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Physiol. Genomics 21:351-361, 2005. doi:10.1152/physiolgenomics.00244.2004

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Functional genomic characterization of delipidation elicited by *trans*-10, *cis*-12-conjugated linoleic acid (t10c12-CLA) in a polygenic obese line of mice

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Submitted 18 October 2004; accepted in final form 23 February 2005

House, Ralph L., Joseph P. Cassady, Eugene J. Eisen, Thomas E. Eling, Jennifer B. Collins, Sherry F. Grissom, and Jack Odle. Functional genomic characterization of delipidation elicited by *trans*-10, *cis*-12-conjugated linoleic acid (t10c12-CLA) in a polygenic obese line of mice. *Physiol Genomics* 21: 351–361, 2005; doi: 10.1152/physiolgenomics.00244.2004.—Gene expression was measured during t10c12-CLA-induced body fat reduction in a polygenic obese line of mice. Adult mice ($n = 185$) were allotted to a 2×2 factorial experiment consisting of either nonobese (ICR-control) or obese (M16-selected) mice fed a 7% fat, purified diet containing either 1% linoleic acid (LA) or 1% t10c12-CLA. Body weight (BW) by day 14 was 12% lower in CLA- compared with LA-fed mice ($P < 0.0001$). By day 14, t10c12-CLA reduced weights of epididymal, mesenteric, and brown adipose tissues, as a percentage of BW, in both lines by 30, 27, and 58%, respectively, and increased liver weight/BW by 34% ($P < 0.0001$). Total RNA was isolated and pooled (4 pools per tissue per day) from epididymal adipose (days 5 and 14) of the obese mice to analyze gene expression profiles using Agilent mouse oligo microarray slides representing >20,000 genes. Numbers of genes differentially expressed by greater than or equal to twofold in epididymal adipose (days 5 and 14) were 29 and 125, respectively. It was concluded that, in adipose tissue, CLA increased expression of uncoupling proteins (1 and 2), carnitine palmitoyltransferase system, tumor necrosis factor- α ($P < 0.05$), and caspase-3 but decreased expression of peroxisome proliferator-activated receptor- γ , glucose transporter-4, perilipin, caveolin-1, adiponectin, resistin, and Bcl-2 ($P < 0.01$). In conclusion, this experiment has revealed candidate genes that will be useful in elucidating mechanisms of adipose delipidation.

apoptosis; cell biology; gene expression; lipid metabolism; obesity

OVER THE PAST 20 YR, the incidence of obesity has reached epidemic proportions. According to the 1999–2000 National Health and Nutrition Examination Survey, ~64% of United States adults ≥ 20 yr old are overweight or obese (<http://www.cdc.gov/nchs/products/pubs/pubd/hestats/obese/obse99.htm>) (28). In 2003, Sturm (53) reported that extreme obesity [body mass index (BMI) ≥ 40] quadrupled between 1986 and 2000 from 1 in 200 adults to 1 in 50. Additionally, within the same time period, there was an increase by a factor of five of Americans with a BMI ≥ 50 , from 1 in 2,000 to 1 in 400 (53). Internationally, the World Health Organization reported that from 1995 to 2000, the number of obese adults worldwide increased from 200 million to over 300 million adults (<http://www.who.int/nut/obs.htm>). In

2003, \$75 billion (US dollars) was spent on medical treatment ascribed to obesity, one-half of which was paid for by Medicare and Medicaid (23).

The rapid increase in the prevalence of obesity has attracted much attention to the delipidative effects of conjugated linoleic acid (CLA). Found naturally in ruminant products (such as beef and cheese), the CLA family consists of several conjugated and stereoisomeric variations of linoleic acid (*cis*, *cis*- $\Delta^{9,12}$ -octadecadienic acid). Arising from anaerobic bacterial biohydrogenation of linoleic acid and α -linolenic acid obtained from plant material (43), the predominant (80–90%) natural form is the *cis*-9,*trans*-11-CLA (c9t11-CLA) isomer, also called rumenic acid. Experiments conducted using a synthetic mixture of c9t11- and t10c12-CLA (usually a 1:1 ratio) have shown that CLA has beneficial effects against cancer (5), diabetes (55), atherosclerosis (38), and obesity (61). However, effects of CLA differ among species.

Delipidative effects of CLA were observed in the ICR line of mice, which displayed a 60% decrease in body fat after 4–5 wk of feeding (44). This experiment has been replicated in different lines of mice, and similar conclusions were drawn (43). The t10c12-CLA isomer is predominantly responsible for delipidative activity observed with mixed (c9t11/t10c12) CLA treatment (21, 27, 43, 45). Consequently, our work focused solely on the effects of t10c12-CLA on adiposity.

CLA may impart its delipidative effects by increasing energy expenditure (64), apoptosis (40, 58), fatty acid oxidation (21), and lipolysis (9), as well as decreasing stromal vascular cell (preadipocyte) differentiation (11, 20) and lipogenesis (8, 10, 21). Gene expression studies using quantitative real-time RT-PCR (qRT-PCR) or Northern blot analysis have confirmed these observations (see Supplementary Table S1; available at the *Physiological Genomics* web site).¹ On the basis of this published literature examining CLA effects on murine white adipose tissue, in conjunction with the general effect of CLA in reducing adiposity, we hypothesized several genes that would be affected by t10c12-CLA supplementation, recognizing that not all of these genes are highly expressed in white adipose tissue. These a priori hypotheses are outlined in Table S1. In general, genes expected to be downregulated included those involved in anabolic lipid metabolism, anti-apoptosis, and the adipokines. Genes expected to be upregulated included those involved in catabolic lipid metabolism and those that are

Article published online before print. See web site for date of publication (<http://physiolgenomics.physiology.org>).

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¹The Supplemental Material for this article (Supplemental Tables S1–S5) is available online at <http://physiolgenomics.physiology.org/cgi/content/full/00244.2004/DC1>.



pro-apoptotic. Various transcription factors were projected to increase or decrease, depending on their specific function.

To our knowledge, this is the first experiment to utilize functional genomic techniques to analyze broad effects of CLA in mice. To more closely parallel the major human obesity condition, we utilized the M16 polygenic obese line of mice (18, 50). The M16 line was selected over 27 generations for rapid postweaning gain from an outbred ICR albino population and exhibits positive correlated responses in body weight and percent body fat and is hyperglycemic and hyperinsulinemic (19, 26). Thus we conducted a study using dietary t10c12-CLA as a probe in combination with a polygenic obese mouse model to identify candidate genes associated with adipose metabolism.

MATERIALS AND METHODS

Diet Composition

Purified AIN93G pelleted diets (Harlan Teklad, Madison, WI) were formulated with either 1% t10c12-CLA or linoleic acid as a treatment control. Diet fatty acid content was analyzed and confirmed in our laboratory (data not shown). The t10c12-CLA was kindly donated by the BASF (Ludwigshafen, Germany), and linoleic acid (LA) was purchased from Nu-Chek-Prep (Elysian, MN). Purity of these samples was analyzed in our laboratory, confirming that LA and t10c12-CLA were 99 and 92% pure, respectively. Inert red and blue dyes were incorporated into the pellets to ensure that mice were administered their respective diet at all times.

Mouse Selection and Treatment

The Institutional Animal Care and Use Committee of North Carolina State University approved the animal protocols. To ensure that mice had attained $\geq 95\%$ of their mature body mass, dietary treatment began when they reached 9 wk of age (18). The experiment was conducted with a total of 185 male mice and was split into two replicates (Fig. 1). Mice from each line (obese and nonobese) were fed the t10c12-CLA and LA diets according to a 2×2 factorial design. Mice were individually housed in 10.5-by-6.5-in. polypropylene cages in a windowless, humidity-controlled

room maintained at 21°C, on a 12:12-h day-night cycle. Water was available at all times, and mice were allowed ad libitum access to feed. The LA diet was fed to all animals during a 7-day acclimation period before treatment. Body weights of individual mice were recorded on *days 2, 5, 8, 11, and 14* after onset of treatment. Feed disappearance during each interval was recorded to allow calculation of individual daily feed intake. Mice were euthanized by CO₂ asphyxiation, and epididymal, mesenteric, and scapular brown adipose tissues as well as liver tissue samples were collected from mice chosen at random on *days 0, 5, and 14* of the study. Dissected tissue samples were weighed, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

Lipids were extracted from epididymal, mesenteric, and brown adipose tissues taken from obese and nonobese animals at *day 14*, and fatty acids were quantified by gas-liquid chromatography as reported by Averette-Gatlin et al. (2).

Total RNA was isolated from adipose and liver tissue, using both TriReagent (Sigma, St. Louis, MO) and the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). RNA was then purified following the Qiagen RNeasy Mini kit manufacturer's protocol (<http://www1.qiagen.com/literature/handbooks/INT/rnalit.aspx#rnymicro>), incorporating modifications that have been previously reported (60). Total RNA concentration was measured by spectrophotometrically analyzing a 1:20 solution of RNA diluted in Tris-diethyl pyrocarbonate water at 260 nm. Integrity was verified electrophoretically with 3 μg of RNA on a 1% native agarose gel. Fluorescence was visualized after staining with ethidium bromide.

Total RNA was labeled by direct reverse transcriptional incorporation of cyanine 3 (Cy3)- and cyanine 5 (Cy5)-labeled dCTP, using an oligo(dT) primer. The Agilent Fluorescent Direct Label kit protocol was used with 10 μg of starting material (<http://www.agilent.com>). Hybridizations were for 16 h in a rotating hybridization oven, using the Agilent 60-mer-oligo microarray processing protocol (<http://www.agilent.com>). Slides were subsequently washed with a $6\times$ SSC-0.005% Triton X-102 wash solution for 10 min at room temperature, followed by a second wash with a solution of $0.1\times$ SSC-0.005% Triton X-102 for 5 min. Slides were dried under a nitrogen stream and scanned with an Agilent G2565BA microarray scanner (<http://www.agilent.com>).

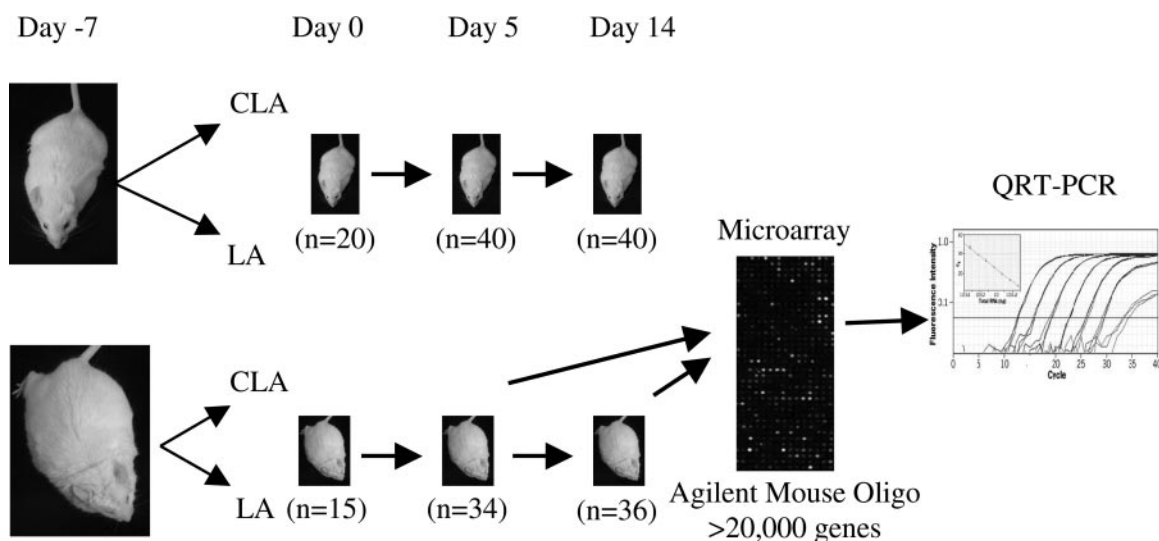


Fig. 1. Experimental scheme outlining the random allocation of mice into conjugated linoleic acid (CLA) and linoleic acid (LA) dietary treatment groups over the course of the 14-day trial. Tissues were collected on *days 0, 5, and 14* of the trial from nonobese (ICR) and obese (M16) mice. Epididymal adipose tissue taken from obese mice on *days 5 and 14* was subjected to microarray analysis, and select genes were further examined using quantitative real-time RT-PCR (qRT-PCR).

Microarray Design

Functional genomic analysis was conducted on RNA samples isolated from epididymal adipose extracted on *days 5* and *14*. Because line-by-treatment interactions were not detected ($P > 0.05$) in growth and fat depot weights, only tissues from obese mice (M16) were screened. A total of 70 samples were pooled into 4 groups (per treatment per day) totaling 16 samples and yielding a total of 8 microarray slides (4 slides per tissue per day; Fig. 2). Therefore, each observation was replicated on four arrays, with a fluor reversal applied to two of the four replicate slides (Fig. 2). Agilent Mouse Oligo microarray slides (G4121A; Agilent Technologies, Palo Alto, CA) containing >20,000 probes, designed in collaboration with the NIEHS Toxicogenomics Research Consortium and Paradigm Genetics (Research Triangle Park, NC), were used for the analysis. The 60-mer oligonucleotide probes were oriented in the sense (5'-3') direction and spotted using Agilent's SurePrint fabrication technology, which utilizes an industrial-scale inkjet printer that synthesizes oligonucleotide probes in situ on glass wafers, which are then scribed onto bar-coded 1-by-3-in. glass slides. A complete probe list (G4121A) can be found at <http://dir.niehs.nih.gov/microarray/chips.htm>.

Microarray Data Collection

Gene expression data were obtained with the use of Agilent G2567AA Feature Extraction software, using defaults for all parameters except ratio terms, which were changed according to Agilent protocol to fit the direct labeling procedure. Files and images, including error values and *P* values, were exported from the Agilent Feature Extraction software and loaded into Rosetta Resolver (version 3.2, build 3.2.2.0.33; Rosetta Biosoftware, Kirkland, WA). Four arrays for each sample pair, including fluor reversals, were combined into ratio experiments in Rosetta Resolver (52). Intensity plots were produced for each ratio experiment, and genes specifically output by this software were considered "signature genes" if $P < 0.01$. *P* values reflecting both microarray slide and biological variation were calculated using the Rosetta Resolver error model (52). In compliance with the "minimum information about microarray experiments" (MIAME) (6), the raw data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and have been as-

signed the following GEO accession numbers: GSM27145, GSM27154, GSM27155, GSM27158, GSM27162, GSM27164, GSM27166, GSM27168, and GSE1580.

qRT-PCR

Two genes of interest, caveolin-1 (*cav-1*) (NM_007616) and caspase-3 (*casp-3*) (NM_009810), were further analyzed by qRT-PCR. These genes were chosen because they represented spots of high (*cav-1*) and low (*casp-3*) intensity on the microarray and are associated with lipid metabolism (*cav-1*) and apoptosis (*casp-3*). β -Actin (X03672) was chosen as a normalization standard housekeeping gene. A 1- μ g aliquot of total RNA was reverse transcribed into cDNA, using an oligo(dT) primer (Roche Applied Science, Indianapolis, IN) and the Qiagen Omniscript Reverse Transcriptase kit (Qiagen), according to the manufacturer's protocol. RNase inhibitor (Roche Applied Science) was included in the reaction. Each treatment by day (e.g., CLA \times day 5) was replicated using at least 14 animals for each gene. Primers were designed to span introns to prevent amplification of possible genomic DNA contamination. DNASTAR PrimerSelect software (DNASTAR, Madison, WI) was used to design the following primers: NM_007616 (*cav-1*), forward 5'-ACGCGCACACCAAGGAG-3', reverse 5'-CA-AAGTAAATGCCCCAGATGAG-3'; NM_009810 (*casp-3*), forward 5'-AATGGGCCTGTTGAACTGAAAAAG-3', reverse 5'-CCTGT-TAACGCGAGTGAGAATGTG-3'; X03672 (β -actin), forward 5'-CGGCCAGGTCATCACTATTG-3', reverse 5'-GCTAGGAGCCA-GAGCAGTAATC-3'.

qPCR was carried out with 2 μ l of cDNA in a 20- μ l total reaction, using Qiagen's Quantitect SYBR Green PCR kit (Qiagen), following the manufacturer's protocol. Negative controls were included on each 96-well plate. Fluorescence measurements were recorded in real time with an OPTICON real-time thermocycler (MJ Research, Waltham, MA). Melting curve analysis was used to verify primer quality (no primer dimers or nonspecific amplification). Amplification also was verified electrophoretically using 2 μ g of cDNA on a 2% agarose gel (MetaPhor; Cabrex BioScience, Rockland, ME).

Statistical Analysis

Phenotypic analysis. This experiment followed a 2 \times 2 factorial, with two lines of mice [obese (M16) and nonobese (ICR)] fed either a treatment or control diet (t10c12-CLA vs. LA) (Fig. 1). Male mice

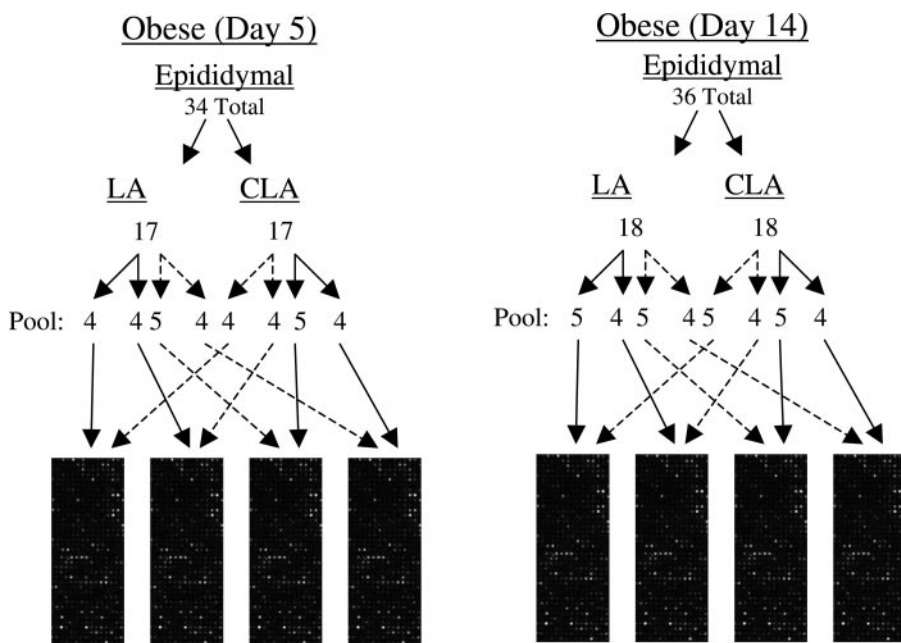


Fig. 2. Experimental strategy diagramming RNA isolation and analysis from epididymal adipose tissue (*days 5* and *14*). Samples were pooled and labeled with Cy-3 (solid arrows) or Cy-5 (broken arrows) before hybridization to Agilent microarray slides. Nos. indicate the no. of animals or RNA samples.

were allocated to each treatment group over two replicates, using a completely randomized design. Time effects on body weight and individual daily feed intake were analyzed using repeated measures ANOVA. Differences between mouse lines and dietary treatments were calculated by use of the SAS general linear model procedure (SAS Institute, Cary, NC). Fixed effects included line, treatment, interval (time points at which body weight and individual daily feed intake measurements were recorded), and replicate. Values were represented using least square means (LSM); *P* value indicated significance of the LSM difference between treatments.

Microarray and qRT-PCR data analysis. A population of control spots was present on each slide and was used to calculate population statistics of spot intensities and background region, using a 99% level of confidence. Normalization was conducted using Agilent G2567AA Feature Extraction software (version 6.1.1). A detailed description of algorithms that were utilized can be found at <http://www.chem.agilent.com/scripts/literaturePDF.asp?iWHID=37629>.

Differentially expressed genes identified by Rosetta Resolver were screened against our hypothesized genes of interest (Supplementary Table S1). An initial analysis for genes not included in our hypotheses was conducted, using Ingenuity Pathway software (Mountain View, CA). Analysis of the data sets (*day 5* and *day 14* epididymal signature genes) was conducted by increasing stringency with each iteration (2-fold change, *P* < 0.001). A detailed description of the software can be accessed at <http://www.ingenuity.com>. Additionally, signature gene lists from both time points were compared with each other to determine genes common to both data sets. These data sets were also compared with the GeneSpring (Silicon Genetics, Redwood, CA) database to identify genes associated with apoptosis, metabolism, and transcription factors.

Relative quantification was calculated by use of the $2^{-\Delta\Delta CT}$ method (37). The cycle threshold (C_T) values represented the cycle number at which fluorescence of the sample exceeded threshold (threshold was determined by multiplying the standard deviation of the baseline by 10).

RESULTS

Tissue Weight Changes Associated with t10c12-CLA

Time-dependent reductions in body weight of t10c12-CLA-fed mice vs. LA-fed mice were similar in obese and nonobese lines (line \times treatment interaction, *P* > 0.7; Fig. 3). Differences in body weights of t10c12-CLA- vs. LA-fed mice were detected after *day 5*, and weights diverged progressively to *day 14*.

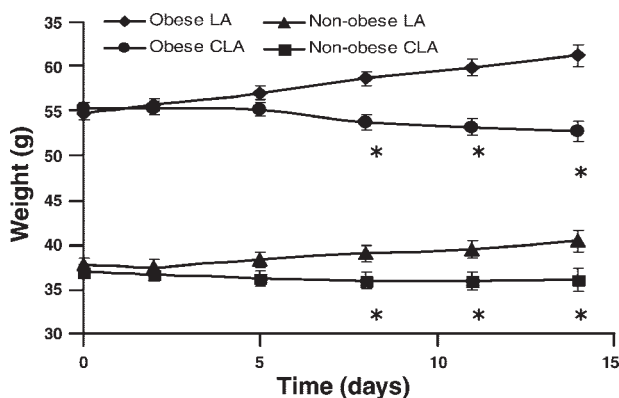


Fig. 3. Body weights of obese and nonobese mice fed either t10c12-CLA or LA. Values represent least square means \pm SE. Line main effect: *P* < 0.0001. Dietary treatment main effect: *P* < 0.0006. Line-by-treatment interaction: *P* > 0.7. *Differs from LA control at similar time and within genetic line.

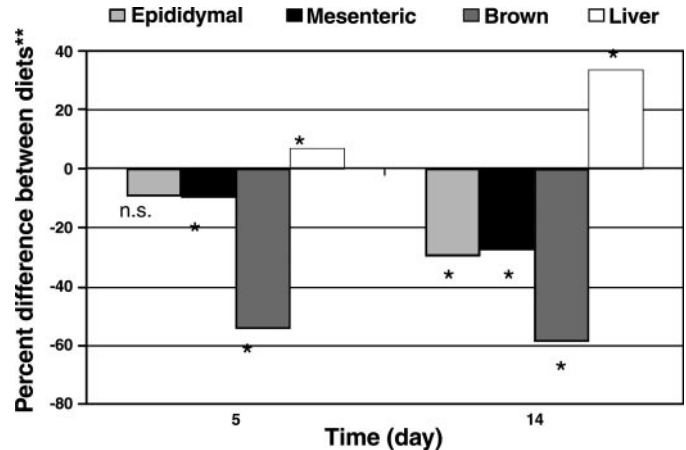


Fig. 4. Tissue weight differences (epididymal, mesenteric, brown adipose, and liver tissue) between t10c12-CLA- and LA-supplemented mice, expressed as a percentage of body weight. NS, not significant. *CLA effect: *P* < 0.0001. **100(CLA - LA)/LA.

Differences in feed intake between t10c12-CLA and LA-fed mice were observed after the second day (obese: 1.61 ± 0.24 g, *P* < 0.0001; nonobese: 0.70 ± 0.22 g, *P* < 0.05) and persisted throughout the trial. However, differences decreased as the trial progressed and by *day 14* were 1.19 ± 0.22 g (*P* < 0.0001) and 0.48 ± 0.21 g (*P* < 0.05) for obese and nonobese lines, respectively. To test whether reduced feed intake could account for reduced body weight (and fat pad mass), we conducted a covariance analysis of feed intake on body and tissue weights. After adjustment of the data for differences in feed intake, differences between the LA- and t10c12-CLA-fed groups remained, indicating that feed intake could not completely account for the difference in body weight (or fat mass).

The effect of t10c12-CLA on differences between tissue weights was similar in both lines of mice (line \times treatment, *P* > 0.05; Fig. 4) and increased progressively over the duration of the trial. Epididymal and mesenteric adipose tissues changed similarly, progressively decreasing in mice supplemented with t10c12-CLA. By *day 14* there was 30% less epididymal adipose tissue (*P* < 0.0001; Fig. 4) and 27% less mesenteric adipose tissue (*P* < 0.0001) in t10c12-CLA-fed mice. By *day 5* there was 54% (*P* < 0.0001) less brown adipose in t10c12-CLA-fed mice, and by *day 14* the difference increased marginally to 58% (*P* < 0.0001). There was an \sim 33% (*P* < 0.0001) increase in liver weight by *day 14* (Fig. 4), with 61% more fat present in the liver of t10c12-CLA-fed mice vs. LA-fed mice (*P* < 0.001; data not shown).

Fatty Acid Composition of Tissues

The t10c12-CLA isomer was only present in adipose depots of mice fed the t10c12-CLA-supplemented diet (Table 1). The ratio of 16:0/16:1 increased in all three fat pads of mice fed t10c12-CLA, driven largely by reductions in 16:1. However, no change in 18:0/18:1 was observed except for a decrease in brown adipose tissue (data not shown). Reduced 14:0 was observed in all three fat pads of t10c12-CLA-fed mice; however, both 18:2, *cis*-9, *cis*-12 and 20:1, *cis*-11 + 18:3, *cis*-9, *cis*-12, *cis*-15 decreased in epididymal (Table 1) and mesenteric adipose tissues (not shown) but increased in brown

Table 1. Fatty acid profile of epididymal adipose tissue of obese and nonobese mice fed either t10c12-CLA or LA for 14 days

Fatty Acid	Obese		Nonobese		P Values			
	LA	CLA	LA	CLA	Pooled SE	Mouse line	Dietary treatment	Line X treatment
14:0	1.61	1.30	2.32	1.13	0.15	0.0820	<0.0001	0.0071
16:0	18.93	20.13	19.73	19.80	0.54	0.6662	0.2474	0.2986
16:1, c9	6.32	4.66	5.62	2.95	0.31	0.0006	<0.0001	0.1141
18:0	1.98	2.00	2.38	3.44	0.15	<0.0001	0.0013	0.0018
18:1, t9	0.60	0.51	0.25	0.70	0.24	0.5262	0.1428	0.0288
18:1, c9	27.65	31.16	32.40	28.99	1.84	0.0011	<0.0001	0.0212
18:2, c9, 12	29.07	27.06	38.26	28.14	1.13	0.0001	<0.0001	0.0013
18:2, t10c12	n.d.	0.59	n.d.	0.87	0.71	0.0150		
20:1, c11 + 18:3, c9, 12, 15	2.34	1.65	3.09	1.98	0.13	0.0005	<0.0001	0.1365
			<i>Ratio</i>					
16:0/16:1	3.10	4.40	3.57	6.78	0.22	<0.0001	<0.0001	0.0002
18:0/18:1	0.06	0.06	0.10	0.10	0.01	<0.0001	0.8775	0.3031

Obese and nonobese values (g/100 g fatty acids) are least square means (LSM). P values indicate significance of LSM difference between treatments for each fatty acid. LA, linoleic acid; CLA, conjugated linoleic acid; c, cis; t, trans.

adipose tissue (not shown) of mice supplemented with t10c12-CLA.

Microarray and Pathway Analysis

Output from Rosetta Resolver resulted in 1,030 (4.9%) and 1,229 (5.9%) (see GEO accession no. GSE1580; <http://www.ncbi.nlm.nih.gov/geo/>) genes differentially expressed (P < 0.01) in day 5 and day 14 epididymal adipose tissue, respectively. Within these, 29 (0.14%) and 125 (0.60%) genes were expressed greater than or equal to twofold in epididymal adipose (days 5 and 14, respectively).

Comparisons of our signature gene data sets (all genes P < 0.01, output from Rosetta Resolver) for the epididymal fat pad with the GeneSpring database resulted in 15 genes matching GeneSpring’s apoptosis database on both day 5 and day 14 (Fig. 5A); 49 genes for day 5 and 75 genes for day 14 matched GeneSpring’s metabolism database (Fig. 5B), and 48 genes for day 5 and 38 genes for day 14 matched genes coding for transcription factors in the GeneSpring database (Fig. 5C). Although day 5 and day 14 epididymal fat pads both shared 15 genes with the GeneSpring apoptosis database, only 2 of the 15 were shared between days 5 and 14 (Table 2); 6 genes were shared between time points in the metabolism comparison (Table 2) and 5 in the transcription factor comparison (Table 2).

Ingenuity pathway analysis of day 14 epididymal adipose tissue yielded several genes of interest, including the peroxisome proliferator-activated receptor-γ (PPAR-γ), which has been previously reported to decrease in expression (22, 25, 31). Potentially novel CLA-gene interactions that resulted from this analysis included cav-1 (proposed to function in free fatty acid and triglyceride transport and storage) and casp-3 (part of the apoptotic pathway). Cav-1 was also present in the day 5 pathway analysis, implicating it as a potentially early responder to CLA treatment.

Table 3 includes genes identified by our analyses as differing among treatments (P < 0.01) and a comparison of the change in gene expression with our a priori hypotheses (listed in Supplemental Table S1). Overall, anti-apoptotic and lipid storage/transport genes had a tendency to be downregulated, whereas genes that were pro-apoptotic and involved in fatty

acid oxidation tended to be upregulated. While we did not observe a change in leptin mRNA concentration, the adipokines adiponectin and resistin were downregulated. In addition to a reduction in PPAR-γ expression, we observed a decrease in CCAAT/enhancer-binding protein-α (C/EBPα) and the retinoic acid receptor (RXR). We also observed a dramatic reduction (~7-fold) in mRNA concentration of the adipocyte marker adipsin. Results from our microarray analysis confirm observations that have been previously reported with glucose transporter-4 (GLUT4) (9, 54), perilipin (9), adiponectin (62), and uncoupling protein-2 (UCP-2) (16, 51, 54, 58, 63). Additionally, we propose potentially novel CLA-gene interactions in adipose tissue with cav-1, phosphofructokinase-2, pyruvate dehydrogenase, diacylglycerol acyltransferase, adipsin, resistin, B-cell lymphoma-2 (Bcl-2), casp-3, and cytochrome c (see Table 3 for a complete list).

The relative increase (days 5 and 14) in cav-1 and no change (day 5) or decrease (day 14) in casp-3 mRNA concentration was confirmed using qRT-PCR (Table 4). Cav-1 at day 5 was downregulated twofold (P < 0.001), and no significant difference was detected for casp-3, in congruence with the array data. We also found that cav-1 was downregulated at day 14 ~1.55-fold (P < 0.05), and that casp-3 was upregulated ~1.41-fold (P < 0.05) (Table 4). The qRT-PCR results were similar to the array results.

DISCUSSION

Phenotypic Effects

Effects of CLA on fat pad reduction in mice are well established; however, the mechanism of action remains unclear. The t10c12-CLA isomer appears to be solely responsible for reduction of fat pad weight (21, 27, 43, 45), and, as a result, the t10c12-CLA isomer was selected as a probe for this study. In agreement with previous reports (15, 27, 43, 56, 57), we observed reduced body weight gain in both lines of mice fed t10c12-CLA. Feed intake also was reduced in the t10c12-CLA-supplemented group (P < 0.0001), an issue that is a source of controversy in the literature, with some groups reporting little to no effect (3, 15, 56, 63) and others reporting a reduction (27, 51, 64). However, studies employing pair feeding (27, 51)



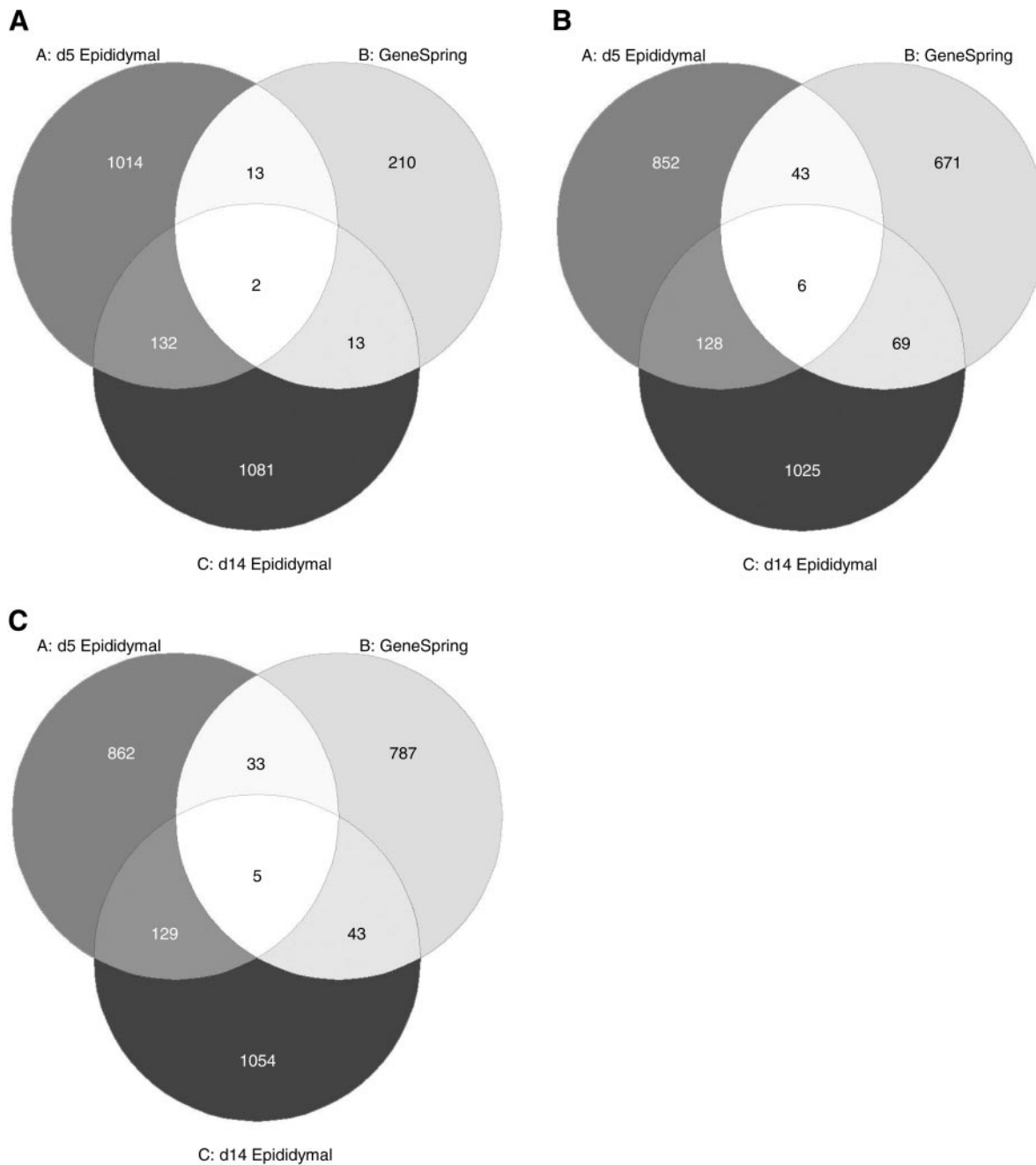


Fig. 5. Differential gene expression in epididymal white adipose tissue of obese mice elicited by 1% dietary CLA. Comparisons of “signature gene” data sets ($P < 0.01$), after 5 and 14 days of feeding, with the GeneSpring database of genes associated with apoptosis (A), metabolism (B), and transcription factor (C). d, Day. A: $A \cap B$, 15 genes (Supplemental Table S2a); $C \cap B$, 15 genes (Supplemental Table S2b); $A \cap C$, 134 genes (Supplemental Table S5); $A \cap B \cap C$, 2 genes (Table 2). B: $A \cap B$, 49 genes (Supplemental Table S3a); $C \cap B$, 75 genes (Supplemental Table S3b); $A \cap C$, 134 genes (Supplemental Table S5); $A \cap B \cap C$, 6 genes (Table 2). C: $A \cap B$, 38 genes (Supplemental Table S4a); $C \cap B$, 48 genes (Supplemental Table S4b); $A \cap C$, 134 genes (Supplemental Table S5); $A \cap B \cap C$, 5 genes (Table 2).

confirm a significant decrease in fat pad mass despite equal energy intake. Accordingly, covariance analysis of feed intake on body and tissue weights in our study yielded a similar finding, that the difference between the LA- and t10c12-CLA-fed groups remained, indicating that feed intake could not completely account for the loss in fat pad mass or difference in body weight. Although it is possible that CLA has an adverse organoleptic quality, it more likely alters metabolism in such a

way as to impart a reduction in feed intake. Interestingly, this effect was rapid, with reductions noted by *day 2*.

Our observations of adipose tissue reduction and increased hepatic lipid further confirm previous reports in the literature (13, 33, 56). Interestingly, t10c12-CLA had the same effect on adipose tissue and body weight in both lines of mice, indicating that t10c12-CLA effects are independent of genetic strain. Hargrave et al. (27) reported a similar observation in mice

Table 2. Differentially expressed genes in epididymal adipose of obese mice fed CLA

Gene Description*	Accession No.
<i>Apoptosis</i>	
TRB-3 (TRB-3)	BC012955
Bcl-2-related ovarian killer protein (Bok)	NM_016778
<i>Metabolism</i>	
Galactosidase, alpha (Gla)	NM_013463
Low density lipoprotein receptor-related protein-1 (Lrp1)	NM_008512
Malic enzyme, supernatant (Mod1)	NM_008615
ATP-binding cassette, subfamily D (ALD), member 2 (Abcd2)	NM_011994
Similar to carbonyl reductase-3, clone MGC:41226	BC028763
Acetyl-coenzyme A synthetase-1 (AMP forming) (Acas1)	NM_019811
<i>Transcription factors</i>	
SRY-box containing gene 6 (Sox6)	NM_011445
SRY-box containing gene 7 (Sox7)	NM_011446
Clone MGC:38361 IMAGE:5344900	BC027771
Notch gene homolog 4, (<i>Drosophila</i>) (Notch4)	NM_010929
Synuclein, alpha interacting protein (synphilin) (Sncaip)	NM_026408

*Identified genes were shared among *day 5* and *day 14* "signature gene" data sets ($P < 0.01$) and the GeneSpring database of genes associated with apoptosis, metabolism, and transcription factors.

selected for high or low energy expenditure. Miner et al. (40) reported a significant genetic line by treatment interaction in fasted mice; however, the authors noted that the interaction was small in magnitude and was not significant in refed mice.

Isomers and metabolites of CLA incorporate into phospholipid and neutral lipid fractions of different tissues (4). Studies analyzing effects of CLA on fatty acid composition in different tissues of several species have shown that it significantly alters fatty acid profiles. Excluding 16:0 and 16:1, we did not observe a significant difference in fatty acid levels between treatments (Table 1). In mice, lipogenesis occurs predominantly in liver tissue, which may partially explain the minor differences we observed. Also, net lipogenesis in these mice near maintenance is less likely than in growing and lactating animals, and this could impact effects on fatty acid profiles.

An increase in the ratio of palmitate to palmitoleate (16:0/16:1) and stearate to oleate (18:0/18:1) with t10c12-CLA supplementation is often observed (12, 17, 35), attributable to a reduction in stearoyl-CoA desaturase-1 (SCD-1) activity. Our results show an increase in the ratio of 16:0/16:1, but we were unable to detect a significant increase in 18:0/18:1. Because SCD-1 is predominantly expressed in liver tissue, our observations may be due to secondary effects of CLA interactions with the liver. A study conducted by Xu et al. (66) analyzing early effects of CLA (4 days) did not detect any changes in the ratios of 16:0/16:1 and 18:0/18:1 in adipose tissue, further indicating that this modulation may be due to downstream effects. A recent experiment using SCD-1 null mice showed that t10c12-CLA exerts its delipidative effects independently of SCD-1 (32); however, in the same study, control mice (SCD1^{+/+}) fed t10c12-CLA had an increase in the ratio of 16:0/16:1 and a decrease in ratio of 18:0/18:1. The authors suggest that the decrease in 18:0/18:1 is due to an increase in SCD-2 gene expression (predominantly expressed in brain and adipose tissue); they also report that t10c12-CLA had no effect on SCD-1 gene expression in adipose tissue. Although we did not detect a significant decrease in 18:0/18:1 in epididymal and mesenteric adipose tissues, results from our microarray analysis confirm these observations. That is, we detected an ~1.3-

fold induction in SCD-2 ($P < 0.05$; Table 3) expression in t10c12-CLA-fed mice, with no significant change in SCD-1 expression between treatment groups. The duration of the Kang et al. (32) study was 4 wk, whereas our experiment only lasted 2 wk, which may explain why we did not find a significant reduction in 18:0/18:1. Given these results, it seems probable that CLA is exerting its delipidative effects through multiple mechanisms.

Gene Expression Effects

To further explore mechanisms associated with t10c12-CLA supplementation, we conducted a functional genomic analysis of epididymal adipose tissue extracted on *days 5* and *14* of the trial from the obese line of mice. Following models previously proposed for potential CLA mechanisms (11, 43), we hypothesized that genes associated with lipid metabolism and apoptosis would be modulated by t10c12-CLA. Specifically, genes associated with lipogenesis (anabolic) and anti-apoptosis would be downregulated, and those involved with fatty acid oxidation, lypolysis (catabolic), and pro-apoptosis would be upregulated. Several studies have analyzed the expression of genes associated with these functions and are summarized in Supplemental Table S1. The microarray data confirmed most of our hypotheses and results previously reported. Additionally, our analyses identified novel genes that are modulated by t10c12-CLA supplementation.

The gene differentially expressed to the greatest degree in our analysis was UCP-1 (~12-fold) (Table 3); however, this is a gene that is predominantly expressed in brown adipose tissue, and the magnitude of increase in expression may be due to a low initial concentration, rendering it biologically unimportant. However, a study conducted by Tsukiyama-Kohara et al. (59) showed that eukaryotic translation initiation factor 4E-binding protein-1 (Eif4ebp1)^{-/-} mice had smaller white fat pads and increased metabolic rate, with no change in individual daily feed intake. Additionally, white adipose tissue adopted a multilocular appearance similar to that of brown adipocytes and expressed UCP-1 (~6-fold) (59). Eif4ebp1 is a gene that codes for eIF4E-binding protein-1 (4EBP1), part of a family of 4EBPs that bind to eIF4E, thereby preventing its association with eIF4F and preventing translation of a subset of genes, including the PPAR- γ coactivator-1 (PGC1) (59). We did not detect a significant change in PGC1, but we observed a reduction in Eif4ebp1 mRNA concentration (~1.5-fold reduction; Table 3). Furthermore, we observed a decrease in phosphatidylinositol 3-kinase (PI3-kinase) expression at *day 5* (~1.1-fold; Table 3). The PI3-kinase signaling pathway is a mechanism by which extracellular stimuli are able to alter eIF4F activity (24). The increase in UCP-1 coupled with a decrease in Eif4ebp1 expression, as well as the similar phenotypic response that we observed in our t10c12-CLA-fed mice, may indicate that CLA is working in part through an eIF4-dependent mechanism. Recently, Brown et al. (8) showed that one of the ways by which t10c12-CLA reduced triglyceride content in human adipocytes was through activation of the mitogen-activated protein kinase/extracellular signal-related kinase (MEK/ERK) signaling pathway. This was coupled with hypersecretion of IL-6 and IL-8, and they proposed that this induction occurred through the autocrine/paracrine actions of these adipokines (8). Although we did not detect a change in



Table 3. Comparison of microarray results with a priori hypotheses regarding the effects of t10c12-CLA on genes associated with lipid metabolism and adipocyte biology

Gene Name	Accession No.	Hypothesis	Observed Results (Fold Change, CLA vs. LA)	
			Day 5	Day 14
<i>Lipid metabolism</i>				
Glycerol-3-phosphate acyltransferase	NM_008149	↓		1.59
Diacylglycerol acyltransferase	NM_026713	↓		-1.24
Perilipin	AK031445	↓		-1.48
Caveolin	NM_007616	NA	-1.96	-1.71
Eukaryotic translation initiation factor 4E binding protein-1	NM_007918	NA	-1.77	-1.40
Phosphatidylinositol 3-kinase	NM_008839	NA	-1.33	
GLUT4	NM_009204	↓		-2.33
Phosphofructokinase-2	NM_133232	↓		-2.24
Phosphoenolpyruvate carboxykinase	NM_011044	NA		-1.52
Pyruvate dehydrogenase	NM_008810	↓	-1.49	
Acetyl-CoA carboxylase	BC022940	↓		1.44
Malic enzyme	NM_008615	↓	1.30	1.79
Isocitrate dehydrogenase-2	NM_008322	↓	-1.79	-2.41
Phospholipase A2	NM_012044	↑	1.54	3.13
Carnitine palmitoyl transferase I (L)	AF017175	↑		1.54
Carnitine palmitoyl transferase I (M)	NM_009948	↑		1.18
Acyl-carnitine translocase	NM_020520	↑		1.33
Uncoupling protein-1	NM_009463	↑		12.85
Uncoupling protein-2	NM_011671	↑	1.32	1.49
Stearoyl-CoA desaturase-2	NM_009128	NA		1.33*
<i>Adipokines</i>				
Adiponectin	NM_009605	↓		-3.68
Adipsin	NM_013459	↓		-7.79
Resistin	BB609634	↓		-2.81
IL-6	NM_031168	NA		1.76
<i>Transcription factors</i>				
PPAR-γ	NM_011146	↓		-1.70
Retenoic acid receptor	NM_009107	NA		-1.29
CAAT/enhancer binding protein-α	NM_007679	↓	-1.65	
<i>Apoptosis</i>				
TNF-α	NM_013693	↑	1.11*	1.36†
Cytochrome <i>c</i>	AK077566	↑		1.81
Caspase-3	NM_009810	↑		1.38
Bcl-2	NM_016778	↓	-1.58	-1.44

Fold changes are significant at $P < 0.01$, unless stated otherwise (blank space represents no significant change). L, liver; M, muscle; PPAR, peroxisome proliferator-activated receptor; NA, not applicable (genes not included in a priori hypotheses; see Supplemental Table S1); ↑, upregulated; ↓, downregulated. * $P < 0.05$. † $P = 0.07$.

IL-8 message, we found that IL-6 was upregulated in day 14 epididymal adipose tissue (Table 3). Therefore, t10c12-CLA may be imparting its effects through signal transduction mechanisms.

Our data showed a significant increase in message for enzymes that catalyze the initial steps of fatty acid biosynthesis, such as acetyl-CoA carboxylase at day 14 and malic

enzyme at days 5 and 14 (Table 3), but failed to show a change in fatty acid synthase. These data are in conflict with previously reported observations (9, 32, 36, 46, 58) and our hypothesis that t10c12-CLA works by decreasing lipogenesis. However, because lipogenesis in the mouse primarily occurs in the liver, with very little occurring in adipose tissue, it is probable that the delipidative effect of t10c12-CLA in the mouse may be

Table 4. Comparison of microarray and qRT-PCR results

Gene	Microarray Results (Fold Change)				qRT-PCR Results (Fold Change)			
	Day 5	P value	Day 14	P value	Day 5	P value	Day 14	P value
Caveolin-1	-1.96	<0.01	-1.71	<0.01	-2.00	<0.001	-1.49	<0.05
Caspase-3	-1.01	>0.05	1.38	<0.01	1.10	>0.05	1.41	<0.05

For microarray results, statistical significance was determined with Rosetta Resolver software for microarray data. For quantitative real-time RT-PCR (qRT-PCR) results, statistical significance was determined by a 1-sided *t*-test for qRT-PCR. Relative expression for qRT-PCR analysis was determined by the $2^{-\Delta\Delta CT}$ method (37).

working through mechanisms other than lipogenesis (at least in adipose tissue). Indeed, we observed that t10c12-CLA had a negative effect on glucose transport (GLUT4) in adipose tissue and transport of triglycerides and fatty acids for storage in lipid droplets (cav-1) (Table 3).

Studies conducted on 3T3-L1 adipocytes and human adipocytes have confirmed a reduction in the size of the lipid droplet upon CLA supplementation (8, 12, 31). Additionally, negative effects of t10c12-CLA on perilipin (protein associated with intracellular lipid droplets) would add credence to the suggestion that lipid droplet morphology is being compromised. Our analysis showed a decrease in cav-1 mRNA expression (Table 3). Adipose tissue has a high abundance of caveolae, accounting for ~30% of the surface area of an adipocyte (49). Razani et al. (49) showed that cav-1 null mice were resistant to diet-induced obesity despite being hyperphagic. Mice were unable to convert lipoprotein triglycerides to lipid droplet form for storage (49). Additionally, there may be a reciprocal relationship between cav-1 and the MEK/ERK pathway (34). Therefore, if t10c12-CLA stimulates MEK/ERK signaling, as proposed by Brown et al. (8), then this may be the mechanism by which cav-1 expression is reduced. This observation may indicate that t10c12-CLA reduces adipose tissue, at least partially, through a caveolin-dependent mechanism and adds credence to the proposal that it is working through the MEK/ERK signaling pathway. In contrast to Ryder et al. (51), we observed a decrease in phosphoenolpyruvate carboxykinase (PEPCK) mRNA concentration (Table 3). The presence of PEPCK may not be expected in adipose tissue, as it is predominantly expressed in liver and kidney. However, an experiment conducted on mice mutated to abolish expression of PEPCK in white adipose tissue showed they had reduced triglyceride deposition (42).

Collectively, it appears that the delipidative effects of t10c12-CLA in mice are occurring through reduced fatty acid and triglyceride translocation and storage, as well as decreased glucose availability, more so than affecting endogenous lipogenesis. Furthermore, CLA may reduce triglyceride content by increasing fatty acid oxidation (21, 44). This is further evident by an increase in the activity of carnitine palmitoyl transferase (CPT) (44, 48), a mitochondrial membrane-bound protein essential for shuttling long-chain fatty acids into the mitochondria where they undergo β -oxidation. Our results confirm these

observations on a genetic level (Table 3). We observed an increase in message for both liver and muscle isoforms of CPT-1 as well as an increase in acyl-carnitine translocase. Currently, the reported effects of CLA on lipolysis are conflicting. Several in vitro studies have reported an increase in lipolysis in response to CLA (9, 44); however, our data are in agreement with a study that reported no effect in vivo in mice (66). Therefore, we agree that fatty acid oxidation, not lipolysis, is indeed one mechanism by which t10c12-CLA imparts its delipidative effects.

Among genes associated with cell biology, we observed an increase in expression of tumor necrosis factor- α (TNF- α) and a decrease in expression of PPAR- γ , adipsin, resistin, and adiponectin (Table 3). Previous reports regarding the effect of CLA on adiponectin are inconsistent (41, 62). It has been shown that adiponectin levels are negatively correlated with BMI (1, 29, 30, 65); therefore, it may be expected that adiponectin levels would increase with weight loss. However, adiponectin mRNA is expressed predominantly in mature adipocytes, and it has been demonstrated that differentiation dramatically increases its expression (30). Negative effects of t10c12-CLA on preadipocyte differentiation are well established (7-9, 58) and are represented in this study by a reduction in PPAR- γ , RXR, and C/EBP- α mRNA concentrations (Table 3); therefore, a reduction in adiponectin expression may be a result of decreased differentiation. Additionally, adiponectin may increase insulin sensitivity (1, 30, 67); however, the opposite has been reported in mice fed t10c12-CLA (14, 58), and this may be due to decreased adiponectin expression. The precise mechanism of action of adiponectin remains unclear.

Several groups have reported an increase in apoptotic activity in adipose tissue upon t10c12-CLA supplementation (20, 27, 40, 57, 58); however, studies analyzing this potential interaction on a genetic level have been sparse. Our results confirm a report by Tsuboyama-Kasaoka et al. (57) that CLA increases TNF- α expression (Table 3). TNF- α is a cytokine that induces lipolysis and adipocyte dedifferentiation as well as apoptosis of pre- and mature adipocytes (47). A study using colon SW480 tumor cells showed that t10c12-CLA increased casp-3 activity, reduced Bcl-2 expression, and increased cytosolic cytochrome *c* (39). We show that t10c12-CLA works similarly in adipose tissue by increasing casp-3 and cytochrome *c* expression as well as by decreasing Bcl-2 expression

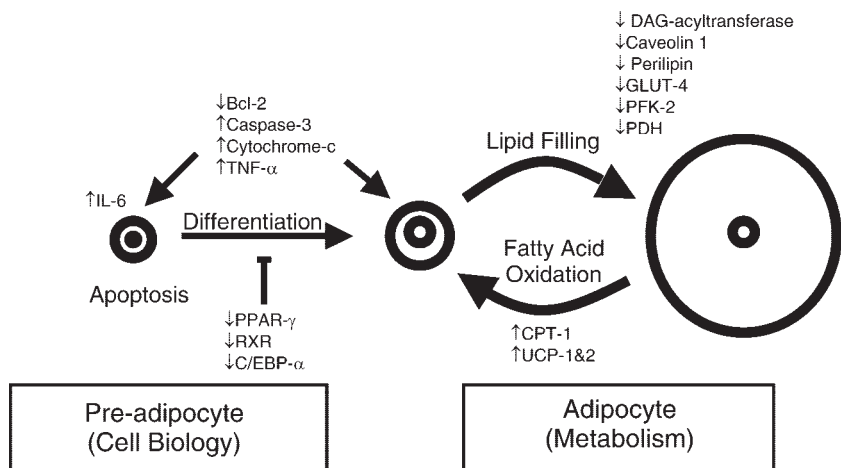


Fig. 6. Pleotrophic effects of t10c12-CLA on gene expression profile in murine white adipose tissue. Arrows indicate direction of message change after treatment with 1% dietary CLA. Bcl, B-cell lymphoma; PPAR, peroxisome proliferator-activated receptor; RXR, retinoic acid receptor; C/EBP, CCAAT/enhancer-binding protein; DAG, diacylglycerol; PFK, phosphofructokinase; PDH, pyruvate dehydrogenase; CPT, carnitine palmitoyl transferase; UCP, uncoupling protein.

(Table 3). Therefore, we confirm on a genetic level the increase in apoptosis reported by Miner et al. (40) and Hargrave et al. (27), based on internucleosomal DNA degradation measurements. It therefore seems probable that t10c12-CLA is additionally imparting its delipidative effects through an apoptotic mechanism.

In summary, we suggest that, at the genetic level, t10c12-CLA is imparting its pleiotropic anti-adipogenic effects by inhibiting preadipocyte differentiation and increasing fatty acid oxidation and apoptosis, as well as preventing storage and translocation of fatty acids and triglycerides into lipid droplets (Fig. 6). Additionally, it seems highly probable that these effects arise through t10c12-CLA induction of signal transduction mechanisms. We also propose that little if any effect is being contributed by induction of lipolysis or inhibition of de novo lipogenesis in murine white adipose tissue.

This experiment is unique in that, to our knowledge, it is the first to utilize functional genomic techniques to characterize gene expression during CLA-induced degradation of body fat. In addition to genes previously reported, this experiment has identified potentially novel gene candidates that are involved in mechanisms associated with lipid metabolism and apoptosis that are affected by dietary intake of t10c12-CLA. The long-range goal of this research is to better understand mechanisms associated with obesity and identify genes that may be targeted for pharmacological development. We believe that the power of the nutrigenomic approach, coupled with the potent effects of CLA presented herein, provide valuable insight toward achieving this goal.

ACKNOWLEDGMENTS

We thank Dr. Ben Corl for conducting the fatty acid analysis and Peggy Overton for coordinating the phenotypic data collection. BASF kindly donated the t10c12-CLA used in this experiment.

GRANTS

The North Carolina Agricultural Research Service supported this work.

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