Dietary *trans-10,cis-12* conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse

Lionel Clément,* Hélène Poirier,* Isabelle Niot,* Virginie Bocher,† Michèle Guerre-Millo,§ Stéphane Krief, Bart Staels,† and Philippe Besnard[1,*]**

Physiologie de la Nutrition,* Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation (ENSBANA) FRE2328 CNRS-CESG/Université de Bourgogne F- 21000, Dijon, France; Département d'Athérosclérose/U545 INSERM,† Institut Pasteur de Lille, F-59019 Lille and Faculté de Pharmacie, Université de Lille II, Lille, France; U.465 INSERM,§ Institut Biomédical des Cordeliers, F-75006 Paris, France; and Bioprojet Biotech,** 4 rue du Chesnay-Beauregard, F-35760 Saint Grégoire, France

Abstract Conjugated linoleic acids (CLA) are a class of positional, geometric, conjugated dienoic isomers of linoleic acid (LA). Dietary CLA supplementation results in a dramatic decrease in body fat mass in mice, but also causes considerable liver steatosis. However, little is known of the molecular mechanisms leading to hepatomegaly. Although *c9,t11*- and *t10,c12*-CLA isomers are found in similar proportions in commercial preparations, the respective roles of these two molecules in liver enlargement has not been studied. We show here that mice fed a diet enriched in *t10,c12*-CLA (0.4% w/w) for 4 weeks developed lipoatrophy, hyperinsulinemia, and fatty liver, whereas diets enriched in *c9,t11*-CLA and LA had no significant effect. In the liver, dietary *t10,c12*-CLA triggered the ectopic production of peroxisome proliferator-activated receptor γ (PPARγ), adipocyte lipid-binding protein and fatty acid transporter mRNAs and induced expression of the sterol responsive element-binding protein-1a and fatty acid synthase genes. In vitro transactivation assays demonstrated that *t10,c12*- and *c9,t11*-CLA were equally efficient at activating PPARα, β/δ, and γ and inhibiting liver-X-receptor. Thus, the specific effect of *t10,c12*-CLA is unlikely to result from direct interaction with these nuclear receptors. Instead, *t10,c12*-CLA-induced hyperinsulinemia may trigger liver steatosis, by inducing both fatty acid uptake and lipogenesis.—Clément, L., H. Poirier, I. Niot, V. Bocher, M. Guerre-Millo, S. Krief, B. Staels, and P. Besnard. **Dietary *trans-10,cis-12* conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. *J. Lipid Res.* 2002.** 43: **1400–1409.**

Supplementary key words conjugated linoleic acid • peroxisome proliferator-activated receptors • liver-X-receptors • sterol responsive element-binding proteins • hyperinsulinism • liver steatosis

Conjugated linoleic acids (CLA) are a class of positional and geometric conjugated dienoic isomers of linoleic acid (LA) found in dairy products, in bovine and ovine meat, and in partially hydrogenated vegetable oils. Although *c9,t11*-CLA (also known as rumenic acid) is the main CLA isomer present in food, it is found in almost equal proportions with the *t10,c12*-CLA isomer in commercial CLA preparations. The effects of CLA have been thoroughly studied in various animal models (1, 2). CLA have anticarcinogenic properties in both mice and rats (3), and delay the onset of atherosclerosis in rabbits (4) and hamsters (5). CLA-enriched diets also cause rapid, massive changes in body composition, particularly in the mouse, in which a decrease in fat stores associated with an increase in lean body mass has been reported (6, 7). Much is now known about the physiological basis of the CLA-induced decrease in adipose tissue mass. CLA supplementation leads to an increase in energy expenditure (7), which may be secondary to a stimulation of sympathetic nervous activity (8). CLA reduce lipid uptake and storage in 3T3-L1 adipocytes by inhibiting lipoprotein lipase (9, 10) and stearoyl-CoA desaturase-I (11) activities. Finally, it has been suggested that the decrease in adipose tissue involves an apoptotic mechanism linked to an increase in tumor necrosis factor α production (12). Recent studies with purified isomers have strongly suggested that CLA-induced fat loss is dependent on the *t10,c12*-CLA isomer in the mouse (13).

Another consequence of dietary CLA supplementation in mice is massive liver enlargement (12, 14–16). However, the cellular and molecular mechanisms involved in this process are unknown. It has been suggested that the effects of CLA on the liver may be partially controlled by

Manuscript received 8 January 2002 and in revised form 19 June 2002.
DOI 10.1194/jlr.M20008-JLR200

Abbreviations: ALBP, adipocyte lipid-binding protein; CLA, conjugated linoleic acid(s); FAS, fatty acid synthase; FAT/CD36, fatty acid transporter; LA, linoleic acid; L-FABP, liver fatty acid-binding protein; LXR, liver-X-receptor; PEPCK, phosphoenol-pyruvate carboxykinase; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol responsive element-binding protein; 22R-CS, 22(R)-hydroxycholesterol; WAT, white adipose tissue.

[1] To whom correspondence should be addressed.
e-mail: pbesnard@u-bourgogne.fr

peroxisome proliferator-activated receptor α (PPARα) (17), a nuclear receptor known to regulate lipid metabolism in this organ (18). Indeed, both *c9,t11*-CLA and *t10,c12*-CLA have been shown to activate PPARα in transfection assays (17). Consistent with this finding, an isomeric CLA mixture induced the expression of typical PPARα target genes encoding proteins involved in hepatic lipid transport (liver fatty acid-binding protein or L-FABP) and catabolism (acyl-CoA oxidase, and cytochrome P450 4A1) (19). However, the role of PPARα in CLA-mediated steatosis remains to be clarified.

Other transcription factors in addition to PPARα, such as liver-X-receptors (LXRs) and sterol responsive element-binding protein 1 (SREBP1), play a critical role in hepatic lipid metabolism by controlling de novo fatty acid synthesis (20, 21). It was recently suggested that the balance within the cell between oxysterols and polyunsaturated fatty acids (PUFA), which interfere with LXR activation in vitro, is a crucial determinant of hepatic lipogenesis (22). It has also been established that SREBP1 is a major regulator of this pathway.

This study was designed to explore the effects of dietary supplementation with purified CLA isomers. The effects of purified *c9,t11*-CLA and *t10,c12*-CLA were investigated in mice fed an isomer enriched-diet for 4 weeks. We found that the *t10,c12*-CLA isomer was responsible for CLA-induced lipoatrophy and liver steatosis. A profound change in the pattern of hepatic gene expression, favoring lipid accumulation, was observed in mice fed a diet rich in *t10,c12*-CLA. In vitro transactivation assays showed that this effect on gene expression was not mediated by the direct activation of PPARs or LXRs. Instead, it may have been triggered by the marked increase in circulating insulin levels induced by dietary *t10,c12*-CLA.

MATERIALS AND METHODS

Experimental protocols

French guidelines for the use and care of laboratory animals were followed. C57Bl/6J mice weighing 22.5 ± 0.1 g at the beginning of the experiment were individually housed in a controlled environment (constant temperature, humidity, and darkness from 8 AM to 8 PM). The food intake and body mass gain of each mouse were monitored at regular intervals.

To explore the effects on body composition of the two main CLA isomers found in commercial preparations, female mice were fed ad libitum for 4 weeks on a semi-synthetic diet (UAR, France) containing either 2.4% sunflower oil (control diet), or 2% sunflower oil plus 0.4% linoleic acid (LA diet; Sigma), or highly purified CLA isomers (i.e., *c9,t*-CLA, or *t10,c12*-CLA diets; Natural Lipids Ltd, Norway) (**Table 1**). The diets were freshly prepared every day. We used females rather than males because they are more responsive to CLA supplementation (6). Anesthetized animals were bled by sectioning auxiliary vessels. The mice were killed, and liver and periuteral white adipose tissue (WAT) was collected, weighed, then rapidly frozen in liquid nitrogen and stored at −80°C.

TABLE 1. Composition of diets

Ingredients	Control	LA	*c9,t11*-CLA	*t10,c12*-CLA
			g/100g	
Sunflower oil	2.4	2.0	2.0	2.0
Fatty acids				
LA (99% of purity)		0.4		
c9,t11-CLA (91.6% of purity)			0.4	0.004
t10,c12-CLA (96.2% of purity)			0.008	0.4
Casein	13	13	13	13
Carbohydrates	48.7	48.7	48.7	48.7
Cellulose	3.3	3.3	3.3	3.3
Mineral+Vitamin mix	4	4	4	4

Northern blotting

Total RNA was extracted from liver and WAT by the phenol-chloroform-LiCl method (23). It was subjected to electrophoresis in a 1% agarose gel and transferred to a GeneScreen membrane (NEN) as previously described (24). cDNA probes were obtained from various sources: the liver fatty acid-binding protein (L-FABP) cDNA was obtained from J. I. Gordon (Washington University, St Louis MO), adipocyte lipid-binding protein (ALBP) cDNA and fatty acid transporter (FAT/CD36) cDNA were obtained from P.A. Grimaldi (INSERM U460, Nice, France), fatty acid synthase (FAS) cDNA was obtained from P. Ferré (INSERM U465, Paris, France) and phosphoenol-pyruvate carboxykinase (PEPCK) cDNA was obtained from C. Forest (INSERM U530, Paris, France). The probes were labeled with [α^{32}P]dCTP (3000 Ci/mmol; Amersham) using a megaprime kit (Amersham). A 24-residue oligonucleotide specific for rat 18S rRNA was used as a control to ensure that equivalent amounts of RNA were loaded and transferred. This oligonucleotide was 5′ end-labeled using T_4 polynucleotide kinase and [γ^{32}P]ATP (3000 Ci/mmol, Amersham).

Real-time quantitative RT-PCR

cDNA was synthesized by reverse transcription of 5 μg of total RNA in a total volume of 20 μl using random hexamers and murine Moloney leukemia virus reverse transcriptase (Life Technologies). Real-time quantitative RT-PCR was then performed with 50 ng of reverse transcription products (diluted in 5 μl of 1× Sybr Green buffer), with 200 nM sense and antisense primers (Genset) in a final volume of 25 μl, using Sybr Green PCR core reagents in an ABI PRISM 7700 Sequence Detection System instrument (Applied Biosystems). As we used Sybr Green to determine amplification-associated fluorescence for real-time quantitative RT-PCR, it was important to check that the fluorescence generated was not overestimated due to contamination resulting from residual genomic DNA amplification (using controls without reverse transcriptase) and/or from the formation of primer dimers (controls with no DNA template or reverse transcriptase). RT-PCR products were also analyzed by electrophoresis in an ethidium bromide-stained agarose gel to check that a single amplicon of the expected size was indeed obtained. 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase amplifications were used to assess variability in the initial quantities of cDNA. Relative quantification for any given gene, expressed as fold variation over control, was calculated by determining the difference between the cycle threshold (C_T) of the given gene in the control (A) and treated (B) samples, using the $2^{-\Delta}(C_{TA}-C_{TB})$ formula, according to manufacturer's protocol. CT values are expressed as means of triplicate measurements. The sense and antisense primers used were: GGGAGCCTGAGAAACGGC and GGGTCGGGAGTGGGTAATTT for 18S, GGCCATCCACAGTCTTCTGG and ACCACAGTCCATGCCATCACTGCCA for GAPDH, GCGC-

CATGGACGAGCTG and TTGGCACCTGGGCTGCT for SREBP1a, GGAGCCATGGATTGCACATT and GCTTCCAGAGAGGAGGCCAG for SREBP1c, CCCTTGACTTCCTTGCTGCA and GCGTGAGTGTGGGCGAAT for SREBP2, AGGCCGAGAAGGAGAAGCTGTTG and TGGCCACCTCTTTGCTCTGCTC for PPARγ.

Transfection assays

Transient transfections were performed in undifferentiated human enterocyte-like Caco-2 cells (passage 40). These cells were cultured in 60 mm dishes at 37°C, under a humidified atmosphere (95% air/5% CO_2) in DMEM supplemented with 20% FCS, 4 mM L-glutamine, 1% non essential amino acids, 50 mg/ml streptomycin, and 200 IU/ml penicillin. One day before transfection, Caco2 cells were treated with 0.5% trypsin and 0.25 mg/ml EDTA, then replated in 6-well plates. They were supplied with fresh medium supplemented with 10% delipidated FCS (Sigma) 4 h before transfection. Cells were typically cotransfected with 4 μg of L-FABP promoter construct (25) and with 0.1 μg of pSG5 effector plasmid expressing full-length cDNAs for mouse PPARα (26), PPARβ/δ (27), PPARγ (28), or pSG5 alone. We included 1 μg of the CMVβ-gal plasmid, in which expression of the β-galactosidase reporter gene is driven by the cytomegalovirus promoter/enhancer, as an internal control of transfection efficiency. Transfection was carried out by the calcium-phosphate method (29). Experiments were performed with 100 μM LA, *c9,t11*-CLA, or *t10,c12*-CLA complexed with 12.5 μM fatty acid-free BSA in DMEM supplemented with 10% delipidated FCS. Cells were harvested 24 h after induction. Cell extracts were prepared and assayed for β-galactosidase (β-Gal) and chloramphenicol acetyltransferase (CAT) activities. All points correspond to triplicate determinations.

PPAR activation assays

We evaluated the effects of LA and CLA isomers on PPAR transactivation by carrying out transient transfection assays with a vector encoding chimeric proteins comprising the DNA-binding site of the yeast transcription factor Gal4 fused to the ligand-binding domain of human PPARα, PPARβ/δ, or PPARγ and a reporter vector containing five copies of the Gal4-responsive element cloned upstream from the Herpes simplex thymidine kinase promoter and the luciferase gene, as previously described (30). Briefly, COS-1 cells were transfected by incubation for 2 h at 37°C in culture medium without fetal calf serum (FCS), with the cationic lipid RPR 120535B, 20 ng/well of reporter vector (pG5TkpGL3), and 100 ng/well of expression vector (pGal4-PPARαDEF, pGal4-PPARβ/δDEF, or pGal4-PPARγDEF). One nanogram per well of pRL-CMV (Promega, Madison, WI) was used as a control for transfection efficiency. Cells were treated for 36 h with vehicle alone (0.1% DMSO v/v) or with various concentrations of LA, *c9,t11*-CLA, or *t10,c12*-CLA (10 to 200 μM). Activation efficiency was compared in the presence and absence of specific agonists of PPARα, PPARβ/δ, and PPARγ: Wy14643 (50 μM), MW166 Check (10 μM), and BRL 49653 (10 μM), respectively. At the end of the experiment, the cells were washed once with ice-cold 0.15 M NaCl in 0.01 M sodium phosphate buffer (pH 7.2), and luciferase activity was determined with the Dual-Luciferase™ reporter assay system (Promega, Madison, WI). The protein content of the extract was determined by Bradford's assay using the kit from Bio-Rad (Bio-Rad, Munich, Germany).

LXRα activation assay

One day before transfection, HEK 293 cells were plated in DMEM supplemented with 10% FCS, in 24-well plates, at a density of 6×10^4 cells/well. Transfection mixtures contained 50 ng of TkpGl3 reporter plasmid, which carried five copies of the Gal4 response element, and 10 ng of a chimeric Gal4 construct containing the ligand-binding domain of LXRα (or 10 ng of insertless plasmid as a control). We added 50 ng of the β-gal pSVβ-gal construct for standardization of the results. Cells were transfected by lipofection, involving incubation for 2 h with RPR-120535B in serum-free medium. The medium was then replaced with DMEM supplemented with 10% FCS and various concentrations of LA, *c9,t11*-CLA, or *t10,c12*-CLA (10 to 100 μM), or the ethanol vehicle alone. The cells were incubated for 16 h, after which we added 10 μM 22(R)-hydroxycholesterol (22R-CS) and incubated the cells for a further 20 h. Cell extracts were prepared and assayed for luciferase activity. Results were standardized on the basis of β-galactosidase activity. All points correspond to triplicate determinations.

Biochemical assays

The total lipid content of livers was determined by Delsal's method (31). Blood glucose concentration was determined by enzymatic methods (Biotrol Diagnostics). Plasma insulin and leptin levels were determined by radioimmunoassay (CIS Bio and Linco, respectively).

Statistical analysis

The results are expressed as means ± SE. The significance of differences between groups was determined by carrying out Student's *t*-test.

RESULTS

CLA effects on energy intake and body mass

CLA supplementation led to a significant decrease in daily energy intake (15.6 ± 0.3 Kcal/mouse/day for *c9,t11*-CLA, and 16.5 ± 0.7 for *t10,c12*-CLA versus 23.3 ± 1.5 in controls, $P < 0.001$). This effect has been reported in previous studies (9) and is not CLA-specific. Indeed, a similar decrease was also found in mice fed a diet supplemented with LA (16.9 + 0.7 Kcal/mouse/day, $P < 0.05$ vs. controls). The addition of LA and CLA as fatty acids rather than as triglycerides might lead to sensorial and/or post-ingestive problems, resulting in partial aversion for these diets (7). A short-term decrease in body mass occurred in mice fed a diet rich in *t10,c12*-CLA, but no significant difference was found at the end of the experiment (data not shown).

CLA-mediated changes in body composition are specific to the *t10,c12*-CLA isomer

In contrast to the results obtained for LA- and *c9,t11*-CLA-enriched diets, the diet enriched in *t10,c12*-CLA resulted in a dramatic decrease in the mass of peri-uteral WAT (**Fig. 1A**). The abundance of the mRNAs encoding adipocyte lipid-binding protein (ALBP, also termed aP2) and fatty acid synthase (FAS), two proteins known to be involved in fatty acid uptake and accumulation in the adipocyte, was also markedly lower in mice fed this diet than in other mice (Fig. 1B). Similarly, only the *t10,c12*-CLA diet triggered a massive enlargement of the liver (3.1-fold increase, $P > 0.001$), which displayed the typical features of a fatty liver: pale color and accumulation of intracellular lipids (**Fig. 2**).

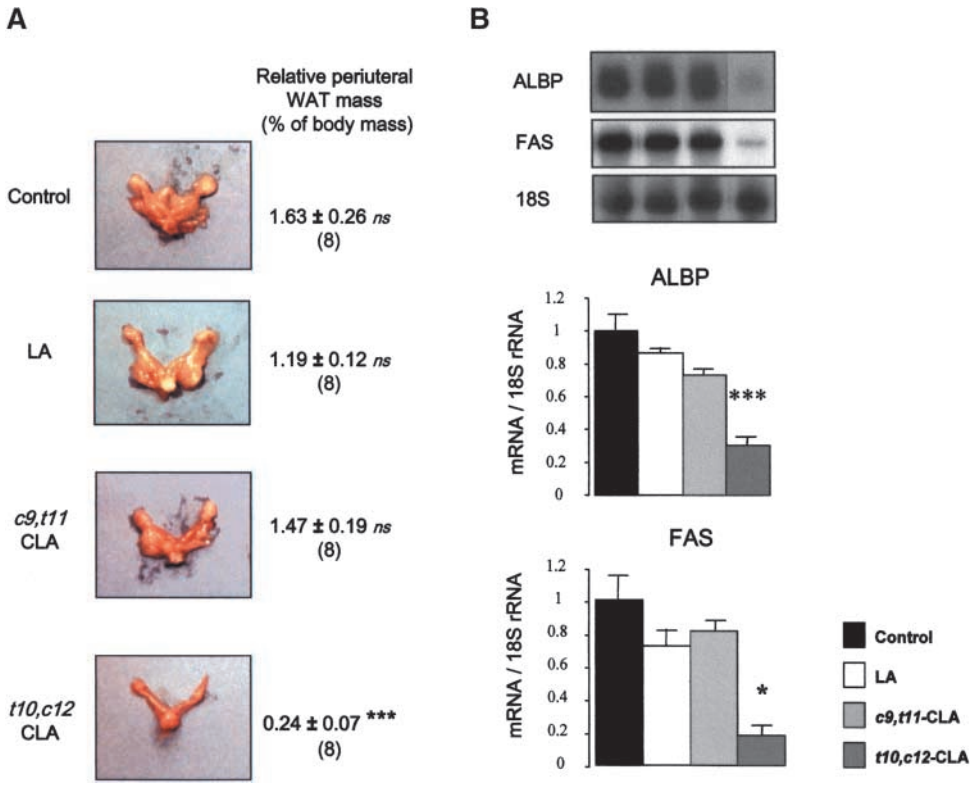

Fig. 1. *t10,c12*-CLA is responsible for changes in white adipose tissue (WAT). Female C57Bl/6J mice fed for 4 weeks on diets containing 2.4% (w/w) sunflower oil alone (Control), or 2% sunflower oil plus 0.4% linoleic acid (LA), or 0.4% *c9,t11*-CLA, or 0.4% *t10,c12*-CLA. A: Typical gross changes and relative periuteral WAT mass. B: Northern blots and bar graph indicating changes in adipocyte lipid-binding protein (ALBP) and fatty acid synthase (FAS) mRNA levels. The changes with respect to the control were calculated after correcting for loading differences on the basis of 18S rRNA levels. Means ± SE, n = 5. $*P < 0.05$; $***P < 0.01$.

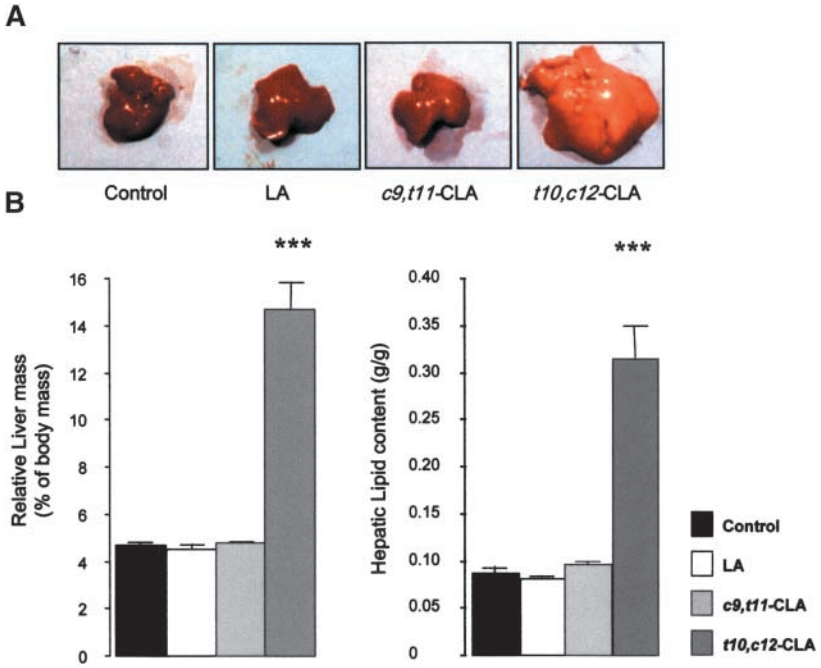

Fig. 2. Liver steatosis is induced by *t10,c12*-CLA supplementation. Female C57Bl/6J mice were fed for 4 weeks on diets containing 2.4% (w/w) sunflower oil alone (Control), or 2% sunflower oil plus 0.4% linoleic acid (LA), 0.4% *c9,t11*-CLA, or 0.4% *t10,c12*-CLA. A: Typical gross changes. B: Relative liver mass and hepatic lipid content. Means ± SE, n = 8. $***P < 0.01\%$.

LA, t10,c12-CLA and *c9,t11*-CLA exert similar effects on PPARα, β/δ, and γ and LXRα activation

We investigated whether the isomer-specific CLA-mediated effect on adipose tissue and liver lipid metabolism was due to the direct activation of PPARs by means of a sensitive and specific assay involving chimeric proteins comprising the DNA-binding site of the yeast transcription factor Gal4 fused to the ligand-binding domain of each of the three known PPAR isoforms (PPARα, PPARβ/δ, and PPARγ) and a reporter gene system driven by five copies of the Gal4 response element inserted upstream from the luciferase gene (30). LA and the two CLA isomers were found to be potent PPARα activators, whereas PPARβ/δ was activated to a lesser extent and the effect on PPARγ activation was negligible (**Fig. 3**). Consistent with the results obtained by Moya-Camarena et al. (17), *c9,t11*-CLA appeared to be more efficient than *t10,c12*-CLA at activating PPARα. Indeed, 200 μM *t10,c12*-CLA was required to obtain the same level of PPARα activation obtained with 50 μM *c9,t11*-CLA (Fig. 3). Thus, the direct activation of PPARs by *t10,c12*-CLA cannot account for the induction of fatty liver by this compound. However, CLA may upregulate typical PPARα target genes. We investigated this possibility by cotransfecting Caco-2 cells with a construct consisting of the L-FABP promoter cloned upstream from a CAT reporter gene and PPAR expression vectors. The PPARα isoform gave the greatest increase in L-FABP promoter activity. Moreover, slightly higher levels of PPARα-mediated transactivation of the L-FABP promoter were obtained in the presence of *c9,t11*-CLA than with *t10,c12*-CLA or LA (**Fig. 4A**). In vivo, both *c9,t11*-CLA- and *t10,c12*-CLA-enriched diets induced a significant increase in liver L-FABP mRNA levels, whereas the LA diet did not (Fig. 4B). The accumulation of CLA in the liver and/or the transformation of CLA into more active metabolites may account for this difference.

PUFA are known to inhibit the lipogenic pathway. The molecular basis of this regulation was recently described and involves competitive inhibition between physiological LXR agonists (oxysterols) and PUFA for the LXR ligand-binding domain, leading to the inhibition of SREBP1c induction by LXR, a crucial step in lipogenesis (22). We investigated the effects of CLA on LXR activity by cotransfecting cells with a construct encoding a Gal-4 DNA-binding domain fused to the ligand-binding domain of LXRα and a reporter plasmid in which expression of the reported gene was driven by a Gal4-responsive element. The efficiency of this assay was assessed. We observed a 2.5-fold increase in luciferase activity if the specific physiological LXR agonist 22(R)-hydroxycholesterol (22R-CS) was used alone, and 42% inhibition if this molecule was used in association with LA (**Fig. 5**). Dose-dependent inhibition of reporter gene transactivation by 22R-CS was observed in the presence both *c9,t11*-CLA and *t10,c12*-CLA,

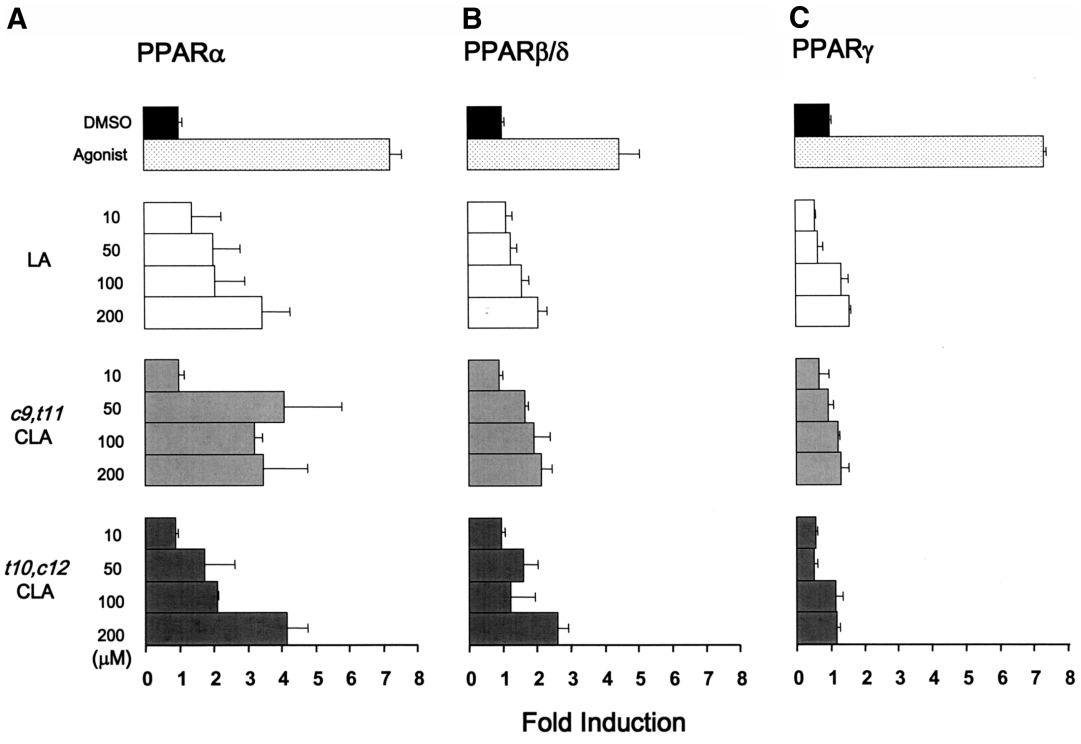

Fig. 3. Effects of linoleic acid (LA), *c9,t11*-CLA, and *t10,c12*-CLA on the activity of PPARα, PPARβ/δ and PPARγ chimeras. COS-1 cells were cultured and transfected with plasmids expressing the Gal4-hPPARα, Gal4-hPPAR, and Gal4-hPPARγ chimeras and a reporter plasmid carrying five copies of the DNA-binding element of the yeast transcription factor Gal4 cloned upstream from the thymidine kinase promoter and the luciferase reporter gene. Cells were treated for 36 h with vehicle (0.1% DMSO, v/v), LA, *c9,t11*-CLA, or *t10,c12*-CLA (1 to 200 μM). Cell extracts were then analyzed for luciferase activity and protein content.

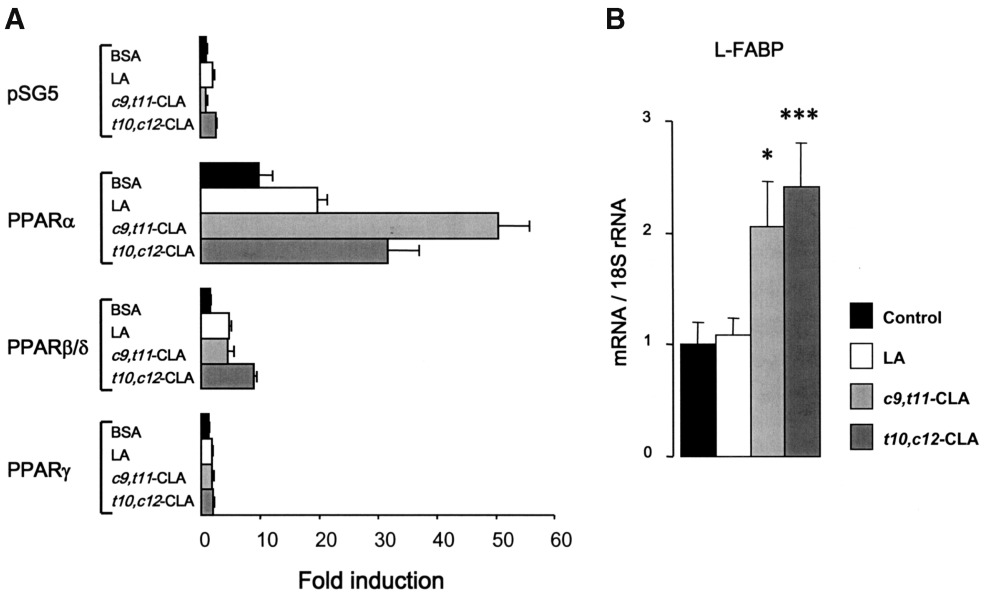

Fig. 4. Comparison of the effect of linoleic acid (LA), *c9,t11*-CLA, or *t10,c12*-CLA on the regulation of a typical PPARα target gene. A**:** Caco-2 cells were transiently cotransfected with a construct in which the L-FABP promoter was cloned upstream from the CAT reporter gene, and with plasmids encoding murine PPARα, PPARβ/δ, or PPARγ. Cells were treated as described in the Materials and Methods section, with 100 μM LA or purified isomers, 24 h before harvesting. Control cultures (solid bar) received only the vehicle (12.5 μM BSA). Transcriptional activity is expressed as CAT activity standardized with respect to β-galactosidase activity. Means ± SE, n = 3. B: Female C57Bl/6J mice were fed for 4 weeks on diets containing 2.4% (w/w) sunflower oil alone (Control), 2% sunflower oil plus 0.4% linoleic acid (LA), or 0.4% *c9,t11*-CLA, or 0.4% *t10,c12*-CLA. Liver fatty acid-binding protein (L-FABP) mRNA levels were analyzed by northern blotting. Changes with respect to the control were calculated after correcting for loading differences according to18S rRNA levels. Means ± SE, n = 5. $*P < 0.05$; $***P < 0.001$.

demonstrating that the two CLA exerted inhibitory effects on LXR activation similar to those of LA (Fig. 5).

t10,c12-CLA supplementation modifies the expression of hepatic genes

Substantial *t10,c12*-CLA-mediated modifications of hepatic gene expression may be responsible for liver steatosis. We investigated this possibility by studying the levels of expression of genes encoding transcription factors, lipid-binding proteins, and enzymes known to play a significant role in lipid metabolism. LA- and *c9,t11*-CLA-enriched diets had no significant effect on the pattern of expression of the genes studied. By contrast, supplementation with the *t10,c12*-CLA isomer quadrupled PPARγ mRNA levels. This upregulation was accompanied by the robust induction of two typical PPARγ target genes: those encoding the fatty acid transporter (FAT/CD36) and the ALBP, known to be involved in LCFA uptake in adipocytes. A slight but significant increase in liver sterol responsive element-binding protein 1a (SREBP1a) mRNA levels was also observed. By contrast, expression of the phosphoenol-pyruvate carboxykinase (PEPCK) gene, which encodes a key gluconeogenetic enzyme, decreased significantly following *t10,c12*-CLA treatment (**Fig. 6**). Finally, other mRNAs, encoding PPARα, PPARβ/δ, SREBP1c, SREBP2 (Fig. 6), and liver-X-receptors (LXRα, LXRβ, data non shown), showed no clear change in level.

t10,c12-CLA-fed mice are markedly hyperinsulinemic

The hepatic phenotype developed by mice fed the *t10,c12*-CLA-enriched diet (fatty liver associated with the overproduction of PPARγ and FAS) is very similar to that found in fat-depleted transgenic aP2/SREBP1c (32), A-ZIP/F1 (33), and aP2/DTA (34) mice. Expression of the PPARγ (35) and FAS genes is inducible by insulin and an inverse correlation between plasma leptin and insulin levels has been found in these lipoatrophic mouse models. We therefore decided to assay these two hormones. Leptin is secreted by both white and brown adipose cells as a function of fat accumulation. As expected, the depletion of fat stores specifically mediated by *t10,c12*-CLA supplementation was associated with a significant decrease in plasma leptin levels, which was not reproduced in mice fed the LA- or *c9,t11*-CLA-enriched diet. (**Fig. 7A**). A dramatic increase in non fasting plasma insulin levels was observed in mice fed the *t10,c12*-CLA-enriched diet, these mice displaying insulin concentrations 10 times higher than mice fed control, and LA- or *c9,t11*-CLA-enriched diets (Fig. 7B). Despite this marked hyperinsulinemia, plasma glucose concentration remained within the normal range in these mice (Fig. 7C).

DISCUSSION

The possible beneficial effects of CLA supplementation in decreasing body fat mass have received a great deal of

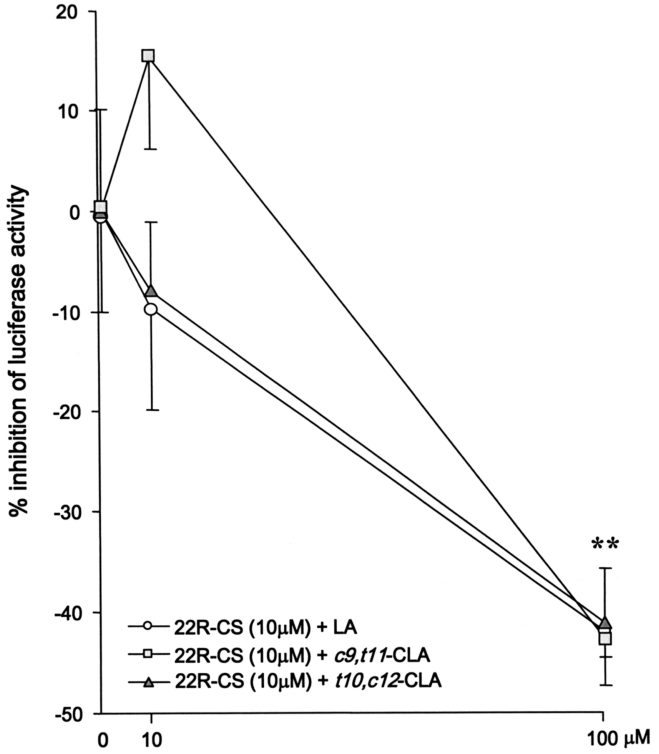

Fig. 5. CLA inhibits LXR activation by 22R-hydroxycholesterol. HEK293 cells were cotransfected with the Gal4-driven luciferase reporter construct, a plasmid encoding the Gal4 DNA-binding domain fused to the ligand-binding domain of LXR, and a control plasmid, pSV-βGal. The cells were then incubated with 0 μM, 10 μM, or 100 μM linoleic acid (LA), *c9,t11*-CLA or *t10,c12*-CLA. Ten micromoles of 22(R)-hydroxycholesterol (22R-CS) was added 16h later and cells were incubated for a further 20 h. Luciferase activity was measured and standardized according to β-galactosidase activity. Means ± SE, n = 3. $^{**}P < 0.01$ versus 22R-CS.

attention, but the potentially adverse effects of CLA on the liver and insulin balance have been largely ignored. This is paradoxical because CLA-mediated hepatomegaly and/or hyperinsulinemia have been observed in several animal studies (2) and a trend toward an increase in insulin levels has been reported in humans (36). The effects of CLA are especially dramatic in the C57Bl/6J mouse strain, in which chronic supplementation with a 1% equimolar mixture of the *c9,t11*-CLA and *t10,c12*-CLA isoforms induces a marked loss of body fat and massive fatty liver accompanied by marked hyperinsulinemia (12). This study provides the first demonstration that the *t10,c12*-CLA isomer is responsible for this remarkable phenotype, which closely resembles the lipoatrophic diabetes syndrome found in transgenic fat-depleted aP2/SREBP1c (32), aP2/DTA (33), and A-ZIP mice (34). It also provides evidence for profound *t10,c12*-CLA-mediated changes in the pattern of hepatic gene expression, contributing to fat accumulation in the liver. Indeed, the strong and specific induction of genes expressed at only very low levels in the normal liver, such as those encoding PPARγ, FAT/CD36, and ALBP, was observed in mice fed the *t10,c12*-CLA-enriched diet. Similar hepatic overexpression of the PPARγ gene has also been reported in fat-less A-ZIP/F-1 and aP2/DTA transgenic mice and in *ob/ob* mice, suggesting that it is a specific feature of steatotic livers (37). As FAT/CD36 and ALBP are cellular lipid-binding proteins, their overproduction is likely to increase fatty acid uptake capacity in the liver. The increase in FAS mRNA levels in *t10,c12*-CLA fed mice demonstrates that the lipogenic activity of the liver is also specifically induced by this CLA isomer. This effect may be accounted for by the concomitant induction of the SREBP1a gene, which is known to be involved in regulation of the hepatic lipogenic program (38).

It was recently suggested that the observed CLA-mediated changes in body composition result from the direct activation of PPARs (17). The lack of reproduction of a lipoatrophic diabetes-like syndrome in mice fed a diet enriched in *c9,t11*-CLA, even though this isomer can also bind and activate PPARα and PPARβ/δ, is not consistent with this hypothesis. Indeed, the upregulation of typical PPARα and PPARβ/δ target genes, such as L-FABP (25), by both *t10,c12*-CLA and *c9,t11*-CLA clearly dissociates this activity from hepatic fat accumulation. This conclusion is consistent with recent data obtained in PPARα-null mice. Indeed, although the CLA-dependent activation of PPARα target genes is not reproduced in the liver of PPARα$^{-/-}$ mice, the absence of PPARα does not preclude the reduction of body fat mass and liver enlargement (39). LXR is another transcription factor known to be involved in regulation of the rate-limiting enzymes of lipogenesis. LXRα activation assays have demonstrated that *t10,c12*-CLA-induced fat storage in the liver cannot be accounted for by a specific agonist effect of this CLA isomer. Moreover, both the *c9,t11*-CLA and *t10,c12*-CLA isomers inhibit LXRs in a similar manner to PUFA (22). Thus, our data demonstrate that *t10,c12*-CLA-mediated liver steatosis is not dependent on the specific activation/inhibition of PPARα, β/δ and γ, or LXRα.

Thus, the train of events leading to *t10,c12*-CLA-induced alterations in the liver remains unclear, but is probably indirect. Several lines of evidence strongly suggest that the liver steatosis occurring in *t10,c12*-CLA-fed mice is secondary to hyperinsulinemia, which causes high levels of FA uptake and synthesis (Fig. 7). First, fatty liver was not observed in *c9,t11*-CLA-fed mice, which remained normoinsulinemic (Figs. 2 and 7). In these conditions, levels of expression of the PPARγ and FAS genes were low and similar to those in mice fed the control diet. Second, in CLA-fed mice and in aP2-SREBP1c and A-ZIP/F1 fat-less transgenic mice, fat deposition in the liver is reversed if blood insulin and leptin levels are normalized by systemic leptin infusion (12, 40, 41). Third, hyperinsulinemia is associated with the induction of PPARγ gene expression in the liver and with liver steatosis in several mouse models (37). Fourth, insulin is known to upregulate PPARγ gene expression in adipocytes (35) and to induce FAS gene expression in the liver (42, 43). Finally, and most importantly, the downregulation of PEPCK strongly suggests that the livers of *t10,c12*-CLA-fed mice remain sensitive to insulin.

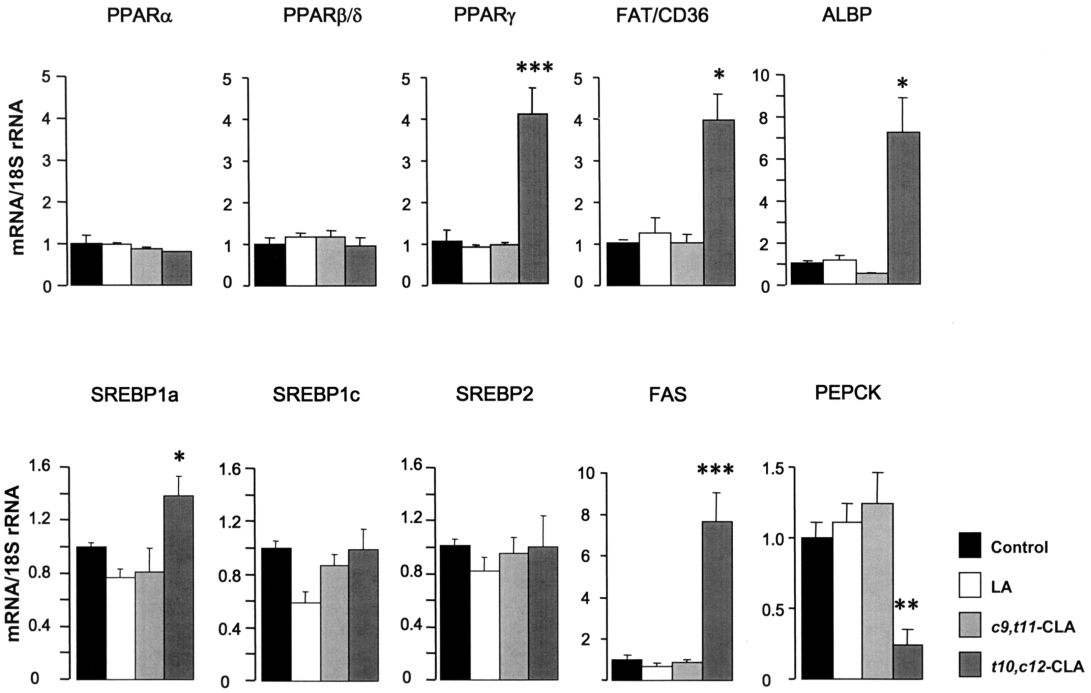

Fig. 6. CLA supplementation leads to changes in the level of expression of genes encoding transcription factors and proteins involved in lipid uptake and metabolism. Female C57Bl/6J mice were fed for 4 weeks on diets containing 2.4% (w/w) sunflower oil alone, or 2% sunflower oil plus 0.4% linoleic acid, 0.4% *c9,t11*-CLA, or 0.4% *t10,c12*-CLA. mRNA levels were analyzed by northern blotting (PPARα, PPARβ/δ, FAS, PEPCK, FAT/CD36, ALBP) or by real-time quantitative RT-PCR (PPARγ, SREBP1a, 1c, and 2). Changes with respect to the control were calculated after correcting for loading differences on the basis of 18S rRNA levels. Means ± SE, n = 5. $*P < 0.05$; $**P < 0.025$; $***P < 0.001\%$.

The cause of the dramatic hyperinsulinemia triggered by the *t10,c12*-CLA-enriched diet remains to be determined. Further experiments are required to determine whether CLA supplementation alters insulin secretion by pancreatic β-cells.

CLA have been found to affect body composition in several animal models including mice, rats, hamsters, rabbits, chickens, and pigs. However, it is unclear whether the lipoatrophic effects of *t10,c12*-CLA isomer found in the C57Bl/6J mouse can be extrapolated to other species. Indeed, the response to CLA appears to be highly species-specific, with mice generally more sensitive than other rodent species (44). Moreover, within a single species, differences between strains may be observed. For instance, lev-

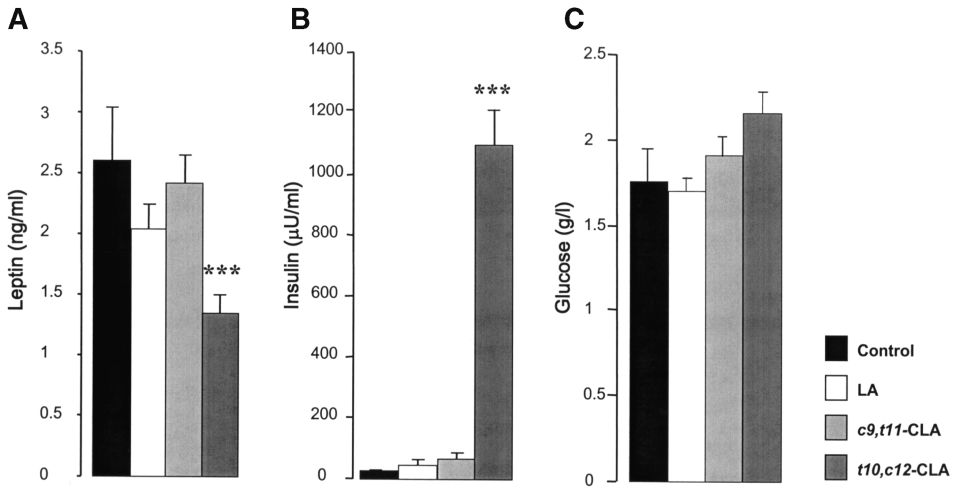

Fig. 7. *t10,c12*-CLA triggers changes in plasma leptin and insulin levels. Female C57Bl/6J mice were fed for 4 weeks on diets containing 2.4% (w/w) sunflower oil alone (Control), or 2% sunflower oil plus 0.4% linoleic acid (LA), 0.4% *c9,t11*-CLA, or 0.4% *t10,c12*-CLA. Means ± SE, n = 8. $***P < 0.001\%$.

els of fat accumulation in the liver appear to be higher in C57BL/6J, CD-I, and AKR/J mice than in SENCAR mice (12, 14, 43, and data presented here). In a recent review, Pariza et al. attributed these differences in CLA responsiveness to species-specific body fat turnover, which may be higher in mice than in larger mammals. Indeed, CLA-mediated fat loss appears to be largely dependent on fat turnover because the action of CLA on adipose tissue results in the inhibition of fatty acid uptake by the adipocyte, with no change in the lipolytic activity of the cell (1). Therefore, the lack of a clear effect of CLA on body fat mass in some species may be due to low levels of fat turnover during the duration of the experimental period (1). In humans, few clinical studies have been carried out and the results available are not readily comparable. For instance, no significant change in body fat mass and energy expenditure was found in healthy women (45) subjected to CLA supplementation (3 g/d for 64 d). By contrast, a more intense CLA treatment (3.4 or 6.8 g/d for 12 weeks) was found to be positively associated with a significant decrease in body fat mass in overweight and obese humans (46). Regarding the detrimental effects of *t10,c12*-CLA on the liver demonstrated in the C57Bl/6J mouse strain, the lack of reliable data for humans necessitates further investigations before any conclusions can be drawn as to the possible clinical value of CLA supplementation with a commercial mixture as a means of weight management.

This work was supported by research grants from Danone Institute France and the Groupe Lipides et Nutrition (GLN) to P.B. We thank Jocelyne André, Isabelle Lefrère, and Marie Claude Monnot for technical assistance.

REFERENCES

1. Pariza, M. W., Y. Park, and M. E. Cook. 2001. The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* **40:** 283–298.
2. Kelly, G. S. 2001. Conjugated linoleic acid: a review. *Altern. Med. Rev.* **6:** 367–382.
3. Durgam, V. R., and G. Fernandes. 1997. The growth inhibitory effect of conjugated linoleic acid on MCF-7 cells is related to estrogen response system. *Cancer Lett.* **116:** 121–130.
4. Lee, K. N., D. Kritchevsky, and M. W. Pariza. 1994. Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis.* **108:** 19–25.
5. Nicolosi, R. J., E. J. Rogers, D. Kritchevsky, J. A. Scimeca, and P. J. Huth. 1997. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. *Artery.* **22:** 266–277.
6. Park, Y., K. J. Albright, W. Liu, J. M. Storkson, M. E. Cook, and M. W. Pariza. 1997. Effect of conjugated linoleic acid on body composition in mice. *Lipids.* **32:** 853–858.
7. West, D. B., J. P. Delany, P. M. Camet, F. Blohm, A. A. Truett, and J. Scimeca. 1998. Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. *Am. J. Physiol.* **275:** R667–R672.
8. Ohnuki, K., S. Haramizu, K. Oki, K. Ishihara, and T. Fushiki. 2001. A single oral administration of conjugated linoleic acid enhanced energy metabolism in mice. *Lipids.* **36:** 583–587.
9. Park, Y., K. J. Albright, J. M. Storkson, W. Liu, M. E. Cook, and M. W. Pariza. 1999. Changes in body composition in mice during feeding and withdrawal of conjugated linoleic acid. *Lipids.* **34:** 243–248.
10. Lin, Y., E. Schuurbiers, S. Van der Veen, and E. A. De Deckere. 2001. Conjugated linoleic acid isomers have differential effects on triglyceride secretion in Hep G2 cells. *Biochim. Biophys. Acta.* **1533:** 38–46.
11. Choi, Y., Y. Park, M. W. Pariza, and J. M. Ntambi. 2001. Regulation of stearoyl-CoA desaturase activity by the trans-10,cis-12 isomer of conjugated linoleic acid in HepG2 cells. *Biochem. Biophys. Res.* **284:** 689–693.
12. Tsuboyama-Kasaoka, N., M. Takahashi, K. Tanemura, H. J. Kim, T. Tange, H. Okuyama, M. Kasai, S. Ikemoto, and O. Ezaki. 2000. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes.* **49:** 1534–1542.
13. Park, Y., J. M. Storkson, K. J. Albright, W. Liu, and M. W. Pariza. 1999. Evidence that the trans-10,cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. *Lipids.* **34:** 235–241.
14. DeLany, J. P., F. Blohm, A. A. Truett, J. A. Scimeca, and D. B. West. 1999. Conjugated linoleic acid rapidly reduces body fat content in mice without affecting energy intake. *Am. J. Physiol.* **276:** R1172–R1179.
15. DeLany, J. P., and D. B. West. 2000. Changes in body composition with conjugated linoleic acid. *J. Am. Coll. Nutr.* **19:** 487S–493S.
16. West, D. B., F. Y. Blohm, A. A. Truett, and J. P. DeLany. 2000. Conjugated linoleic acid persistently increases total energy expenditure in AKR/J mice without increasing uncoupling protein gene expression. *J. Nutr.* **130:** 2471–2477.
17. Moya-Camarena, S. Y., J. P. Vanden Heuvel, and M. A. Belury. 1999. Conjugated linoleic acid activates peroxisome proliferator-activated receptor α and β subtypes but does not induce hepatic peroxisome proliferation in Sprague-Dawley rats. *Biochim. Biophys. Acta.* **1436:** 331–342.
18. Schoonjans, K., J. Peinado-Onsurbe, A-M. Lefebvre, R. A. Heyman, M. Briggs, S. Deeb, B. Staels, and J. Auwerx. 1996. PPARα and PPARγ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* **15:** 5336–5348.
19. Belury, M. A., S. Y. Moya-Camarena, K-L. Liu, and J. P. Vanden Heuvel. 1997. Dietary conjugated linoleic acid induces peroxisome-specific enzyme accumulation and ornithine decarboxylase activity in mouse liver. *J. Nutr. Biochem.* **8:** 579–584.
20. Peet, J., B. A. Janowski, and D. J. Mangelsdorf. 1998. The LXRs: a new class of oxysterol receptors. *Curr. Opin. Genet. Dev.* **8:** 571–575.
21. Shimano, H. 2000. Sterol regulatory element-binding protein-1 as a dominant transcription factor for gene regulation of lipogenic enzymes in the liver. *Trends Cardiovas. Med.* **10:** 275–278.
22. Yoshikawa, T., H. Shimano, N. Yahagi, T. Ide, M. Amemiya-Kudo, T. Matsuzaka, M. Nakakuki, S. Tomita, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, A. Takahashi, H. Sone, J. Osuga Ji, T. Gotoda, S. Ishibashi, and N. Yamada. 2002. Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. *J. Biol. Chem.* **277:** 1705–1711.
23. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
24. Besnard, P., A. Mallordy, and H. Carlier. 1993. Transcriptional induction of the fatty acid binding protein gene in mouse liver by bezafibrate. *FEBS Lett.* **327:** 219–223.
25. Poirier, H., I. Niot, M. C. Monnot, O. Braissant, C. Meunier-Durmort, P. Costet, T. Pineau, W. Wahli, T. M. Willson, and P. Besnard. 2001. Differential involvement of peroxisome-proliferator-activated receptors alpha and delta in fibrate and fatty-acid-mediated inductions of the gene encoding liver fatty-acid-binding protein in the liver and the small intestine. *Biochem. J.* **355:** 481–488.
26. Issemann, I., and S. Green. 1990. Activation of members of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature.* **347:** 645–649.
27. Amri, E-Z., F. Bonino, G. Ailhaud, N. A. Abumrad, and P. A. Grimaldi. 1995. Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes. *J. Biol. Chem.* **270:** 2367–2371.
28. Kliewer, S. A., B. M. Forman, B. Blumberg, E. S. Ong, U. Borgemeyer, D. J. Mangelsdorf, K. Umesono, and R. M. Evans. 1994. Differential expression and activation of a family of murine peroxisome proliferator activated receptors. *Proc. Natl. Acad. Sci. USA.* **91:** 7355–7359.
29. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. In Molecular

Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Sping Harbor, NY.
30. Raspe, E., L. Madsen, A. M. Lefebvre, I. Leitersdorf, L. Gelman, J. Peinado-Onsurbe, J. Dallongeville, J. C. Fruchart, R. Berge, and B. Staels. 1999. Modulation of rat liver apolipoprotein gene expression and serum lipid levels by tetradecylthioacetic acid (TTA) via PPARalpha activation. *J. Lipid Res.* **40:** 2099–2110.
31. Delsal, J. L. 1944. Nouveau procédé d'extraction des lipides du sérum par le méthylal. Application aux microdosages du cholestérol total, des phospholipides et des protéines. *Bull. Soc. Chim. Biol.* **26:** 99–105.
32. Shimomura, I., R. E. Hammer, J. A. Richardson, S. Ikemoto, Y. Bashmakov, J. L. Goldstein, and M. S. Brown. 1998. Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev.* **12:** 3182–3194.
33. Moitra, J., M. M. Mason, M. Olive, D. Krylov, O. Gavrilova, B. Marcus-Samuels, L. Feigenbaum, E. Lee, T. Aoyama, M. Eckhaus, M. L. Reitman, and C. Vinson. 1998. Life without white fat: a transgenic mouse. *Genes Dev.* **12:** 3168–3181.
34. Burant, C. F., S. Sreenan, K. Hirano, T. A. Tai, J. Lohmiller, J. Lukens, N. O. Davidson, S. Ross, and R. A. Graves. 1997. Troglitazone action is independent of adipose tissue. *J. Clin. Invest.* **100:** 2900–2908.
35. Vidal-Puig, A. J., R. V. Considine, M. Jimenez-Linan, A. Werman, W. J. Pories, J. F. Caro, and J. S. Flier. 1997. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J. Clin. Invest.* **99:** 2416–2422.
36. Medina, E. A., W. F. Horn, N. L. Keim, P. J. Havel, P. Benito, D. S. Kelley, G. J. Nelson, and K. L. Erickson. 2000. Conjugated linoleic acid supplementation in humans: effects on circulating leptin concentrations and appetite. *Lipids.* **35:** 783–788.
37. Boelsterli, U. A., and M. Bedoucha. 2002. Toxicological consequences of altered peroxisome proliferator-activated receptor gamma (PPARgamma) expression in the liver: insights from models of obesity and type 2 diabetes. *Biochem. Pharmacol.* **63:** 1–10.
38. Shimano, H., J. D. Horton, R. E. Hammer, I. Shimomura, M. S. Brown, and J. L. Goldstein. 1996. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J. Clin. Invest.* **98:** 1575–1584.
39. Peters, J. M., Y. Park, F. J. Gonzalez, and M. W. Pariza. 2001. Influence of conjugated linoleic acid on body composition and target gene expression in peroxisome proliferator-activated receptor alpha-null mice. *Biochim. Biophys. Acta.* **1533:** 233–242.
40. Shimomura, I., R. E. Hammer, S. Ikemoto, M. S. Brown, and J. L. Goldstein. 1999. Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature.* **401:** 73–76.
41. Gavrilova, O., B. Marcus-Samuels, L. R. Leon, C. Vinson, and M. L. Reitman. 2000. Leptin and diabetes in lipoatrophic mice. *Nature.* **403:** 850; discussion 850–851.
42. Tobin, K. A., S. M. Ulven, G. U. Schuster, H. H. Steineger, S. M. Andresen, J. A. Gustafsson, and H. I. Nebb. 2002. Liver X receptors as insulin-mediating factors in fatty acid and cholesterol biosynthesis. *J. Biol. Chem.* **277:** 10691–10697.
43. Shimano, H. 2001. Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. *Prog. Lipid Res.* **40:** 439–452.
44. Moya-Camarena, S. Y., and M. A. Belury. 1999. Species differences in the metabolism and regulation of gene expression by conjugated linoleic acid. *Nutr. Rev.* **57:** 336–340.
45. Zambell, K. L., N. L. Keim, M. D. Van Loan, B. Gale, P. Benito, D. S. Kelley, and G. J. Nelson. 2000. Conjugated linoleic acid supplementation in humans: effects on body composition and energy expenditure. *Lipids.* **35:** 777–782.
46. Blankson, H., J. A. Stakkestad, H. Fagertun, E. Thom, J. Wadstein, and O. Gudmundsen. 2000. Conjugated linoleic acid reduces body fat mass in overweight and obese humans. *J. Nutr.* **130:** 2943–2948.