

# Isomer-Dependent Metabolic Effects of Conjugated Linoleic Acid

## Insights From Molecular Markers Sterol Regulatory Element-Binding Protein-1c and LXR $\alpha$

Helen M. Roche,<sup>1</sup> Enda Noone,<sup>1</sup> Ciaran Sewter,<sup>2</sup> Siobhan Mc Bennett,<sup>3</sup> David Savage,<sup>2</sup> Michael J. Gibney,<sup>1</sup> Stephen O'Rahilly,<sup>2</sup> and Antonio J. Vidal-Puig<sup>2</sup>

Conjugated linoleic acid (CLA) is a heterogeneous group of positional and geometric isomers of linoleic acid. This study demonstrates the divergent effects of the *cis*-9 *trans*-11 (c9,t11-CLA) and *trans*-10 *cis*-12 (t10,c12-CLA) isomers of CLA on lipid metabolism and nutrient regulation of gene expression in *ob/ob* mice. The c9,t11-CLA diet decreased serum triacylglycerol ( $P = 0.01$ ) and nonesterified fatty acid (NEFA) ( $P = 0.05$ ) concentrations, and this was associated with reduced hepatic sterol regulatory element-binding protein-1c (SREBP-1c;  $P = 0.0045$ ) mRNA expression, coupled with reduced levels of both the membrane-bound precursor and the nuclear forms of the SREBP-1 protein. C9,t11-CLA significantly reduced hepatic LXR $\alpha$  ( $P = 0.019$ ) mRNA expression, a novel regulator of SREBP-1c. In contrast, c9,t11-CLA increased adipose tissue SREBP-1c mRNA expression ( $P = 0.0162$ ) proportionally to the degree of reduction of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) mRNA ( $P = 0.012$ ). Recombinant TNF- $\alpha$  almost completely abolished adipose tissue SREBP-1c mRNA expression *in vivo*. The t10,c12-CLA diet promoted insulin resistance and increased serum glucose ( $P = 0.025$ ) and insulin ( $P = 0.01$ ) concentrations. T10,c12-CLA induced profound weight loss ( $P = 0.0001$ ) and increased brown and white adipose tissue UCP-2 ( $P = 0.001$ ) and skeletal muscle UCP-3 ( $P = 0.008$ ) mRNA expression. This study highlights the contrasting molecular and metabolic effect of two isomers of the same fatty acids. The ameliorative effect of c9,t11-CLA on lipid metabolism may be ascribed to reduced synthesis and cleavage of hepatic SREBP-1, which in turn may be regulated by hepatic LXR $\alpha$  expression. *Diabetes* 51:2037–2044, 2002

From <sup>1</sup>Molecular Nutrition, Department of Clinical Medicine, Trinity Centre for Health Sciences, St. James's Hospital, Dublin, Ireland; the <sup>2</sup>Department of Clinical Biochemistry and Medicine, Addenbrooke's Hospital, University of Cambridge, Cambridge, U.K.; and the <sup>3</sup>Department of Physiology, Trinity College Dublin, Dublin, Ireland.

Address correspondence and reprint requests to Helen M. Roche, Department of Clinical Medicine, Trinity Centre for Health Sciences, St. James's Hospital, Dublin 8, Ireland. E-mail: hmroche@tcd.ie.

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BAT, brown adipose tissue; CLA, conjugated linoleic acid; DTT, dithiothreitol; FBS, fetal bovine serum; NEFA, nonesterified fatty acid; PUFA, polyunsaturated fatty acid; SREBP, sterol regulatory element-binding protein; TAG, triacylglycerol; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; WAT, white adipose tissue.

The novel fatty acid conjugated linoleic acid (CLA) may have potential as a therapeutic nutrient with respect to dyslipidemia and insulin resistance that is characteristic of the metabolic syndrome. CLA refers to the positional and geometric conjugated dienoic isomers of linoleic acid (C18:2 n-6). CLA is a natural food component found in the lipid fraction of meat and milk and other dairy products (1). The *cis*-9, *trans*-11 CLA isomer (c9,t11-CLA) is the principal dietary form of CLA, but lower levels of the other isomers (*trans*-10, *cis*-12 CLA [t10,c12-CLA], *trans*-9, *trans*-11 CLA, and *trans*-10, *trans*-12 CLA) are also present in CLA food sources (2). Animal feeding studies show that CLA reduces body fat accumulation and increases lean body mass in a dose-responsive manner that is not due to reduced energy intake (3–5). CLA also improves plasma lipid metabolism (6) and inhibits the progression of atherosclerosis (7) in rabbits. The effects of CLA on lipid and glucose metabolism are controversial. Feeding a 50:50 (t10,c12-CLA: c9,t11-CLA) blend of CLA isomers improved insulin sensitivity and glucose tolerance in Zucker diabetic *fa/fa* (ZDF) rats, but c9,t11-CLA had no effect (8,9). In contrast, a CLA-enriched diet induced insulin resistance and hyperlipidemia in C57BL/6J mice (10). In our study, we determined the isomer-specific metabolic effects of two CLA isomers (c9,t11-CLA and t10,c12-CLA) and explored the molecular mechanisms involved. The study was completed in *ob/ob* C57BL-6 mice, a well-characterized model of obesity, hyperlipidemia, and insulin resistance, previously used to characterize the biology of sterol regulatory element-binding protein (SREBP)-1c (11).

The SREBPs are members of the basic helix-loop-helix leucine zipper family of membrane-bound transcription factors that activate the genes involved in lipogenesis and insulin sensitivity (12,13). SREBPs are synthesized as membrane-bound precursor proteins, which become activated when the NH<sub>2</sub>-terminal domain is cleaved and translocates to the nucleus, where it binds to the enhancer promoter region of the target genes. The two isoforms SREBP-1a and -1c are derived from the same gene through

TABLE 1  
Fatty acid composition (wt/wt %) of the control (linoleic acid) and the c9,t11-CLA- and t10,c12-CLA-enriched diets

	Control	c9,t11-CLA	t10,c12-CLA
SFA	40.4	39.0	40.4
MUFA	32.6	34.1	32.6
PUFA	26.7	26.6	26.7
Free fatty acid composition of the PUFA fraction			
18: n-6	6.65	0.27	0.32
c9,t11 CLA	—	4.43	1.03
t10,c12 CLA	—	0.84	4.59
Other CLA isomers	—	0.11	0.30

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid.

the use of alternate transcription promoters that produce different forms of exon 1 (14). The SREBP-1 isoforms regulate fatty acid and triacylglycerol (TAG) synthesis (15). SREBP-1 is a key transcription factor that mediates nutrient regulation of hepatic lipogenesis. In the fed state, SREBP-1c mRNA and protein levels are increased (16), and in SREBP-1 gene knockout mice (SREBP-1<sup>-/-</sup>), there is no induction of lipogenic gene expression upon refeeding (17). Polyunsaturated fatty acids (PUFAs) reduce SREBP-dependent gene expression in CV-1 and Hep G2 cells in a dose-dependent manner, and this effect is greater with increasing chain length and degree of unsaturation of PUFAs (18). In vitro studies show that PUFAs reduce SREBP-1a and -1c mRNA and inhibit proteolytic cleavage of SREBP-1 (19). In vivo studies demonstrate that feeding PUFAs reduces the abundance of mature SREBP-1 in hepatic nuclear extracts and downregulates SREBP-1 responsive gene expression (20). Recent studies have suggested that hepatic SREBP-1c expression is dependent on the nuclear hormone receptor LXR (21) and requires endogenous LXR ligands (22). In vitro PUFAs inhibit SREBP-1c gene transcription by antagonizing the actions of LXRs (23).

The present study shows that two isomers of CLA (c9,t11-CLA and t10,c12-CLA) have opposite effects on lipid metabolism and insulin resistance in *ob/ob* C57BL/6 mice. The c9,t11-CLA isomer improved lipid metabolism, and this was associated with downregulation of hepatic SREBP-1c and LXR $\alpha$  mRNA expression and reduced levels of the nuclear SREBP-1 levels, whereas feeding the t10,c12-CLA isomer induced a profound diabetic state associated with hyperlipemia and had no effect on SREBP-1c and LXR $\alpha$  expression.

## RESEARCH DESIGN AND METHODS

**Animal experiment and nutritional intervention.** The animal feeding experiment was conducted at the BioResources Unit at Trinity College (Dublin, Ireland) according to good animal welfare protocols that comply with European legislation governing the use of animals in research. The mice were exposed to a 12-h light/12-h dark cycle and maintained at a constant temperature of 22°C. Twenty-four 6-week-old male *ob/ob* mice, purchased from Harlan UK, were fed the control diet for 7 days and then were randomly assigned to one of three dietary fatty acid treatments for 4 weeks. The diets contained 131 g fat/kg (30% energy); provided equal amounts of saturated fatty acids, monounsaturated fatty acids, and PUFAs; and were prepared by Unilever (Vlaardingen, the Netherlands), as previously described (24). The PUFA fraction of the control diet contained linoleic acid as free fatty acid, and the CLA-enriched diets contained an equivalent amount of the CLA isomers as free fatty acids (Table 1). The CLA was supplied by Looders Croklaan

(Wormerveer, the Netherlands). Feed consumption and mouse weight were measured weekly. The animals were killed in the fed state. The mice were killed and blood was collected by cardiac puncture. The blood was centrifuged, and the serum was harvested and stored (-20°C). White adipose tissue (WAT), brown adipose tissue (BAT), skeletal muscle, and liver samples were harvested. All tissue samples for RNA analysis were immersed in RNase Later (Ambion, AMS Technology, Cambridgeshire, U.K.), and tissue samples for protein analysis were snap-frozen in liquid nitrogen. All samples were stored at -70°C.

The effect of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) on SREBP-1c expression was investigated in male C57/BL6 mice. At 8:00 A.M., food was withdrawn and the mice were weighed (27.5–30 g). Four mice received an intraperitoneal injection (75  $\mu$ g/kg) of mouse recombinant TNF- $\alpha$  (Sigma-Aldrich, Dorset, U.K.) reconstituted (10  $\mu$ g/ml) in sterile saline. Two control mice received an intraperitoneal injection of an equivalent volume (0.225 ml) of saline. After treatment, the mice were segregated into control and treated cages with water and without food. After 6 h, the animals were killed by cervical dislocation, and the liver and WAT samples were immediately harvested. Tissue samples for RNA analysis were immersed in RNase Later, and tissue samples for protein analysis were snap-frozen in liquid nitrogen. All samples were stored at -70°C.

**Tissue culture.** Human mature adipocytes were isolated from fresh adipose tissue biopsies. Adipose tissue was diced finely and digested in a collagenase solution (Hanks' balanced salt solution containing 3 mg/ml type 2 collagenase (Sigma-Aldrich) and 1.5% BSA) for 1 h in a shaking water bath at 37°C. The mature adipocytes were separated from the stromovascular cells by centrifugation (10 min, 1,500g) of the digestion mixture over dionyl-phthalate oil. Mature adipocytes were cultured in Dulbecco's modified Eagle's medium/Ham's F12 plus 10% fetal bovine serum (FBS) at 37°C, 5% CO<sub>2</sub> for 48 h in the presence (10 ng/ml) or absence of human TNF- $\alpha$  (R&D Systems, Minneapolis, MN). HepG2 cells were grown to confluence in minimum essential medium (Sigma-Aldrich) containing 10% FCS, 100 units of penicillin, and 0.1 mg/ml streptomycin. TNF- $\alpha$  (10 ng/ml) was added to the medium. Control cells were maintained in medium minus TNF- $\alpha$ . After 24 h, total RNA was extracted. Human myoblast cells were grown to confluence in Ham's F10 medium (Gibco, Paisley, U.K.) containing 20% FBS, 100 units of penicillin, and 0.1 mg/ml streptomycin. Differentiation was induced by replacing the medium with  $\alpha$ -minimum essential medium (Gibco) containing 2% FBS, 100 units of penicillin, and 0.1 mg/ml streptomycin. After 15 days postdifferentiation, TNF- $\alpha$  (10 ng/ml) was added to the medium. Control cells were maintained in medium minus TNF- $\alpha$ . After 24 h, total RNA was extracted.

**Biochemical analysis.** Serum TAG (TAG PAP, bioMerieux, Lyon, France), cholesterol (Cholesterol PAP, bioMerieux), glucose (Glucose PAP, bioMerieux), and NEFA (NEFA, Randox Laboratories, Antrim, U.K.) concentrations were determined using enzymatic colorimetric assays on a Technicon RA-XT analyser (Bayer, Dublin, Ireland) according to the manufacturer's instructions. Serum insulin concentrations were measured using a Rat Insulin ELISA kit (Crystal Chem, Chicago, IL) according to the manufacturer's instructions.

**mRNA expression analysis.** Total RNA was extracted from animal tissues and cultured cells using the RNeasy mini extraction kit (Qiagen, West Sussex, U.K.). RNA integrity was assessed with agarose gel electrophoresis and ethidium bromide staining. The RNA sample was quantified by spectrophotometry, diluted to a concentration of 100 ng/ $\mu$ l in RNase-free water, snap-frozen, and stored (-80°C). For synthesizing cDNA, 100 ng of RNA was reverse-transcribed for 1 h at 37°C in a 20- $\mu$ l reaction containing 1 $\times$  RT buffer (50 mmol/l Tris-HCl, 75 mmol/l KCl, 3 mmol/l MgCl<sub>2</sub>, and 10 mmol/l dithiothreitol [DTT]), 100 ng random hexamers, 1 mmol/l dNTPs, and 100 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Reactions in which RNA was omitted served as a negative control. A reaction that contained 400 ng of total RNA was used as the standard. After first-strand cDNA synthesis, this standard was serially diluted 1:2 in RNase-free water to generate a standard curve for the PCR analysis. The accession numbers and sequences of the primers and probes for the target genes are presented in Table 2. Oligonucleotide primers and TaqMan probes were designed using Primer express (version 1.0 Perkin-Elmer Applied Biosystems, Warrington, U.K.). TNF- $\alpha$  mRNA expression was measured using a predeveloped kit (Perkin-Elmer Applied Biosystems). RNA expression was quantified by real-time quantitative PCR in duplicate on an ABI 7700 sequence detection system (TaqMan, Perkin-Elmer Applied Biosystems). Each 25- $\mu$ l reaction contained 2  $\mu$ l of first-strand cDNA, 1 $\times$  PCR Mastermix (Promega), forward and reverse primers, and TaqMan probe. All reactions were performed using the following cycling conditions: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Target gene mRNA expression was normalized to that of the control group and expressed as a proportion of the internal control glyceraldehyde-3-phosphate dehydrogenase (Perkin-Elmer

TABLE 2  
Accession numbers and primer and probe sequences of the target genes

Target gene (accession no.)	Forward primer sequence	Reverse primer sequence	Probe sequence
LXR $\alpha$ (AJ132601)	TCGCAAAATGCCGCCA	TCAAGCGGATCTGTTCTTCTGA	CAGGCATGAGGGGAGGAGTGTGTGCT
SREBP-1a (Shimomura et al., 1997)	CGGTTTTGAACGACATCGAA	TCAAATAGGCCAGGGAAGTCA	ACATGCTTCAGCTCATCAACAACCAAGACA
SREBP-1c (L16995.1)	GCCATGGATTGCACATTGA	TCAAATAGGCCAGGGAAGTCA	ACATGCTTCAGCTCATCAACAACCAAGACA
UCP-2 (U69135)	GGGCCCTCTGGCCCGGGACT	ACCAGCTCAGCACAGTTGACA	CTCCCAAATGTTGCCCGTAAATGCCA
UCP-3 (AF032902)	CCCTGCACTACCCCAACCTTG	GCTTGCCTGGCAATCTTTTG	CGCACAGCTTCCCTCCCTGAACTGAA

Applied Biosystems). Reproducibility of the PCR was examined by measuring the Ct readings of five samples in two different assays. Intra- and interassay coefficients of variation ranged from 0.19 to 4.2% and 0.54 to 3.0%, respectively.

For DNA microarray analysis, 10  $\mu$ g of pooled RNA from a subcohort ( $n = 3$ ) of mice from each dietary group was transcribed into double-stranded cDNA (SuperScript choice system, Gibco) using an oligo-dT primer containing a T7 RNA polymerase promoter. Biotin-labeled cRNA was generated by *in vitro* transcription using the ENZO BioArray RNA labeling kit (Affymetrix UK, High Wycombe, U.K.). cRNA samples were hybridized against Affymetrix Murine Genome 74Av2 arrays (Affymetrix UK). Detection via a streptavidin-labeled fluorochrome with laser scanning was completed according to Affymetrix protocols. Normalization of data for interarray comparisons of gene expression was completed using Affymetrix GeneChip analysis software.

**SREBP-1 immunoblot analysis.** The cytosolic and nuclear protein fractions were isolated as previously described (25) from liver samples. Briefly, 200–400 mg of tissue was chopped finely in 1.5 ml of cold lysis buffer (10 mmol/l Tris-HCl at pH 7.4, 10 mmol/l NaCl, 3 mmol/l MgCl<sub>2</sub>, 0.5% NP-40) with 10  $\mu$ l/ml protease inhibitors (ABESF, Aprotinin, Leupeptin, Bestatin, Pepstatin A, E-64 [Protease Inhibitor Cocktail]; Sigma-Aldrich) and with 10  $\mu$ l/ml phosphatase inhibitors (Na-orthovanadate, Na-molybdate, Na-tartrate, Imidazole [Phosphatase Inhibitor Cocktail 2]; Sigma-Aldrich). Samples were incubated on ice for 15 min and disrupted three times for 10 s at 13,000 rpm with a polytron homogenizer (ULTRA-TURRAX T18 homogenizer, IKA Lab Technology, Lennox, Dublin, Ireland). The homogenate was centrifuged at 2,000 rpm (500g) at 4°C for 10 min in a Beckman TLX-100 ultracentrifuge (Beckman Instruments, Mason Technology, Dublin, Ireland) to pellet the nuclei. Nuclear extracts were obtained by resuspending the nuclear pellets in the nuclear extraction buffer (10 mmol/l HEPES at pH 7.4, 420 mmol/l NaCl, 1.5 mmol/l MgCl<sub>2</sub>, 2.5% glycerol, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT) with 10  $\mu$ l/ml protease inhibitors and 10  $\mu$ l/ml phosphatase inhibitors. Membrane fractions were obtained from the supernatants (membrane/cytosolic extracts) by centrifugation at 75,000 rpm at 4°C for 30 min. The pelleted membrane fractions were resuspended in the membrane resuspension buffer (10 mmol/l Tris-HCl at pH 6.8, 100 mmol/l NaCl, 1% SDS, 1 mmol/l EDTA, 1 mmol/l EGTA) with 10  $\mu$ l/ml protease inhibitors and with 10  $\mu$ l/ml phosphatase inhibitors. Protein concentration of the nuclear extract and membrane fraction was determined using the Bradford assay and quantified at wavelength of 595 nm (Eppendorf Biophotometer, Lennox, Dublin, Ireland). Protein (100  $\mu$ g) from the nuclear extracts and membrane fractions were mixed with 5 $\times$  SDS loading buffer (1 $\times$  loading buffer contains 30 mmol/l Tris-HCl at pH 7.4, 3% SDS, 5% [vol/vol] glycerol, 0.004% [vol/vol] bromophenol blue, 2.5% [vol/vol] b-mercaptoethanol). After boiling for 5 min, aliquots of protein were subjected to 6% SDS-PAGE and transferred onto nitrocellulose membranes. Gels were calibrated with a prestained protein molecular weight marker (BioLabs, Hertfordshire, U.K.). The nitrocellulose membrane was incubated with blotto A (TBS-Tween 0.05) for 2.5 h, then incubated with a 1:100 dilution of rabbit polyclonal SREBP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were washed six times for 10 min in 0.5% TBS-Tween, then incubated with a 1:2,500 dilution of the monoclonal anti-rabbit IgG secondary body (Santa Cruz Biotechnology) for 1.5 h at room temperature. The membranes were washed six times for 10 min in 0.5% TBS-Tween. The horseradish peroxidase was developed after incubation of the membrane in luminescence solution (12 mg of luminol [Sigma-Aldrich], 4 mg of P-iodophenol [Sigma-Aldrich], 0.5 ml of DMSO [BDH], 0.5 ml of H<sub>2</sub>O<sub>2</sub> [Sigma-Aldrich], 50 ml of 100 mmol/l Tris [pH 8.8]) and detected by luminography and developed onto a Kodak film.

**Statistical analysis.** Statistical analyses were performed with Data Desk 4.1 (Data Description, Ithaca, NY). The data were transformed to the natural log (ln) to give the data a normal Gaussian distribution as required. One-way ANOVA, with dietary intervention as the independent variable, was used to investigate differences between treatments. Post hoc statistical analysis was performed using the Scheffe test to identify the significance of the individual dietary treatments. All significant comparisons made are relative to the control group.

## RESULTS

**C9,t11-CLA improves lipid metabolism, whereas the t10,c12-CLA induces insulin resistance and weight loss.** The effects of the CLA isomers on indexes of postprandial lipid and glucose metabolism are presented in Table 3. The c9,t11-CLA diet significantly reduced serum TAG ( $P = 0.0132$ ) and NEFA ( $P = 0.05$ ) concentrations. In contrast, mice fed with t10,c12-CLA isomer became severely insulin resistant, with marked increases in serum

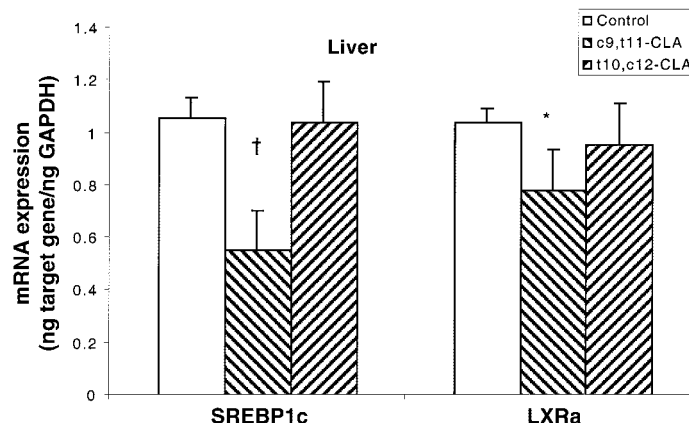
**TABLE 3**  
Effect of the control (linoleic acid), c9,t11-CLA, and t10,c12-CLA treatments on serum lipid, glucose, and insulin metabolism

	Control (n = 8)	c9,t11-CLA (n = 8)	t10,c12-CLA (n = 8)
TAG (mmol/l)	1.30 $\pm$ 0.21	0.95 $\pm$ 0.29 <sup>†</sup>	1.33 $\pm$ 0.36
NEFA (mmol/l)	1.07 $\pm$ 0.19	0.83 $\pm$ 0.09*	1.03 $\pm$ 0.10
Cholesterol (mmol/l)	7.76 $\pm$ 0.78	7.61 $\pm$ 0.72	8.20 $\pm$ 0.74
Glucose (mmol/l)	14.07 $\pm$ 6.54	17.15 $\pm$ 4.81	21.09 $\pm$ 4.88*
Insulin (ng/ml)	2.05 $\pm$ 0.17	3.39 $\pm$ 0.65	15.43 $\pm$ 4.42 <sup>†</sup>

Data are group means  $\pm$  SD. Significantly different from the control diet \* $P < 0.05$ ; <sup>†</sup> $P < 0.01$ .

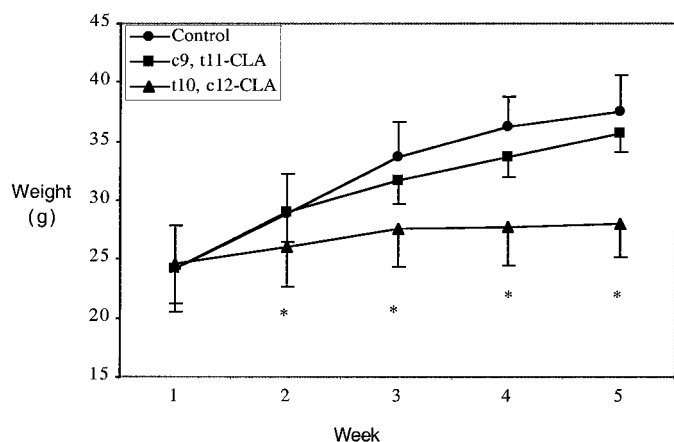
glucose ( $P = 0.025$ ) and insulin ( $P = 0.01$ ) concentrations. It is interesting that serum TAG and NEFA levels were not further increased in the t10,c12-CLA fed *ob/ob* mice despite their marked insulin resistance and severe diabetes. The growth curves (Fig. 1) show that the t10,c12-CLA isomer caused significant ( $P = 0.0001$ ) inhibition of weight gain. The t10,c12-CLA isomer also significantly ( $P = 0.0069$ ) reduced epididymal adipose tissue fat pad weight ( $1.78 \pm 0.16$  g), compared with the control and c9,t11-CLA-fed mice ( $2.30 \pm 0.25$  g and  $2.25 \pm 0.28$  g, respectively).

**Effect of CLA isomers on molecular markers of lipogenesis.** We next explored the potential molecular mechanism behind the alterations in fatty acid metabolism. Mice fed the c9,t11-CLA isomer had reduced hepatic SREBP-1c mRNA expression ( $P = 0.0045$ ), whereas the t10,c12-CLA isomer had no effect (Fig. 2). Hepatic SREBP-1a mRNA expression was five- to eightfold less abundant than the SREBP-1c isoform, and its expression was not significantly altered by either CLA isomer. To explore further the mechanism of SREBP-1c gene regulation, we examined the level of LXR $\alpha$  mRNA, one of the transcription factors known to regulate the expression of SREBP-1c. Using real-time PCR-based quantification, we observed that c9,t11-CLA also reduced hepatic LXR $\alpha$  mRNA expression ( $P = 0.015$ ; Fig. 2). Furthermore, DNA microarray analysis of liver samples from a subcohort ( $n = 3$ ) of mice from each dietary group confirmed that c9,t11-CLA caused an equivalent reduction ( $-1.3$ -fold) in hepatic LXR $\alpha$  expression. DNA microarray analysis also

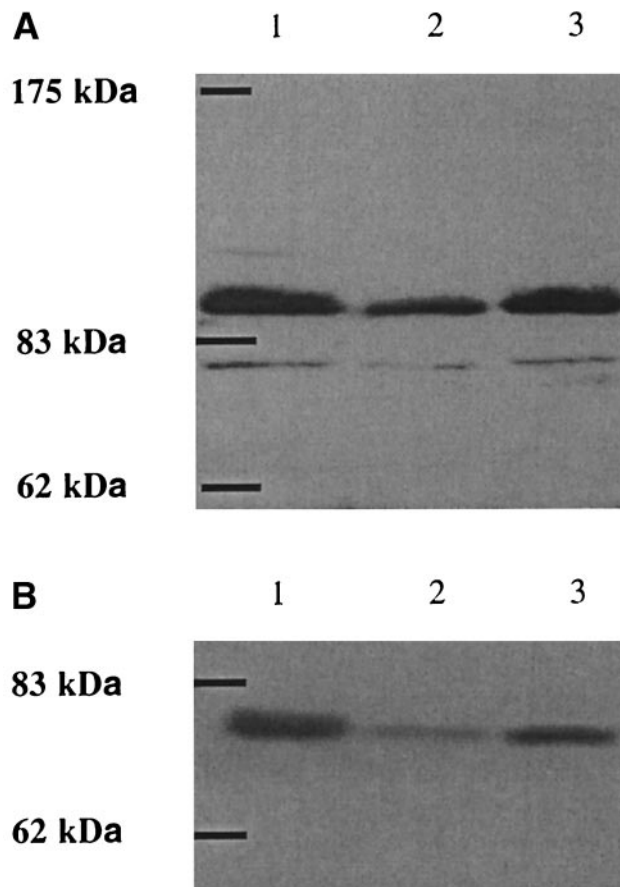


**FIG. 2.** The effect of the control (linoleic acid), c9,t11-CLA and t10,c12-CLA diets on hepatic SREBP-1c and LXR $\alpha$  mRNA expression in male *ob/ob* mice. Values represent group means  $\pm$  SE. Significantly different from the control diet \* $P < 0.05$ ; <sup>†</sup> $P < 0.01$ .

showed that C9,t11-CLA but not the t10,c12-CLA isomer downregulated the expression of another LXR $\alpha$  responsive gene, hepatic ABCA1 ( $-1.6$ -fold); this reduction was equivalent to that for SREBP-1c. Western blot analysis showed that c9,t11-CLA reduced the levels of both the membrane-bound precursor form and the nuclear form of the SREBP-1 protein, suggesting potential regulation of the synthesis and cleavage of SREBP-1 (Fig. 3). We then



**FIG. 1.** The effect of the control (linoleic acid), c9, t11-CLA and t10, c12-CLA diets on weight gain during the 4-week dietary intervention period in male *ob/ob* mice. Values represent group means  $\pm$  SD. Significantly different from the control diet \* $P < 0.0001$ .



**FIG. 3.** Western blot analysis of hepatic cellular membrane precursor (A) and nuclear SREBP-1 protein (B) after the control (linoleic acid) (lane 1), c9,t11-CLA (lane 2), and t10,c12-CLA (lane 3) diets in male *ob/ob* mice.

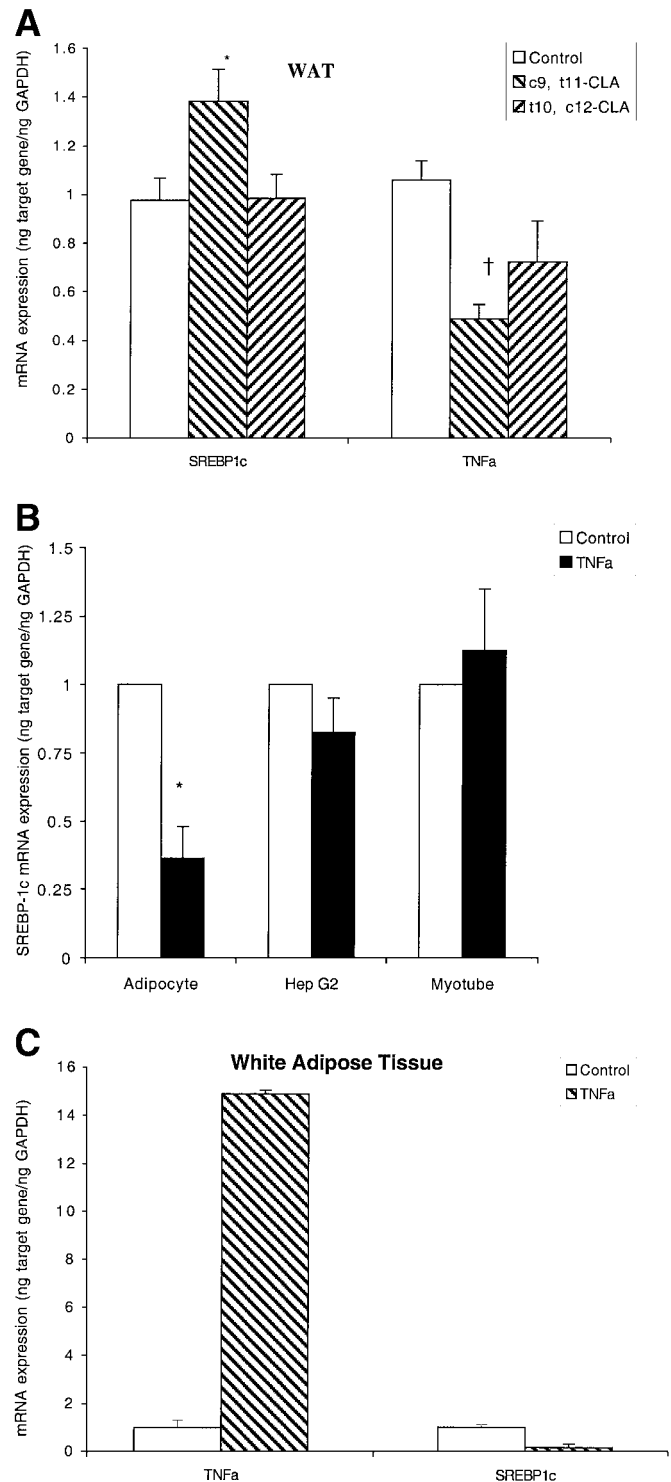
explored the effect of c9,t11-CLA on SREBP-1c in WAT. Whereas c9,t11-CLA markedly reduced hepatic SREBP-1c mRNA, WAT SREBP-1c mRNA expression was significantly increased ( $P = 0.0162$ ; Fig. 4A). This divergent effect of c9,t11-CLA on SREBP-1c mRNA expression in liver and WAT suggest tissue-specific regulation of SREBP-1 expression.

Because other studies have suggested that TNF- $\alpha$ , a known modulator of insulin sensitivity, directly regulates SREBP-1c, we explored whether the effects of CLA on SREBP-1c may be related to changes in TNF- $\alpha$  in liver and WAT. We observed that c9,t11-CLA isomer significantly reduced TNF- $\alpha$  mRNA expression in liver ( $P = 0.0081$ ) and WAT ( $P = 0.012$ ; Fig. 4A). SREBP-1c mRNA expression was inversely related to TNF- $\alpha$  mRNA expression in WAT ( $r = -0.447$ ;  $P = 0.0132$ ). This relationship was not observed in the liver. In vitro TNF- $\alpha$  treatment (10 ng/ml for 48 h) significantly reduced SREBP-1c mRNA expression ( $P = 0.01$ ) in mature adipocytes. This effect seems to be adipocyte-specific because TNF- $\alpha$  treatment (10 ng/ml for 48 h) had no effect on SREBP-1c mRNA expression in HepG2 cells or mature myotubes (Fig. 4B). To investigate further whether TNF- $\alpha$  had a direct effect on WAT SREBP-1c in vivo, mice received an intraperitoneal dose of recombinant TNF- $\alpha$ . In WAT, recombinant TNF- $\alpha$  administration almost completely obliterated SREBP-1c mRNA expression (Fig. 4C).

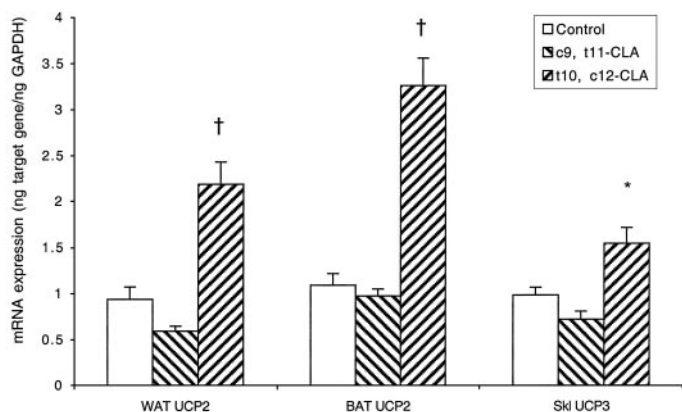
**Effect of CLA on molecular markers of mitochondrial energy expenditure.** The marked catabolic effects of the t10,c12-CLA diet prompted investigation of the mitochondrial uncoupling proteins. Previous studies have shown that a blend of CLA increases oxygen consumption and energy expenditure (5,10). The marked weight loss after the t10,c12-CLA diet could not be ascribed to reduced feed intake. Mean daily feed intake for the c9,t11-CLA and t10,c12-CLA groups was  $55.40 \pm 3.27$  g/d and  $55.34 \pm 4.17$  g/d, respectively. The t10,c12-CLA isomer induced UCPs mRNA expression in an isoform- and tissue-specific manner (Fig. 5). The t10,c12-CLA diet significantly increased UCP-2 mRNA expression in WAT, BAT ( $P = 0.001$ ), and liver ( $P = 0.007$ ; data not shown) and UCP-3 mRNA expression in skeletal muscle ( $P = 0.0008$ ). In contrast, the diet enriched with the c9,t11-CLA isomer did not effect UCP expression.

## DISCUSSION

Dyslipidemia and insulin resistance are common features associated with type 2 diabetes and cardiovascular disease. Recently, several pharmacologic agents known to activate nuclear receptors have been designed to treat the metabolic syndrome. Thus, identifying novel nutrients that could improve lipid and glucose metabolism are an attractive strategy. The biological nature of the effect of CLA in vivo is controversial (26). Our study shows that the divergent metabolic effects of the different CLA isomers may partly account for this. In this article, we demonstrate that the beneficial effects of CLA on lipid metabolism, lower serum TAG and NEFA levels, are restricted to the c9,t11-CLA isomer, whereas the t10,c12-CLA isomer inhibited weight gain, reduced adipose tissue mass, and induced a prodiabetic state. Our study concurs with Ryder et al. (9), who showed that feeding a blend of the two CLA



**FIG. 4.** The relationship between SREBP-1c and TNF- $\alpha$ . **A:** The divergent effects of the control (linoleic acid), c9,t11-CLA and t10,c12-CLA diets on SREBP-1c and TNF- $\alpha$  mRNA expression in WAT in male *ob/ob* mice. Values represent group means  $\pm$  SE. Significantly different from the control diet \* $P < 0.02$ ; † $P < 0.01$ . **B:** The effect of TNF- $\alpha$  (10 ng/ml for 24 h) on SREBP-1c mRNA expression in human adipocytes, HepG2 cells, and human myotubes. Values represent group means  $\pm$  SE of six independent experiments. Significantly different from the control \* $P < 0.01$ . **C:** The effect of murine recombinant TNF- $\alpha$  administration (75  $\mu$ g/kg) on TNF- $\alpha$  and SREBP-1c mRNA expression in WAT. Values represent group means  $\pm$  SE of four independent experiments.



**FIG. 5.** The effect of control (linoleic acid), c9,t11-CLA, and t10,c12-CLA diets on UCP mRNA expression in WAT and BAT and skeletal (skl) muscle in male *ob/ob* mice. Values represent group means  $\pm$  SE. Significantly different from the control diet \* $P = 0.001$ .

isomers (t10,c12-CLA and c9,t11-CLA) improved lipid and glucose metabolism in ZDF rats. Also, that study showed that the c9,t11-CLA isomer alone improved TAG metabolism (9). In contrast, when another group fed C57BL/6J mice a similar blend of CLA isomers, the diet induced a lipotrophic diabetic state (10). Our study shows that this effect is attributable to the t10,c12-CLA isomer. It is probable that the divergent effects of CLA on insulin and glucose metabolism previously reported (9,10) reflects the different metabolic consequences of the anti-obesity effect of t10,c12-CLA between animal models. In the ZDF rat, hyperglycemia is secondary to obesity; therefore, the anti-obesity action of t10,c12-CLA would have partly accounted for improved glucose metabolism. In contrast, in the C57BL/6J mouse, the anti-obesity action of t10,c12-CLA resulted in lipodystrophy that probably induced the prodiabetic state.

Among the several molecular markers evaluated, feeding the novel PUFA (c9,t11-CLA) was associated with lower hepatic SREBP-1c mRNA expression, lower levels of its regulator LXR $\alpha$ , and reduced nuclear levels of the mature SREBP-1 protein. SREBP-1c is a key regulator of hepatic lipogenesis that is modulated by nutritional status and metabolic factors (12,16,17,27). SREBP-1 is also involved in adipogenesis; however, its metabolic role in mature adipocytes is unclear (28). The regulatory effects of PUFA on SREBP-1c and LXR expression have been shown in several recent articles. Shimomura et al. (10) showed that insulin promotes hepatic lipogenesis via SREBP-1c. It has been demonstrated that treating isolated hepatocytes with the PUFA arachidonic acid was sufficient to block insulin-dependent induction of SREBP-1 expression (29). That study also showed that the antilipogenic effect of PUFA is primarily mediated via SREBP-1c (29). A comprehensive investigation of the effect of fatty acids on SREBP-1 showed that PUFA reduced mRNA encoding for both SREBP-1a and SREBP-1c and reduced the precursor and nuclear forms of the SREBP-1 protein in HEK-293 cells (19). Hepatic SREBP-1c mRNA expression requires endogenous LXR ligands (22). It was shown recently that PUFAs inhibit SREBP-1c transcription by inhibiting the activation of LXR in cultured hepatocytes (23). While a SREBP-1c/LXR $\alpha$  knockout mouse model is required to prove that c9,t11-CLA improves TAG and NEFA metabolism in vivo

via this pathway. Nevertheless, our results are highly suggestive in that we show that feeding different isomeric conformations of CLA has different effects on hepatic LXR $\alpha$  mRNA, SREBP-1c mRNA, and SREBP-1 protein expression, whereby feeding c9,t11-CLA reduces their expression, and this is associated with improved TAG and NEFA metabolism.

Our data also indicate that SREBP-1c could be differentially regulated in adipose tissue and liver. Increased adipocyte SREBP-1c expression after the c9,t11-CLA diet is likely to promote fatty acid synthesis and storage as TAG, thereby contributing to lower circulating NEFA and TAG levels. Adipose tissue-specific regulation of SREBP-1c may also be related to different sensitivity of the insulin signaling network to effects of TNF- $\alpha$  (30). Given that CLA reduces macrophage TNF- $\alpha$  expression (31), we explored the potential involvement of TNF- $\alpha$  on SREBP-1c expression. In adipose tissue, we observed a strong inverse relationship between SREBP-1c and TNF- $\alpha$  mRNA expression. In vitro, TNF- $\alpha$  decreases adipocyte SREBP-1c expression (32), and we confirm that this effect is adipocyte-specific because TNF- $\alpha$  has no effect on SREBP-1c mRNA expression in HepG2 cells or human myotubes. Furthermore, we show in vivo that recombinant TNF- $\alpha$  administration almost completely abolished SREBP-1c expression in adipose tissue. Hence, the increase in adipocyte SREBP-1c mRNA expression after the c9,t11-CLA diet may have been associated with reduced adipocyte TNF- $\alpha$  expression and increased insulin sensitivity. It has been suggested that the antilipogenic action of metformin, which reduced hepatic SREBP-1 DNA binding, FAS mRNA expression, and fatty acid synthesis in *ob/ob* mice, was due to lower hepatic TNF- $\alpha$  expression (33). Because TNF- $\alpha$  may have a prolipogenic action in the liver and the c9,t11-CLA diet reduced hepatic TNF- $\alpha$  mRNA, we speculated that TNF- $\alpha$  could have reduced hepatic SREBP-1c expression. However, in vitro studies demonstrate that TNF- $\alpha$  treatment had no effect on SREBP-1c mRNA expression in HepG2 cells. Also in comparison to WAT, recombinant TNF- $\alpha$  administration had little effect on hepatic SREBP-1c expression. This suggests that the effect of c9,t11-CLA on hepatic SREBP-1c-mediated lipogenesis was not mediated by TNF- $\alpha$ .

Our study confirms that only the t10,c12-CLA isomer affects body composition in *ob/ob* mice. The anti-obesity effect of CLA has been ascribed to reduced adipocyte size (34), inhibition of adipocyte proliferation (35), increased adipocyte lipolysis (3) and apoptosis (10), and greater fatty acid oxidation and energy expenditure (5). We show that feeding t10,c12-CLA significantly increased WAT (twofold) and BAT (threefold) UCP-2 and skeletal muscle UCP-3 mRNA expression. UCP-2 and UCP-3 gene expression are induced in situations in which partitioning of energy is directed toward fatty acid oxidation (36). In fact, mitochondrial uncoupling reduces (four- to fivefold) fatty acid synthesis and increases (1.5-fold) oxidation (37). Also, overexpression of UCP-3 in skeletal muscle protects against diet-induced obesity (38). Thus upregulation of UCP-2 and UCP-3 in animals fed t10,c12-CLA reflects a situation of preferential fatty acid oxidation.

In summary, we show that two different isomers of CLA, c9,t11-CLA and t10,c12-CLA, have marked differences on

lipid metabolism, body composition, and gene expression. Feeding the c9,t11-CLA isomer significantly improved lipid metabolism, which may be due to isomer-specific effects on hepatic SREBP-1c and LXR $\alpha$  expression. The c9,t11-CLA isomer also increased adipose tissue SREBP-1c expression, which was associated with lower TNF- $\alpha$  expression. In contrast, feeding a diet enriched with the t10,c12-CLA isomer inhibited fat deposition in this model of obesity; however, it also promoted insulin resistance and hyperlipidemia. Finally, our results illustrate that isomeric modification of fatty acids could be an effective strategy to improve the clinical consequences of the metabolic syndrome.

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