). Western lightning chemiluminescence substrate was purchased from Perkin Elmer Life Science (Boston, MA, USA). One-step reverse transcription-polymerase chain reaction kit used in semi-quantitative mRNA analysis was purchased from Qiagen (Valencia, CA, USA). Immunoblotting buffers, precast gels and gene-specific primers were purchased from Invitrogen (Carlsbad, CA, USA), and ribosomal 18S competimer technology internal standards and DNA-free were purchased from Ambion (Austin, TX, USA). All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All reagents and chemicals used to isolate lipids were of analytic grade or better.

2.2. Sources of FA

Isomers of CLA (+98% pure) were purchased from Matreya (Pleasant Gap, PA, USA). [1-^{14}C]-9,11 CLA and [1-^{14}C]-10,12 CLA (specific activity=53.4 mCi/mmol; radiochemical and isomeric purities >95%) were synthesized by the Service de Chimie Bioorganique et de Marquage, F-91191, Gif sur Yvette, France. They were prepared by stereoselective multistep synthesis involving sequential substitution of 1,2-dicholoroethene as previously described [23]. [^{14}C]-Oleic acid and [^{14}C]-linoleic acid (specific activities of 50 mCi/mmol), [^{14}C]-acetate (specific activity of 57 mCi/mmol) and [^{14}C]-pyruvate (specific activity of 15 mCi/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK). Unlabeled oleic acid, elaidic acid, vaccenic acid and trans vaccenic acid were purchased from Sigma-Aldrich.

2.3. Methods

2.3.1. Culturing of human primary adipocytes

Abdominal white adipose tissue (WAT) was obtained from nondiabetic females, between the ages of 20 and 50 years with a body mass index ≤30 during abdominoplasty with consent from the Institutional Review Board at the University of North Carolina at Greensboro. Tissue was digested using collagenase, and stromal vascular (SV) cells were isolated as previously described [22]. Experimental treatment of cultures containing ~50% preadipocytes and ~50% adipocytes occurred on Day 6 of differentiation. Each experiment was done in duplicate and repeated at least once using a mixture of cells from two to three subjects unless otherwise indicated. These cells were used for data presented in Figs. 6 and 7 due to the collaborative nature of this research.

2.3.2. Culturing of human Simpson–Golabi–Behmel Syndrome cells

Simpson–Golabi–Behmel Syndrome (SGBS) is a rare X-linked disorder characterized by pre- and postnatal overgrowth. The cell strain exhibits a high capacity for adipose differentiation, resulting in mature fat cells which are biochemically and functionally similar to human primary adipocytes [24]. They offer an advantage over human primary adipocytes, because they can be grown and differentiated in much greater quantities. For these reasons and due to the collaborative nature of this research, they were used in all of the experiments performed with the exception of data shown in Figs. 6 and 7. They were generously provided by Dr. Martin Wabitsch. SGBS cells were grown to confluence in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham and supplemented with 10% bovine serum, 33 μM biotin, 17 μM pantothenate, 100 μg/ml streptomycin and 62 μg/ml penicillin. To induce differentiation, SGBS cells were repeatedly washed with phosphate buffered saline (PBS) buffer and cultured in serum-free medium supplemented with 350 nM insulin, 200 pM triiodothyronine, 1 μM cortisol, 2 μM BRL 49653 (rosiglitazone), 0.115 mg/ml 1-methyl-3-isobutylxanthine (IBMX), 0.25 mmol/L dexamethasone (DEX) and 0.01 mg/ml human transferrin for 4 days. After 4 days, the media was removed and replaced with the differentiation media in the absence of BRL 49653, IBMX and DEX. All treatments of these cells started on Day 6 of differentiation. These cells were used for data in Figs. 1-5, 8 and 9.

2.3.3. Preparation of FA

Isomers of CLA, oleic acid, elaidic acid, vaccenic acid and trans vaccenic acid were complexed to FA-free bovine serum albumin (BSA) (>98%, A7030, Sigma-Aldrich) at a 4:1 molar ratio using 1 mM BSA stocks. All cultures were continuously treated with FA which were added fresh with each media change (every 3–4 days).

2.3.4. Complexing of [^{14}C]-FA to BSA

Ten nanomoles of cold FA (e.g., 10,12 CLA) was evaporated under nitrogen gas and 1 μCi of [^{14}-C]-FA added and subsequently evaporated again. Ten microliters of a 1.1-mM KOH solution was added and incubated for 10 min at 37°C, centrifuged at 2000 rpm for 1 min and 90 μl of a 2.5-mM BSA stock was added; the solution was left at room temperature for 2–3 h and then overnight at 4°C and subsequently stored at −20°C.

2.3.5. [^{14}C]-FA incorporation into lipid classes

Following treatments, cultures were incubated for multiple time points with 1 μCi of [^{14}C]-9,11 CLA, [^{14}C]-10,12 CLA, [^{14}C]-oleic acid or [^{14}C]-linoleic acid; harvested; and

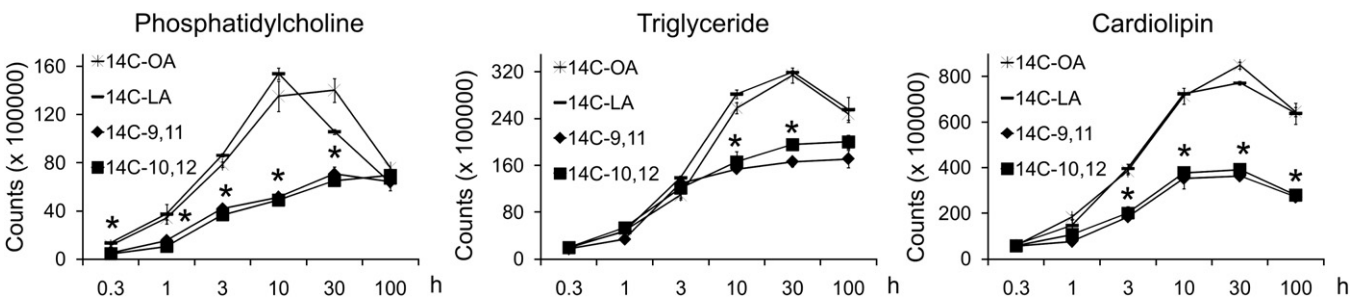

Fig. 2. [^{14}C]-10,12 CLA and [^{14}C]-9,11 CLA incorporate equally into neutral and compound lipids. Cultures of newly differentiated SGBS adipocytes were treated on Day 6 of differentiation with 1 μCi of [^{14}C]-9,11 CLA, [^{14}C]-10,12 CLA, [^{14}C]-9,11 oleic acid (OA) or [^{14}C]-9,11 linoleic acid (LA) for 20 min, 1 h, 3 h, 10 h, 30 h or 100 h. Cultures were then harvested and the lipids were extracted [25]. Neutral and compound lipids were separated by HPTLC in hexane/diethylether/acetic acid (70:30:1, v/v) and chloroform/ethanol/water/triethyl-amine (30:35:7:35, v/v), respectively; incubated overnight on phospho-imager screens; and the spots for phosphatidylcholine, cardiolipin and TG detected and quantified using a Typhoon scanner and ImageQuant TL, respectively. CLA means (±S.E.M., n=2) having an asterisk (*) are significantly (P<.05) different from linoleic and oleic acid for a given time point. Data shown are representative of two individual experiments.

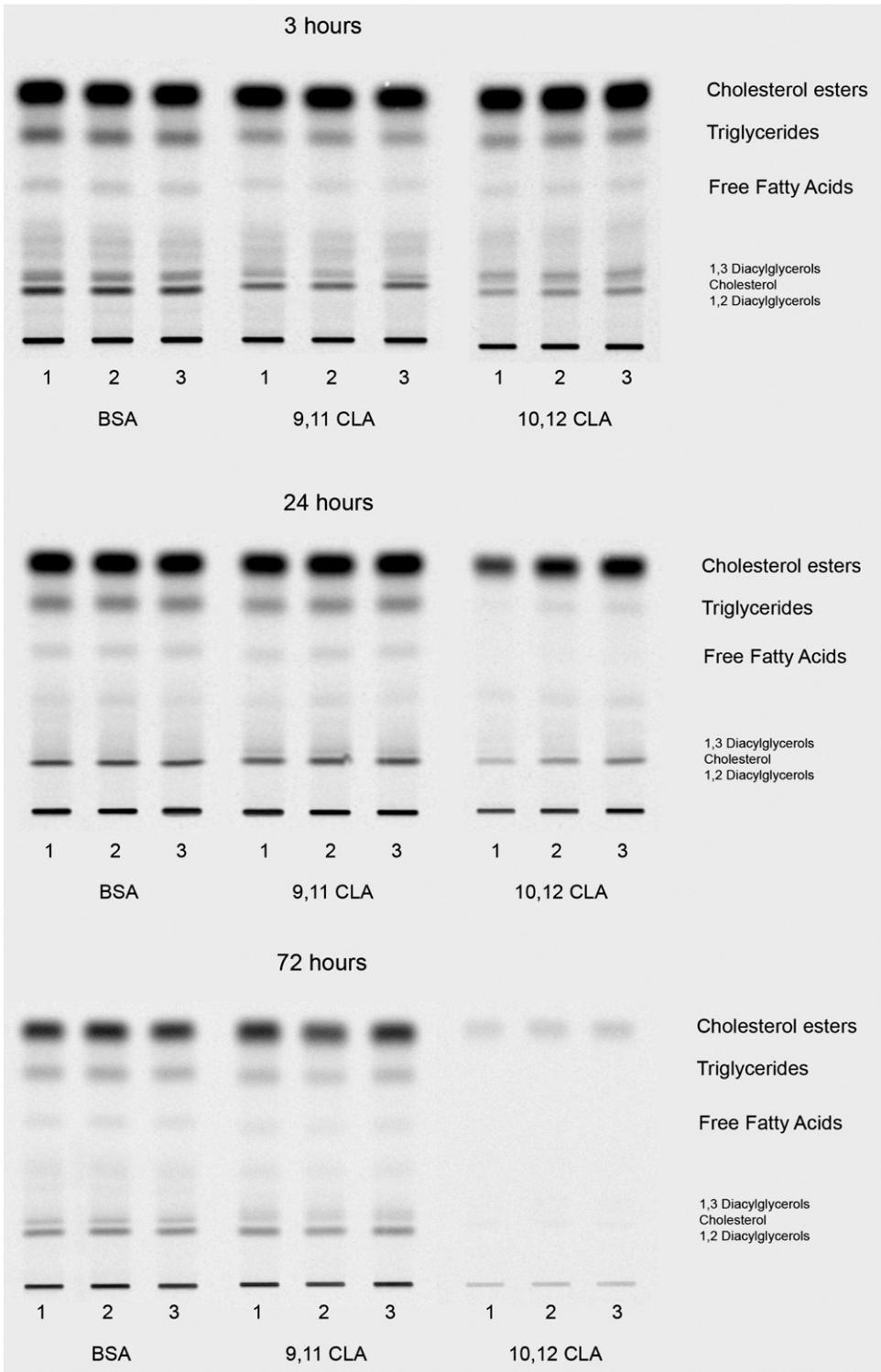

Fig. 3. *Trans*-10, *cis*-12 CLA decreases *de novo* synthesis of neutral lipids. Cultures of newly differentiated SGBS adipocytes were treated on Day 6 of differentiation with BSA, 30 μM 9,11 CLA or 30 μM 10,12 CLA for 3, 24 or 72 h, respectively. Cultures were then incubated with 2 μCi of [^{14}C]-acetate for 240 min and harvested. Lipids were extracted [25], separated by HPTLC in hexane/diethylether/acetic acid (70:30:1 v/v), incubated overnight on phospho-imager screens and the lipid spots were detected. The first three lanes for each treatment represent three different cell culture plates (n=3) and are representative of two independent experiments.

the lipids extracted using the Bligh and Dyer [25] method. Neutral and compound lipids were then separated by high-performance thin layer chromatography (HPTLC) on silica gel 60 plates (Merck KGaA, 50 Darmstadt, Germany) and subsequently developed in hexane/diethylether/acetic acid (70:30:1 v/v) and chloroform/ethanol/water/triethyl-amine (30:35:7:35, v/v), respectively; incubated overnight on phospho-imager screens; and labelled lipids were detected and quantified using a Typhoon scanner and ImageQuant TL (GE Healthcare, Pittsburgh, PA, USA).

2.3.6. De novo synthesis of neutral and compound lipids using [^{14}C]-acetate and [^{14}C]-pyruvate

After treatment, 100 μl of PBS containing 100 nM insulin and 2 μCi [^{14}C]-acetate or 1 μCi [^{14}C]-pyruvate were added to each plate and incubated for 240 min (based on results of a preliminary study). In all cultures, the labeling media was removed and the cells were washed in ice-cold Krebs-Ringer buffer, washed once with ice-cold PBS, harvested in 1 ml PBS (pH 7.4) and immediately frozen at −80°C. For

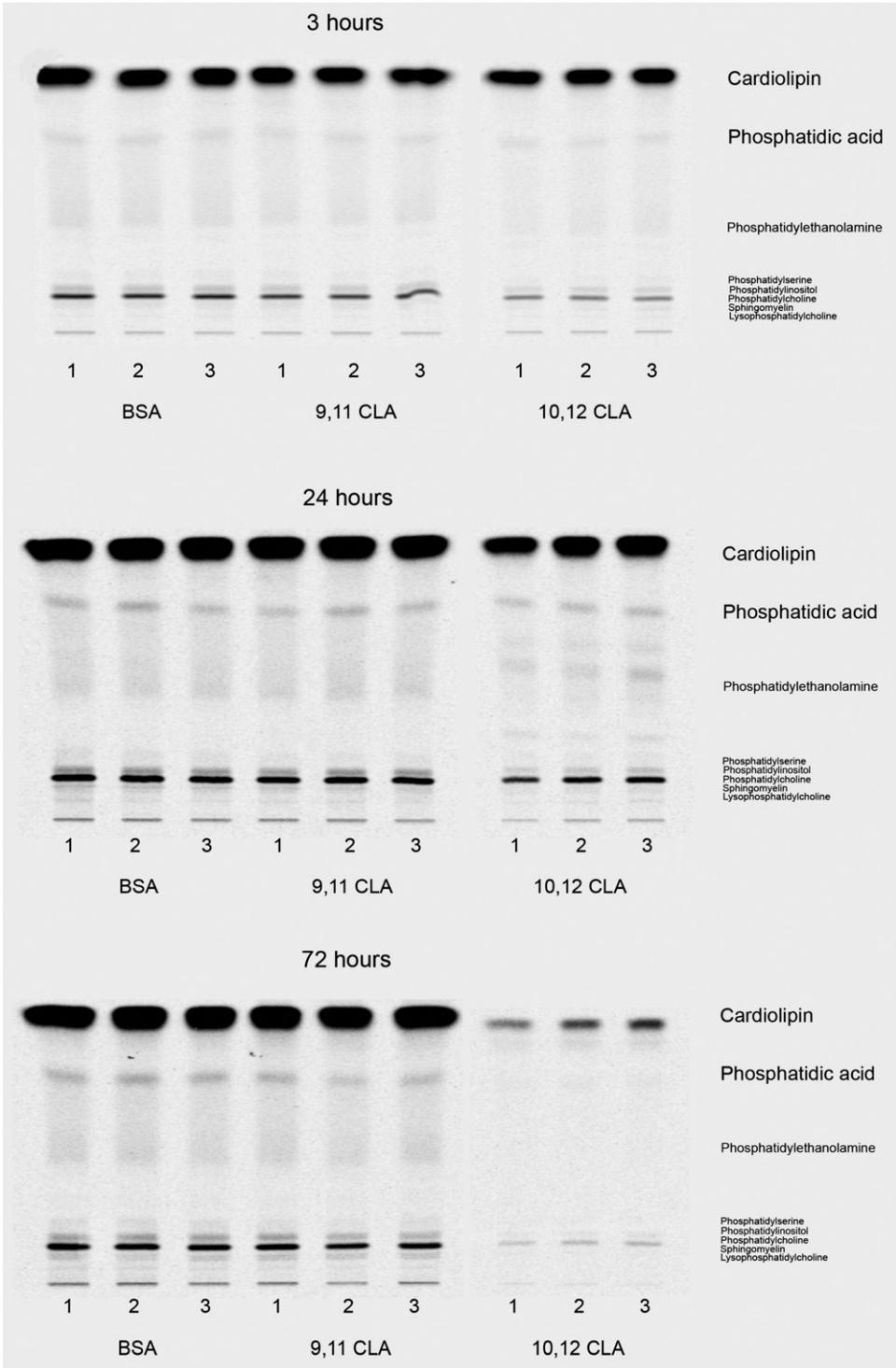

Fig. 4. *Trans*-10, *cis*-12 CLA decreases *de novo* synthesis of compound lipids. Cultures of newly differentiated SGBS adipocytes were treated on Day 6 of differentiation with vehicle (BSA), 30 μM 9,11 CLA or 30 μM 10,12 CLA for 3, 24 or 72 h, respectively. Cultures were then incubated with 2 μCi of [^{14}C]-acetate for 4 h and harvested. Lipids were extracted [25], separated on 2.3% boric acid-treated HPTLC plates in chloroform/ethanol/water/triethylamine (30:35:7:35, v/v), incubated overnight on phospho-imager screens and the lipid spots detected. The first three lanes for each treatment represent three different cell culture plates (n=3) and are representative of two independent experiments.

neutral lipid detection, lipids were extracted [25], separated by HPTLC in hexane/diethylether/acetic acid (70:30:1 v/v), incubated overnight on phospho-imager screens and the lipid spots were detected and quantified using a Typhoon scanner and ImageQuant TL. For compound lipid detection, lipids were extracted [25], separated on boric acid (2.3%)-treated HPTLC plates in chloroform/ethanol/water/triethylamine (30:35:7:35, v/v) and then processed like the neutral lipid procedure. For ceramide detection, extracted lipids were subjected to a mild alkaline hydrolysis to purify the amide-bound lipids, then separated twice by HPTLC in chloroform/methanol/acetic acid (190:9:1 v/v/v), incubated overnight on phospho-imager screens and detected as described above. Protein concentrations were determined using the Qubit fluorometer (Invitrogen) and the Quant-iT protein assay kit as described by the manufacturer. Similar experiments were performed using ^{14}C-pyruvate. Because the results from the ^{14}C-acetate and ^{14}C-pyruvate were similar, only data from the ^{14}C-acetate are shown.

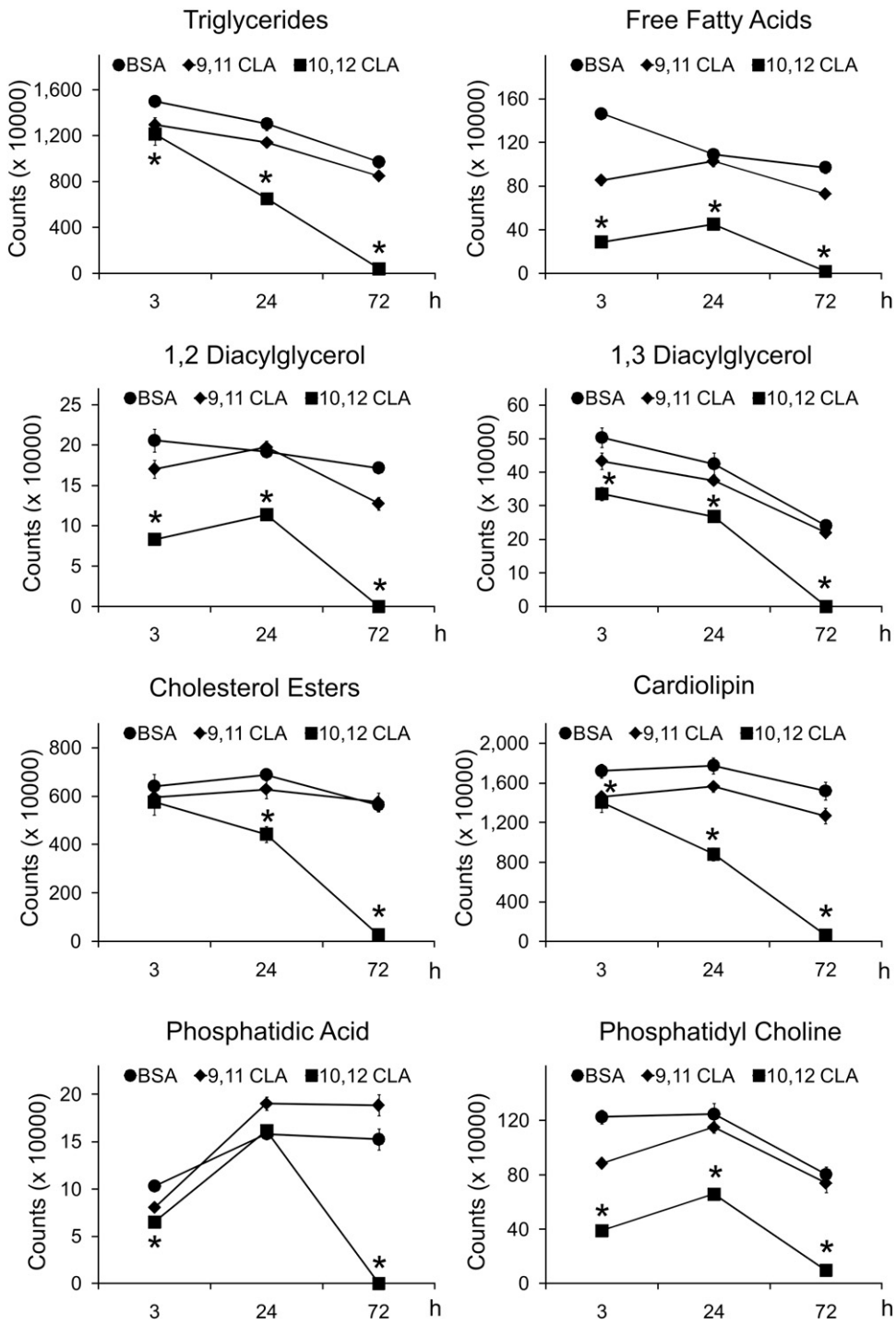

Fig. 5. Quantification of *de novo* synthesis of neutral and compound lipids. The intensities of the HPTLC bands from [^{14}C]-acetate incorporation into specific lipid spots in Figs. 3 and 4 were quantified using a Typhoon scanner and ImageQuant TL and subjected to statistical analyses. 10,12 CLA means (±S.E.M., n=3) having an asterisk (*) are significantly (P<.05) different than the BSA vehicle within a given time point. Data are representative of two independent experiments.

2.3.7. Gas chromatography

For measuring FA profiles, total lipids were saponified, methylated and analyzed using gas chromatography. Briefly, cells were lysed by sonication and the protein concentration was determined. Before extraction, an internal standard C15:0 (pentadecanoic acid) was added. Total cellular lipids were extracted with chloroform/methanol (1:2 v/v) [25]. Aliquots of the lipid extracts were dried under nitrogen gas and converted to FA methyl esters (FAME) by using anhydrous 99.8% MeOH/Methanolic-HCl (3N) (2:1 v/v), heated at 60°C in oil bath for 20 min and identified by comparison to the 68A standard and vaccinate 18:1(n-7) (Larodan, Malmö, Sweden) using GC. GC was performed on a Chrompack CP 9002, equipped with a CPSIL88 FAME 0.25-mm ID capillary column (Chrompack, 51 Middelburg, The Netherlands), and the oven temperature was programmed from 120°C to 220°C, with an increment of 3°C/min. The FA composition of the total lipids was determined (% of total), from which the ratios of 14:1/14:0, 16:1/16:0 and 18:1/18:0 were calculated.

2.3.8. High-performance liquid chromatography

Cells were harvested, centrifuged and the supernatant discarded and cells were stored at −80°C until acyl-CoA extraction. Identification of individual acyl-CoAs by HPLC was performed using standard acyl-CoA mixtures as we described [26].

Table 1
Primers used

Target gene	5′-primer	3′-primer
LXRα	CTTCACGGGAGGAGTGTGTC	CTTCAGTTTCTTCAGGCGGATCT
PPARγ2	AGCAAACCCCTATTCCATGCT	ATCAGTGAAGGAATCGCTTTCTG
SCD-1	CCAACACAATGGCATTCCAG	GGTGGTCACGAGCCCATTC
SREBP-1C	GGAGGGGTAGGGCCAACGGCCT	CATGTCTTCGAAAGTGCAATCC
TBP	GCCCGAAACGCCGAATAT	CCTCATGATTACCGCAGCAAA

LXRα, Liver X receptor; PPARγ2, peroxisome proliferator activated receptor γ2; SCD-1, stearoyl-CoA desaturase-1; SREBP, sterol regulatory element binding protein; TBP, TATA binding protein.

2.3.9. RNA isolation and real-time quantitative PCR

Total RNA was isolated from the cultures using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. RNA was extracted with phenol/1-bromo-3-chloropropane and precipitated with ethanol, dried and resuspended in H_2O. Contaminating genomic DNA was removed by treatment with DNase (DNA-free; Ambion). First-strand cDNA synthesis and real-time quantitative PCR (qPCR) were carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as previously described [22]. Primer sets for SCD-1, LXRα, SREBP-1c, PPARγ and TATA-binding protein (TBP) are shown in Table 1.

2.4. Statistics

Statistical analyses for data in Figs. 2, 5, 6, 7B, 8A and 9B were performed testing the main effects of treatment time and FA type and the interaction of the two (Time×FA) using two-way ANOVA (JMP version 6.03, SAS Institute, Cary, NC, USA). Statistical analyses for data in Figs. 1B–D, 2 and 8B and C were conducted using a one-way ANOVA. Student's *t* tests were used to compute individual pairwise comparisons of least square means ($P<.05$). Data are expressed as the means±S.E.M.

3. Results

3.1. Trans-10, cis-12 CLA, but not other trans FA, delipidates adipocytes

Previous studies have reported that trans FA such as elaidic acid (*trans*-9, C18:1) increase lipolysis [27] and inflammation [28] and reduce PPARγ expression [29] similar to that of 10,12 CLA. To examine whether some or all of the effects of 10,12 CLA are due to the positioning of the trans carbon–carbon double bond in CLA, we treated newly differentiated cultures of SGBS cells with 30 μM elaidic acid (trans 9, C18:1), trans vaccenic acid (trans 11, C18:1) or 10,12 CLA. Cultures treated with 30 μM of oleic acid (cis 9, C18:1) or vaccenic acid (cis 11, C18:1) were included as controls for elaidic and trans vaccenic acid, respectively. Cultures treated with 10,12 CLA for 9 days appeared to contain less lipid (Fig. 1A) and had one of the lowest levels of triglyceride (TG) compared to the other cultures (Fig. 1 B), indicating that these cells were delipidated, as we previously reported for primary human in vitro-differentiated adipocytes [15,21]. Furthermore, 10,12 CLA decreased the C16:1/C16:0 (Fig. 1C) and C18:1/C18:0 ratios (Fig. 1D), although only the C16:1/C16:0 ratio was significantly different than the other FA. Taken together, these data suggest that the position of the trans carbon–carbon bond in 10,12 CLA provides a unique mechanism for reducing the TG content and the MUFA/SFA ratio in human adipocytes compared to other trans FA.

3.2. [^{14}C]-10,12 CLA and [^{14}C]-9,11 CLA incorporate equally into lipids

Although we previously demonstrated that CLA incorporates into neutral and phospholipids during human preadipocyte differentiation [22], the kinetics of CLA's incorporation into specific lipid classes in mature adipocytes has not been determined. Thus, newly differentiated SGBS adipocytes were incubated on Day 6 of differentiation with low levels of [^{14}C]-9,11 CLA and [^{14}C]-10,12 CLA, and their incorporation into specific lipid classes was determined by separating the lipid classes using HPTLC and determining their rate of incorporation into neutral and compound lipids. Cultures were also incubated with low levels of [^{14}C]-oleic and [^{14}C]-linoleic acid as FA controls. Both [^{14}C]-isomers of CLA incorporated into phosphatidylcholine, cardiolipin and TG at similar rates and amounts (Fig. 2). The rates of [^{14}C]-oleic and [^{14}C]-linoleic acid incorporation followed relatively similar patterns (Fig. 2), although they incorporated to a greater extent than the CLA isomers. These differences in incorporation into phosphatidylcholine, cardiolipin and TG became apparent after 0.3, 3 and 10 h, respectively. Incorporation of [^{14}C]-isomers of CLA into other phospholipid classes was not sufficient enough above background to be detected (data not shown). Thus, when given at low levels, CLA isomers readily incorporate into lipid classes equally, albeit at lower levels than oleic and linoleic acids.

3.3. Trans-10, cis-12 CLA decreases de novo lipid synthesis

To investigate the influence of 10,12 CLA on *de novo* lipid synthesis, cultures of newly differentiated SGBS cells were treated on Day 6 of differentiation with BSA, 30 μM 9,11 CLA or 30 μM 10,12 CLA for 3, 24 or 72 h, respectively. Subsequently, cultures were incubated for 4 h, based on the results of a pilot study (data not shown), with either [^{14}C]-acetate or [^{14}C]-pyruvate as substrates. Because the results from both labelled substrates were relatively similar, only data from the [^{14}C]-acetate studies are shown here. Treatment for 3–24 h with 30 μM 10,12 CLA, but not with 9,11 CLA, decreased *de novo* synthesis of neutral lipids (Fig. 3) including cholesterol esters, TG, free FA, cholesterol and diacylglycerols, and compound lipids (Fig. 4) including cardiolipin, phosphatidic acid and phospholipids compared to the BSA vehicle. Quantification and statistical analyses of some of these lipid spots on the HPTLC plates

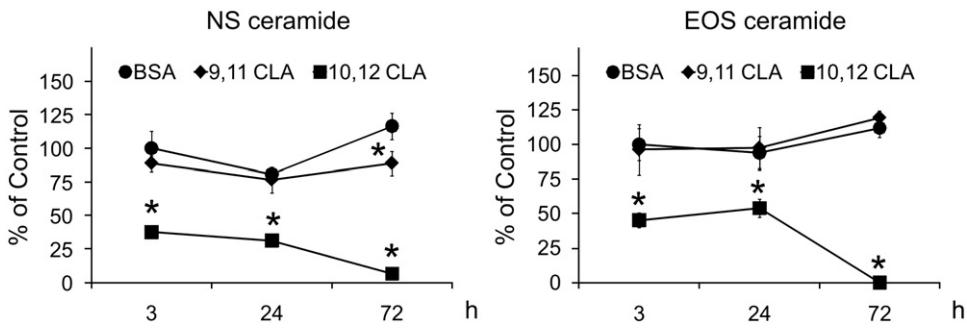

Fig. 6. *Trans*-10, *cis*-12 CLA decreases *de novo* synthesis of ceramides. Cultures of newly differentiated human adipocytes were treated on Day 6 of differentiation with BSA vehicle, 30 μM 9,11 CLA or 30 μM 10,12 CLA for 3, 24 or 72 h, respectively. Cultures were then incubated with 2 μCi of [^{14}C]-acetate for 4 h and harvested. Lipids were extracted [25], subjected to a mild alkaline hydrolysis to purify the amide-bound lipids, then separated twice by HPTLC in chloroform/methanol/acetic acid (190:9:1, v/v/v), incubated overnight on phospho-imager screens, and the lipid spots detected and quantified using a Typhoon scanner and ImageQuant TL. Means (±S.E.M., $n=3$) having an asterisk (*) are significantly ($P<.05$) different than the BSA vehicle within a given time point. Data are representative of two independent experiments. Cer (NS), Nonhydroxy FA ceramide; Cer (EOS), ceramide-containing sphingosine.

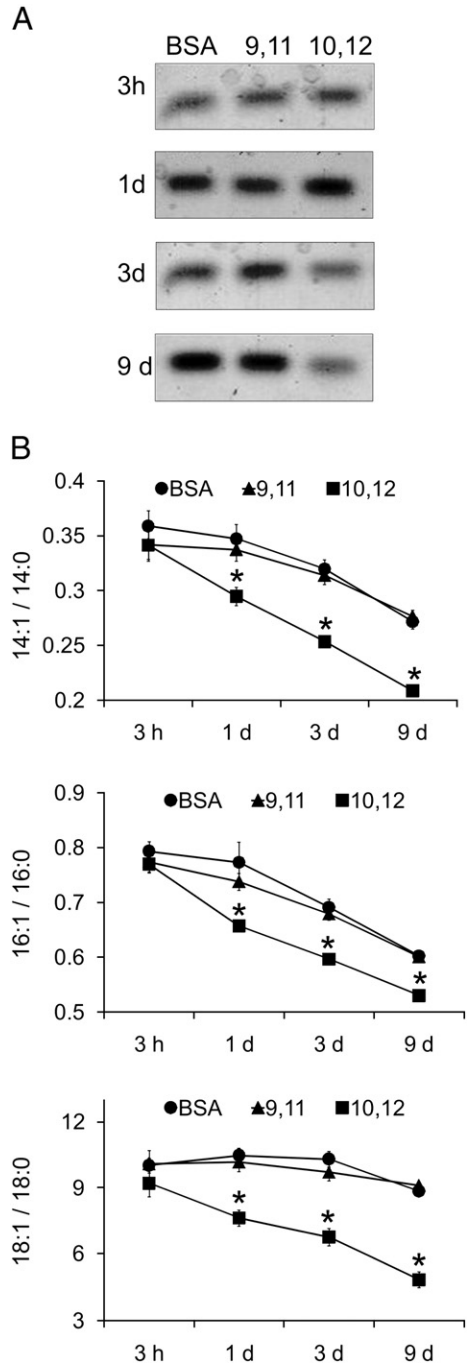

Fig. 7. Acute treatment with *trans*-10, *cis*-12 CLA decreases the TG content and the MUFA/SFA ratio. Cultures of newly differentiated, primary human adipocytes were treated on Day 6 of differentiation with BSA vehicle, 30 μM 9,11 CLA or 30 μM 10,12 CLA for 3 h, 1 day, 3 days, or 9 days, and then total lipids were extracted [25]. (A) Lipids were separated by HPTLC using a neutral lipid separation system consisting of hexane/diethylether/acetic acid (70:30:1). Data shown are representative of three individual experiments. (B) Total lipids were saponified, methylated and analyzed using GC. The FA composition of the total lipids was determined (% of total), from which the ratios of 14:1/14:0, 16:1/16:0 and 18:1/18:0 were calculated. 10,12 CLA means (±S.E.M., n=8) having an asterisk (*) are significantly (P<.05) different than 9,11CLA and BSA vehicle within a time point.

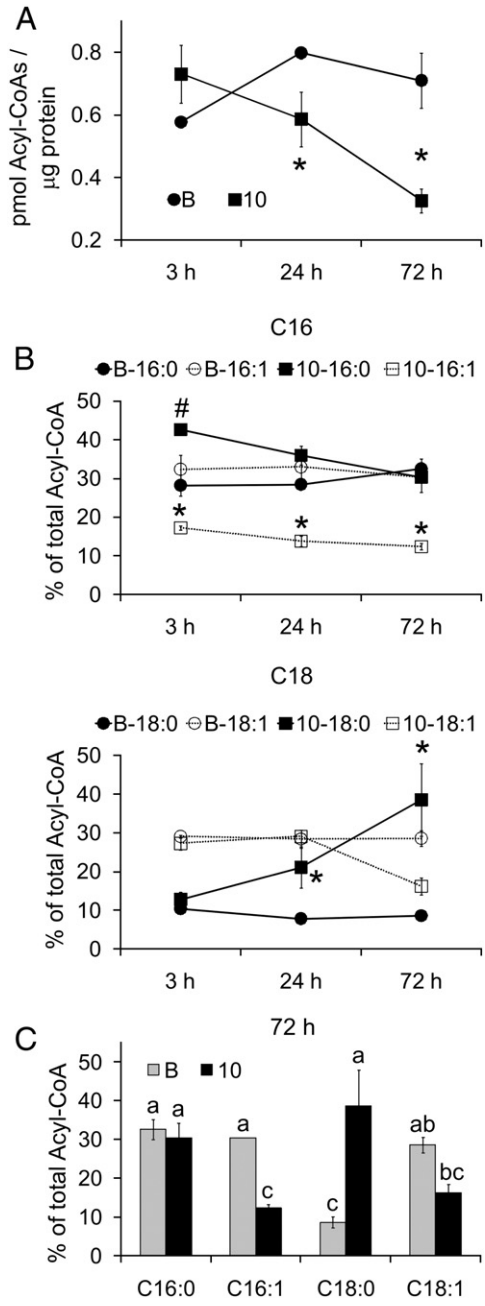

Fig. 8. *Trans*-10, *cis*-12 CLA changes the rates of desaturation in acyl-CoAs. Cultures of newly differentiated SGBS cells were treated on Day 6 of differentiation with BSA vehicle (B) or 30 μM 10,12 CLA [10] for 3, 24 or 72 h. Cultures were then harvested and the acyl-CoAs were extracted and subsequently derivatized to etheno-acyl-CoAs, which were separated and detected by HPLC using a fluorescence detector. After being normalized to equal protein levels, the total amount of acyl-CoAs (panel A) and C16 and C18 acyl-CoAs (Panel B) was calculated using the molecular weight of each individual FA+767.5 (weight of CoA), compared to the C17:0-CoA internal standard. (A) Means (±S.E.M., n=3) having an asterisk (*) are significantly (P<.05) different than the BSA vehicle within a given time point. (B) Means (±S.E.M., n=2) having an asterisk (*) or a pound sign (#) within a FA type (i.e., MUFA is an '*'; SFA is a '#') are significantly different (P>.05) from one another within a given time point. For Panel (C), means (±S.E.M., n=2) not sharing a common superscript are significantly different (P<.05). Data in all panels are representative of two independent experiments.

are shown in Fig. 5. Similarly, *de novo* synthesis of nonhydroxy ceramide (NS) and ceramide-containing sphingosine (EOS), ceramide-containing lipids made from SFA, were lower in cultures treated with 10,12 CLA for 3, 24 and 72 h compared to 9,11 CLA and BSA (Fig. 6). Collectively, these data demonstrate that 10,12 CLA markedly reduces *de novo* synthesis of neutral, phosphoglycerol and complex lipids within 3–24 h in an isomer-specific manner.

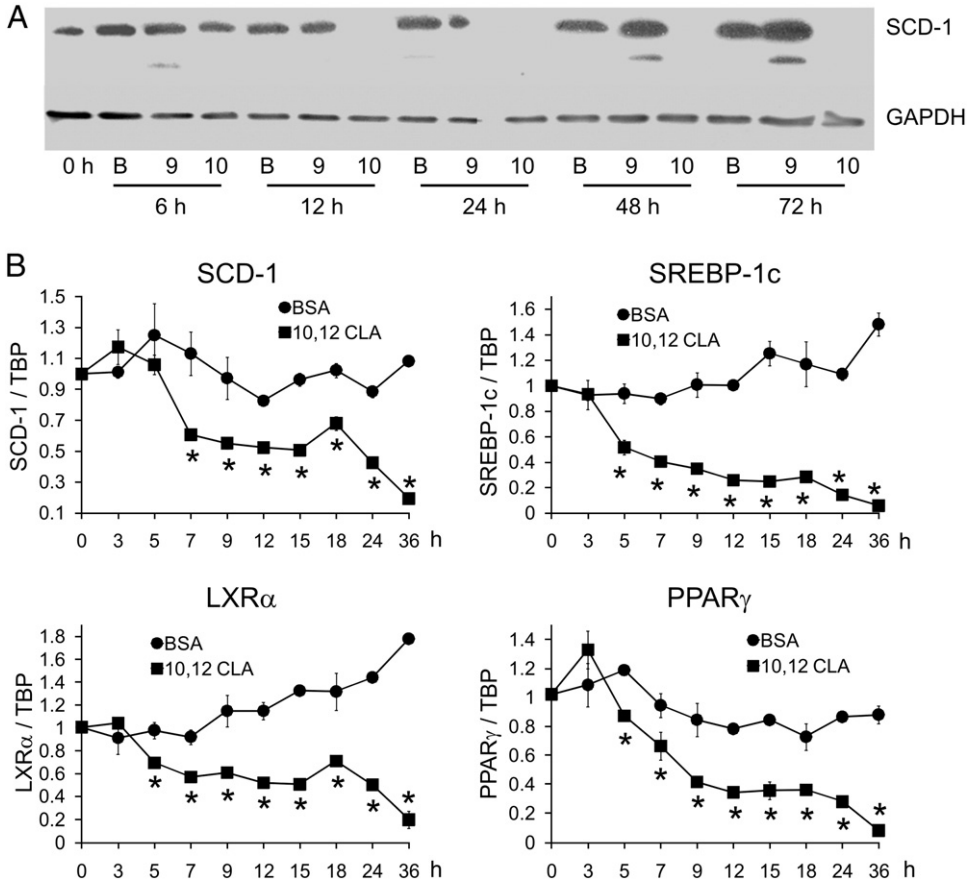

Fig. 9. 10,12 CLA decreases SCD-1 protein and mRNA levels. Cultures of newly differentiated SGBS adipocytes were treated on Day 6 of differentiation with BSA vehicle, 30 μM 9,11 CLA or 30 μM 10,12 CLA over time. (A) Cultures were treated with BSA, 9,11 CLA or 10,12 CLA for 6, 12, 24 , 48 or 72 h. Cells were then harvested and SCD-1 protein and GAPDH levels were determined by immunoblotting. (B) Cultures were treated for 3, 5, 7, 9, 12, 15, 18, 24 or 36 h with either a BSA vehicle or 10,12 CLA. Subsequently, cultures were harvested to determine the mRNA levels of SCD1, SREBP-1c, LXRα, PPARγ and TATA-binding protein (TBP, load control) by qPCR. The expression level of a given gene was calculated after normalization to TBP expression and expressed relative to Day 0 (confluent, noninduced) controls. Means (±S.E.M., $n=2$) having an asterisk (*) are significantly ($P<.05$) different than the BSA vehicle within a given time point. Data shown in Panels (A) and (B) are representative of two individual experiments.

3.4. Trans-10, cis-12 CLA decreases the lipid content and MUFA/SFA ratio

Although we previously demonstrated that chronic treatment with 10,12 CLA reduced the lipid content and MUFA/SFA ratio of differentiating human preadipocytes [22] and in mature human adipocytes (Fig. 1), we did not know the kinetics of this suppression. Therefore, newly differentiated primary adipocytes were treated on Day 6 of differentiation with BSA (vehicle), 9,11 CLA (isomer control) or 10,12 CLA, and harvested after 3 h, 1 day, 3 days or 9 days of treatment. *Trans*-10, *cis*-12 CLA reduced the TG levels (Fig. 7A) after 3 days and the MUFA/SFA ratio (Fig. 7B) after 1 day of treatment compared to 9,11 CLA and the BSA vehicle. Whereas 10,12 CLA decreased total acyl-CoAs (Fig. 8A) after 24–72 h of treatment, it increased the percentage of C18:0 acyl-CoA (Fig. 8B,C), which has been implicated in causing insulin resistance [30].

3.5. Trans-10, cis-12 CLA decreases SCD-1 protein and mRNA levels

Given the important role that SCD-1 plays in desaturating long-chain SFA into MUFA needed for lipid storage and metabolism, we examined the impact of CLA isomers on SCD-1 mRNA and protein levels in mature adipocytes. SGBS adipocytes were treated on Day 6 of differentiation with BSA, 30 μM 9,11 CLA or 30 μM 10,12 CLA, and harvested after 6–72 h for protein or after 3–36 h for mRNA expression. *Trans*-10, *cis*-12 CLA reduced the protein (Fig. 9A) and mRNA levels (Fig. 9B) of SCD-1 after 12 and 7 h of treatment, respectively, compared to 9,11 CLA or BSA vehicle. The mRNA levels of two transcription factors that are essential for the transcriptional regulation of SCD-1, SREBP-1c and LXR were decreased after 5 h of treatment with 10,12 CLA compared to BSA vehicle and 9,11 CLA (Fig. 9B). The mRNA levels of PPARγ, another important regulator of lipogenesis in adipocytes, were similarly decreased by 10,12 CLA. Taken together, these data support the hypothesis that 10,12 CLA decreases lipid metabolism not only by rapidly suppressing the mRNA levels of PPARγ, but also of LXRα and SREBP-1c and their target gene SCD-1, a desaturase required for the synthesis of MUFA essential for neutral and compound lipid synthesis.

4. Discussion

The purpose of this study was to determine the extent to which CLA isomers impacted *de novo* lipid synthesis in human adipocytes. We found that the lipid-lowering effect of 10,12 CLA does not appear to be due to a “structural trans effect”, because several other trans FA did not delipidate adipocytes or alter the MUFA/SFA ratio to the extent of 10,12 CLA. Alternatively, these effects may be due to the position of the trans double bond in the C18:2 structure of CLA. Consistent with these data, we found that 10,12 CLA, but not 9,11 CLA, suppressed *de novo* lipid synthesis within 3–24 h of treatment. This suppression does not appear to be due to impaired incorporation of 10,12 CLA, as both CLA isomers equally incorporate into lipids, albeit at lower levels than other unsaturated FA. Notably, we demonstrated

Table 2
Kinetics of 10,12 CLA-mediated suppression of transcription factors or enzymes that regulate lipogenesis and lipid metabolism in human adipocytes

↓ Regulators of lipogenesis by CLA	↓ Lipid metabolism by CLA				
mRNA or protein	De novo synthesis			MUFA/SFA Ratio	Total lipids
5–12 h	3 h	24 h	72 h	24 h	72 h
LXRα	TG	CE	PA	C14:1/C14:0	TL
SREBP-1c	DAG			C16:1/C16:0	
PPARγ	FFA			C18:1/C18:0	
SCD-1	PC				
	CL				
	CER				

LXRα, liver X receptor α; SREBP-1c, sterol regulatory element binding protein-1c; PPARγ, peroxisome proliferator activated receptor γ; SCD-1, stearoyl CoA desaturase-1; TG, triglyceride; DAG, diacylglycerol; FFA, free fatty acid; PC, phosphatidylcholine; CL, cardiolipin; CER, ceramide; CE, cholesterol ester; PA, phosphatidic acid; TL, total lipids.

that 10,12 CLA decreases the MUFA/SFA ratio within 24 h and the mRNA and protein levels of SCD-1, a delta-9 desaturase essential for MUFA synthesis, metabolism and TG storage, within 7 h in an isomer-specific manner (see Table 2 for the kinetics of these effects). Furthermore, the mRNA levels of LXRα and SREBP-1c were similarly decreased by 10,12 CLA within 5 h. Taken together, these data suggest that 10,12 CLA rapidly reduces SCD-1 abundance and activity in human adipocytes as it does in murine adipocytes [12,31–33], possibly by reducing the levels of LXRα, SREBP-1c and PPARγ that regulate its expression or activity. The rapid 10,12 CLA-mediated reduction in the expression of these lipogenic transcription factors and SCD-1 is likely responsible, in part, for the reduction in MUFA, which could impair neutral and compound lipid synthesis, metabolism or storage.

These and previously published data suggest that 10,12 CLA lowers the lipid content of adipocytes by rapidly (1) decreasing SCD-1 activity, thereby reducing the MUFA needed for neutral and compound lipid synthesis (reviewed in Ref. [34]); (2) decreasing PPARγ activity, thereby reducing the expression of adipogenic and lipogenic proteins needed for lipid biosynthesis (reviewed in Ref. [2]); or (3) increasing inflammatory lipid metabolites or signals that antagonize glucose and FA uptake and subsequent metabolism (reviewed in Ref. [2]).

Stearoyl-CoA desaturase-1 is a central lipogenic enzyme (reviewed in Ref. [34]), and Ntambi et al. first proposed that inhibition of SCD-1 by 10,12 CLA in mice [12,31,32] and in humans [33] is key to the anti-lipogenic effects of 10,12 CLA. Stearoyl-CoA desaturase-1 and diacylglycerol transferase-2 (DGAT2) have been shown to co-localize in the endoplasmic reticulum (ER) and be important for TG synthesis [35]. Dietary and endogenous palmitate (C16:0) and stearate (C18:0) are desaturated by SCD-1 and channeled to DGAT2 for the final step in TG synthesis in the ER. This close association between SCD-1 and DGAT2 enhances the efficiency of TG synthesis [35]. However, 10,12 CLA's reduction in body fat mass was similar in SCD-1 knockout and wild-type mice, suggesting that CLA's antiobesity properties are independent of SCD-1 in mice [36].

We previously demonstrated that long-term treatment (i.e., 12 days) of differentiating primary human preadipocytes with 10,12 CLA decreased the MUFA/SFA ratio and the mRNA levels of SCD-1 [22]. Reductions in SCD-1 mRNA were detected after 6 days of treatment in differentiating human preadipocytes [22]. We found in the current study that 10,12 CLA reduced the mRNA levels of SCD-1, LXRα and SREBP-1c within 5–7 h (Fig. 9B) in mature human adipocytes. Similarly, 10,12 CLA decreased the mRNA expression of SREBP-1c in pig preadipocytes [6]. Conversely, neither 9,11 CLA nor 10,12 CLA affected SREBP-1a or SREBP-1c mRNA levels or the precursor protein in a bovine mammary cell line, whereas the abundance of the mature fragment of SREBP-1a and SREBP-1c protein was significantly reduced by 10,12 CLA, but not by 9,11 CLA [37]. Moreover, 10,12 CLA reduced the mRNA expression for critical genes in the lipid synthesis pathway, whereas 9,11 CLA had no effect. Collectively, these data suggest that 10,12 CLA might also affect the SREBP-1 pathway at the level of proteolytic cleavage rather than affect the availability of the precursor protein.

Alternatively, 10,12 CLA could reduce lipogenesis by inhibiting transporters of glucose (i.e., GLUT4) or FA (i.e., aP2) or esterification enzymes regulated by PPARγ [4,15,22,38], thereby limiting the *de novo* synthesis of SFA for desaturation by SCD-1. Less SFA substrate for SCD-1 would limit its activity (reviewed in Ref. [34]). In agreement with this hypothesis, 10,12 CLA reduced PPARγ mRNA levels within 5 h (Fig. 9B), which could contribute to reduced PPARγ activity. Consistent with these data, we have previously shown that 10,12 CLA reduces PPARγ mRNA [15,22], protein [21,38–40] or activity [21,39] in human adipocytes, and co-supplementation with the PPARγ agonist rosiglitazone attenuates this effect along with preventing insulin resistance and delipidation by 10,12 CLA [21]. *In vivo*, Belury et al. [20,41] have shown that rosiglitazone attenuates many of the adverse effects of CLA treatment including insulin resistance, lipodystrophy or adiponectin depletion.

We speculated that 10,12 CLA produces lipid-borne metabolites like ceramide (reviewed in Ref. [42]) that antagonize PPARγ and insulin sensitivity, because increased PPARγ activity is antagonized by pro-inflammatory mediators like nuclear factor kappa B (NFκB) [43,44], and our previous finding demonstrating that NFκB inhibitors or siRNA targeting p65 attenuates 10,12 CLA's suppression of PPARγ and insulin resistance [38]. However, 10,12 CLA decreased overall *de novo* synthesis of several lipid classes including ceramides, TG and cholesterol esters. Therefore, although 10,12 CLA decreased the ratio of MUFA/SFA, it did not increase the levels of ceramide, a SFA-derived inflammatory lipid that causes insulin resistance (reviewed in Ref. [42]). Consistent with these data, we found that treatment of human adipocytes with ceramide inhibitors (i.e., myriocin, fumonisin B1) did not attenuate 10,12 CLA-mediated induction of inflammatory genes or suppression of adipogenic/lipogenic genes (data not shown). Thus, ceramide does not appear to play a role in 10,12 CLA-mediated inflammation or insulin resistance. In addition, 10,12 CLA reduced total acyl-CoA levels and increased the amount of C18:0 acyl-CoA, which has been implicated in insulin resistance due to impaired oxidation [30].

Based on our previous results and results from this work we suggest that, because of its unique structure, 10,12 CLA or its lipid-borne metabolites initially (e.g., within 5–7 h) alter the activity of lipogenic transcription factors and their respective downstream targets. Our previous results indicate that this could occur through activation of inflammatory signals such as NFκB [38,40] that antagonize LXRα, PPARγ and possibly also SREBP-1. This leads to decreased activity of lipogenic enzymes essential for FA desaturation and incorporation into neutral and compound lipids within 3–24 h. These 10,12 CLA-mediated changes ultimately reduce total cellular lipids within 72 h. Alternatively, 10,12 CLA could also directly affect the activity of desaturases and lipogenic enzymes; however, the kinetics of our data summarized in Table 2 are consistent with 10,12 CLA acting primarily by attenuating the activity of lipogenic transcription factors and their targets, which in turn decrease *de novo* lipid metabolism and lipid accumulation, and not vice versa.

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