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Trans-10,cis-12-CLA dysregulate lipid and glucose metabolism and induce hepatic NR4A receptors

Maria A. Navarro[1,2,3], Lina Badimon[1,3], Cristina Rodriguez[1], Maurizio Gentile[1], Carmen Arnal[3,4], Enda J. Noone[5], Helen M. Roche[5], Jesus Osada[2,3], Jose Martinez-Gonzalez[1]

[1]Centro de Investigacion Cardiovascular (CSIC-ICCC), Hospital de la Santa Creu i Sant Pau, c/Antoni Mª Claret 167, 08025 Barcelona, Spain, [2]Departamento de Bioquimica y Biologia Molecular y Celular, Facultad de Veterinaria, Instituto Aragones de Ciencias de la Salud (Universidad de Zaragoza-Direccion Salud del Gobierno de Aragon), Miguel Servet 177, 50013 Zaragoza, Spain, [3]CIBER de Fisiopatologia de la Obesidad y Nutricion (CIBEROBN), Instituto de Salud Carlos III, Spain, [4]Departamento de Patologia Animal, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain, [5]Nutrigenomics Research Group, UCD Conway Institute, University College, Dublin, Ireland

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1. ABSTRACT

Our aim was to assess the effect of two isomers of conjugated linoleic acids (CLA), *cis*-9,*trans*-11-CLA (c9,t11-CLA) and *trans*-10,*cis*-12-CLA (t10,c12-CLA), on glucose metabolism and hepatic expression of NR4A receptors, key transcription factors regulating gluconeogenesis. ApoE-deficient mice were fed isocaloric, isonitrogenous westernized diets enriched with c9,t11-CLA, t10,c12-CLA or linoleic acid (control diet). Plasma glucose, NEFA, triglyceride and cholesterol concentrations were significantly higher in the t10,c12-CLA group compared with c9,t11-CLA or control group. Plasma insulin concentrations were lowered by c9, t11-CLA compared with either control or t10,c12-CLA group. Hepatic expression of NR4A receptors (Nur77, Nurr1 and NOR-1) was induced by t10,c12-CLA while c9,t11-CLA had not effect. Consistently t10,c12-CLA up-regulated key genes involved in gluconeogenesis including glucose-6-phosphatase, enolase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase. Hepatic expression of NR4A receptors correlated with plasma NEFA, with the expression of their target gene fatty acid transporter (FAT)/CD36 and with the accumulation of fat in the liver. These results suggest that t10,c12-CLA promote dysregulation of lipid and glucose metabolism, at least in part, by an isomer-specific modulation of hepatic expression of NR4A receptors.

2. INTRODUCTION

Conjugated linoleic acids (CLA) are a family of isomers of linoleic acid (18:2n-6) naturally found in foods derived from ruminants such as in meat, milk and dairy products (1). During the last years, considerable attention has been focused on the potential healthy effects of CLA including anti-carcinogenic (2-4), anti-atherogenic (5-7) and cardioprotective (8, 9) properties. Because they reduce body fat in animal models (10-13) and in some cases in humans (14, 15) they have been proposed as weight-loss agents. However, adverse effects were early reported in mice fed CLA-enriched diet including insulin resistance and lipodystrophy (16, 17). The nature of the *in vivo* effects of CLA is controversial since they consist of a mix of isomers with potentially dissimilar properties (18). The most abundant natural CLA isomer is *cis*-9,*trans*-11-CLA (c9,t11-CLA) (1). The average daily intake of c9,t11-CLA is highly dependent on dietary habits ranging from less than 100 mg to more than 500 mg (19, 20). However, commercial CLA supplements usually contain large quantities of *trans*-10,*cis*-12-CLA (t10,c12-CLA) (21). In the last years, t10,c12-CLA has been associated to harmful effects including increase of oxidative stress, inflammation and atherosclerosis as well as hyperglycemia, insulin resistance, lipodystrophy and hepatic steatosis (13, 22-27). The mechanisms underlying the effects of t10,c12-CLA on lipid and glucose metabolism under pathophysiological

conditions have been extensively analyzed and they have been associated to the ability of this isomer to modulate a number of key transcription factors. The physiologic regulation of lipogenic genes in the ruminant lactating mammary gland by t10,c12-CLA is mediated by the sterol regulatory element binding protein (SREBP) (28). Nuclear factor kappaB (NFκB) and peroxisome proliferator-activated receptor-gamma (PPARγ) have been involved in the anti-adipogenic and anti-insulin-sensitizing effects of t10,c12-CLA on adipose tissue (29-31). However, little is known about the mechanisms underlying the isomer-specific effects of CLA on lipid and glucose metabolism in liver.

Glucose homeostasis is maintained through the hormonal regulation of both hepatic glucose production and glucose uptake by muscle and adipose tissue (32). Recently, members of the nuclear receptors subfamily 4 group A (NR4A) have been shown to be transcriptional regulators of hepatic glucose metabolism (33). The NR4A subfamily of nuclear receptors consists of three closely related members: NR4A1 (also known as nerve growth factor-induced clone B [NGFI-B], Nur77 and TR3), NR4A2 (also known as nur-related factor-1 [Nurr1], NOT and RNR1) and NR4A3 (also known as neuron-derived orphan receptor-1 [NOR1], Minor and TEC) (34-36). NR4A receptors modulate gene expression upon binding to a NGFI-B response element (NBRE) or a Nur77 response element (NurRE) present in the promoter of target genes (34, 35). NR4A receptors are physiologically expressed in metabolically active and energy demanding tissues such as liver, heart, skeletal muscle and brain (37, 38). In contrast to classical ligand-activated nuclear receptors the activity of NR4A receptors is controlled mainly at transcriptional level. Indeed, they are early-response genes which expression is induced by extracellular signals, including fatty acids, stress, growth factors and hormones in a cell type-specific manner (34, 36) and in processes such as liver regeneration (39, 40) and after liver ischemia-reperfusion injury (41).

We hypothesized that NR4A receptors would show up-regulation *in vivo* in a model coursing with increase of hepatic neutral lipid stores and hyperglycemia. The aim of the present study was to asses the effects of c9,t11-CLA and t10,c12-CLA on lipid and glucose metabolism in the apolipoprotein E (ApoE)-deficient mice and to determine whether these isomers differentially modulate the hepatic expression of NR4A receptors and key NR4A target genes involved in lipid uptake and gluconeogenesis.

3. MATERIAL AND METHODS

All studies were reviewed and approved by the Ethics Committee for Animal Research of the University of Zaragoza and performed in accordance with the National Institutes of Health guidelines for the use of animals in research laboratories.

3.1. Animals and diets

Homozygous ApoE-deficient male mice (n = 29; 3 month old), bred at the Servicio de Biomedicina y Biomateriales, Zaragoza were housed in sterile filter-top cages in rooms maintained on a 12-h light/12-h dark cycle and had *ad libitum* access to food and water. They were randomly assigned to three treatment groups that were matched for initial fasting plasma cholesterol concentrations. All animals consumed a semi-synthetic high-fat (30% energy) and cholesterol-containing (0.15% w/w) diet that in the three groups provided equal amounts of saturated fatty acids, monounsaturated fatty acids and PUFAs. The PUFA fraction of the diets contained the linoleic acid and the CLA isomers as FFAs. The control diet (n= 10) contained 1.0% (wt/wt) linoleic acid, whereas the others contained an equivalent amount of either c9, t11-CLA (n= 10) or t10, c12-CLA (n= 9), as previously described (22, 27). Diets were stored at –20°C under nitrogen, and fresh supplies of the diets were provided daily. Food consumption, which was determined using metabolic cages (Biosys, Barcelona, Spain), and body weights were recorded weekly throughout the experiment. After the 12-wk intervention period, animals were fasted for 18 h and killed by CO_2 suffocation.

3.2. Biochemical determinations and tissue isolation

At the end of the study fasted animals were killed by suffocation with CO_2. Blood samples were obtained by cardiac puncture, centrifuged at 2500 rpm for 10 min and plasma was collected for Biochemical Determinations. Plasma total triglyceride (TG) and cholesterol concentrations were determined by microtiter assay (Triglyceride Kit 2016647, Roche Diagnostics and Infinity Cholesterol Reagent Kit 401-25P, Sigma Chemical). Plasma NEFA concentrations were determined by a standard commercial kit (Wako, Madrid, Spain). Plasma insulin and glucose concentrations were measured using standard commercial kits (rat/mouse insulin ELISA kit, Linco Research, St. Charles, MO; Glucose RTU, BioMerieux, Lyon, France) and validated with standard controls (Calimat, BioMerieux). Livers were isolated, rinsed in 0.9% saline and cut into small pieces that were either quickly frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction or preserved in formaldehyde and embedded in paraffin wax for histological analysis (13).

3.3. Real-time PCR

Total RNA was isolated using Ultraspec (Biotecx Laboratories) according to the manufacturer's recommendations and was reverse-transcribed with High-Capacity cDNA Archive kit (Applied Biosystems). mRNA levels were quantified using real-time PCR (42, 43). Assays-on-Demand (Applied Biosystems) of TaqMan fluorescent real-time PCR primers and probes were used for different genes as follows: NR4A1 (Mm00439358_m1), NR4A2 (Mm00443056_m1), NR4A3 (Mm00450074_m1), glucose-6-phosphatase (Mm00839363_m1), fructose bisphosphatase 1 (Mm00490181_m1), phosphoenolpyruvate carboxykinase (Mm00440636_m1), pyruvate carboxylase (Mm00500992_m1), enolase (Mm00468267_m1), glycerol kinase (Mm00433907_m1) and fatty acid transporter (FAT)/CD36 (Mm00432398_m1). Results were normalized by 18S ribosomal RNA (4319413E).

Table 1. Effect of control, c9,t11-CLA and t10,c12-CLA diets on plasma NEFA, triglycerides and total cholesterol

	Control (n= 10)	c9, t11-CLA (n= 10)	t10, c12-CLA (n= 9)
NEFA (mmol/l)	0.11 ± 0.03	0.07 ± 0.03*	0.31 ± 0.14†
Triglycerides (mmol/l)	1.78 ± 0.08	1.33 ± 0.15*	4.64 ± 0.6†
Cholesterol (mmol/l)	57.1 ± 3.2	41.1 ± 1.7*	67.1 ± 3.9†

Values are means ± SEM. P <0.05 (Mann Whitney U-test): * versus control and t10,c12-CLA; † versus control and c9,t11-CLA.

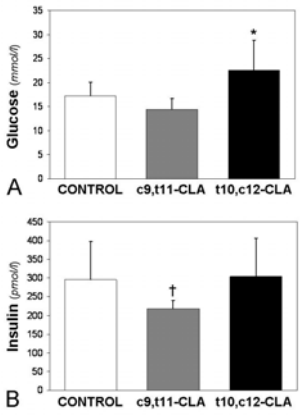

Figure 1. Effect of CLA-enriched diets on plasma levels of glucose (A) and insulin (B). Blood glucose and insulin levels were determined in fasted ApoE-deficient mice fed control (n = 10), c9,t11-CLA- (n = 10) or t10,c12-CLA-enriched diets (n = 9) for 12 wk. Data are shown as mean ± SD. (A) Statistical analysis was carried out by ANOVA. P <0.05: * versus control and c9,t11-CLA-enriched diet. (B) The unpaired t test was used to determine differences in plasma insulin levels P <0.05: † versus control and t10,c12-CLA-enriched diet.

3.4. Histological and immunohistochemical analysis

Sections (4 μm) of hepatic tissue from each mouse were subjected to histological procedures, stained with haematoxylin-eosin and average fat accumulation areas were examined. Images were captured and digitized using a Nikon microscope equipped with a Canon digital camera. Morphometric analyses were performed using NIH Image software. Immunohistochemistry was carried out using a pan-NR4A polyclonal rabbit antibody that recognized the three NR4A receptors (Santa Cruz Biotechnologies; sc-990, 1:50 dilution) (44) followed by detection with a polyclonal goat anti-rabbit IgG coupled to avidin-biotin (1:300 dilution). Sections were counterstained for nuclei with haematoxylin (Panreac).

3.5. Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was carried out by ANOVA (unless otherwise stated) and a value of P < 0.05 was taken to indicate statistical significance. Association between variables was assessed by Spearman correlation test.

4. RESULTS

4.1. Metabolic effects of CLA in ApoE-deficient mice

After the dietary intervention we measured the plasma glucose levels and insulin. Plasma glucose concentration was higher in t10,c12-CLA group compared with control or c9,t11-CLA groups (Figure 1A). Although plasma glucose concentration in the c9,t11-CLA group was lower than in controls they did not differ significantly. Plasma insulin concentrations were lowered by c9, t11-CLA compared with either control or t10,c12-CLA group, while t10,c12-CLA did not significantly modify plasma insulin concentrations compared with the control group (Figure 1B).

The effect of CLA isomers on plasma triglycerides, NEFA and cholesterol is shown in Table 1. At the end of the study t10,c12-CLA fed mice showed significant greater triglycerides, NEFA and cholesterol concentrations compared to those fed c9,t11-CLA or control diets. By contrast, c9,t11-CLA diet significantly reduced plasma triglycerides, NEFA and cholesterol concentrations compared to control diet. t10,c12-CLA fed mice showed significant lower weight gain compared to those fed the c9,t11-CLA or control diets (data not shown) as we reported previously (27).

4.2. Effect of CLA on hepatic expression of NR4A receptors and genes involved in gluconeogenesis

The expression of NR4A receptors in liver was significantly induced by the t10,c12-CLA. This effect was observed by immunostaining using a pan-NR4A antibody (Figure 2A), and was quantified by real-time PCR using specific primers and probes for NR4A1 (Nur77), NR4A2 (Nurr1) and NR4A3 (NOR-1) (Figure 2B). Interestingly, levels of plasma NEFA and triglycerides correlated with hepatic expression of NR4A receptors (Figure 3). Therefore, we analyzed whether the hepatic expression of key genes involved in gluconeogenesis (Figure 4), that are downstream targets of NR4A receptors, were significantly induced by the t10,c12-CLA. Glucose-6-phosphatase, enolase, phosphoenolpyruvate carboxykinase, pyruvate carboxylase and glycerol kinase were differentially modulated by the different CLA isomers. As figure 5 shows, all genes analyzed, except fructose bisphosphatase 1 and glycerol kinase, were significantly induced by t10,c12-CLA. The highest induction was observed on glucose-6-phosphatase (5-fold). In contrast, c9,t11-CLA significantly inhibited the expression of enolase, pyruvate carboxylase and glycerol kinase. These results indicate an isomer-selective effect of CLA on the transcriptional control of the gluconeogenic pathway.

4.3. Effect of CLA on hepatic content of fat and gene expression

The liver of the ApoE-deficient mice from the control group showed accumulation of vacuoles of triglycerides (Figure 6A). c9,t11-CLA-enriched diet substantially alleviated the fatty infiltrates while t10,c12-CLA-enriched diet dramatically increased the size of this vacuoles. The lipid area was significantly greater after feeding the t10,c12-CLA-enriched diet compared to control

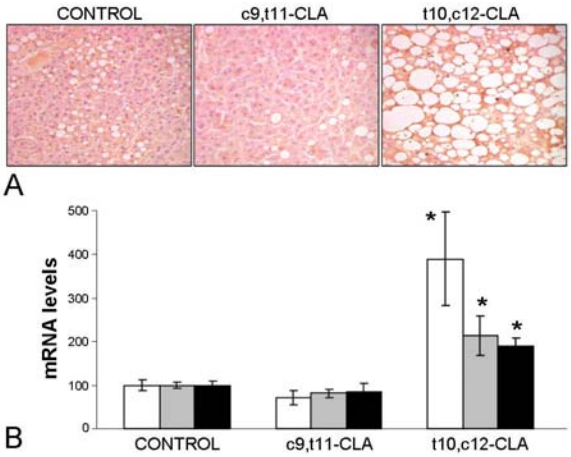

Figure 2. Effect of CLA-enriched diets on the expression of NR4A receptors in liver. Hepatic expression of NR4A receptors in ApoE-deficient mice fed control (n = 10), c9,t11-CLA- (n = 10) or t10,c12-CLA-enriched diets (n = 9) for 12 wk was analyzed by immunohistochemical analysis (A) and real-time PCR (B). Nur77 (NR4A1; white bars), Nurr1 (NR4A2; shaded bars) or NOR-1 (NR4A3; black bars) mRNA levels (relative to control) are shown. Data are shown as mean ± SEM. Statistical analysis was carried out by ANOVA. $P < 0.05$: * versus control and c9,t11-CLA-enriched diet.

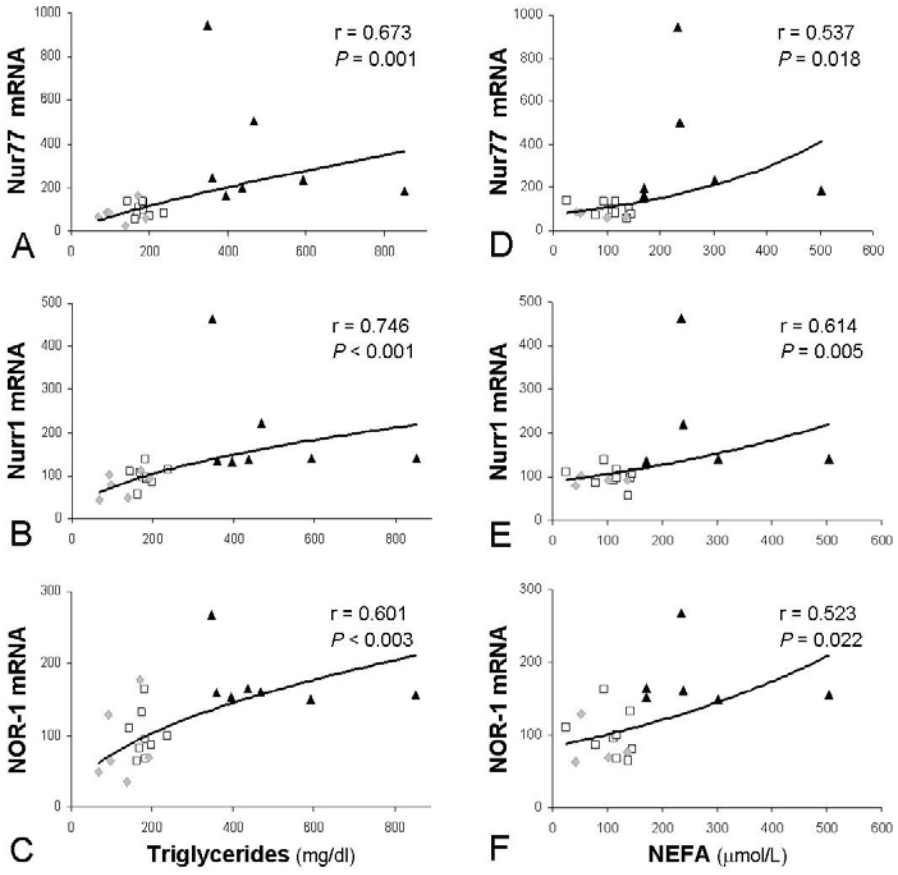

Figure 3. Correlation between hepatic expression of NR4A receptors and plasma levels of triglycerides and NEFA. Results of Spearman correlation test between plasma levels of triglycerides (A-C) or NEFA (C-D) and mRNA levels of NR4A receptors (Nur77 [A and D], Nurr1 [B and E] and NOR-1 [C and F]) in the liver (arbitrary units) in ApoE-deficient mice consuming control (squares), c9, t11-CLA (diamonds) and t10, c12-CLA (triangles) enriched diets.

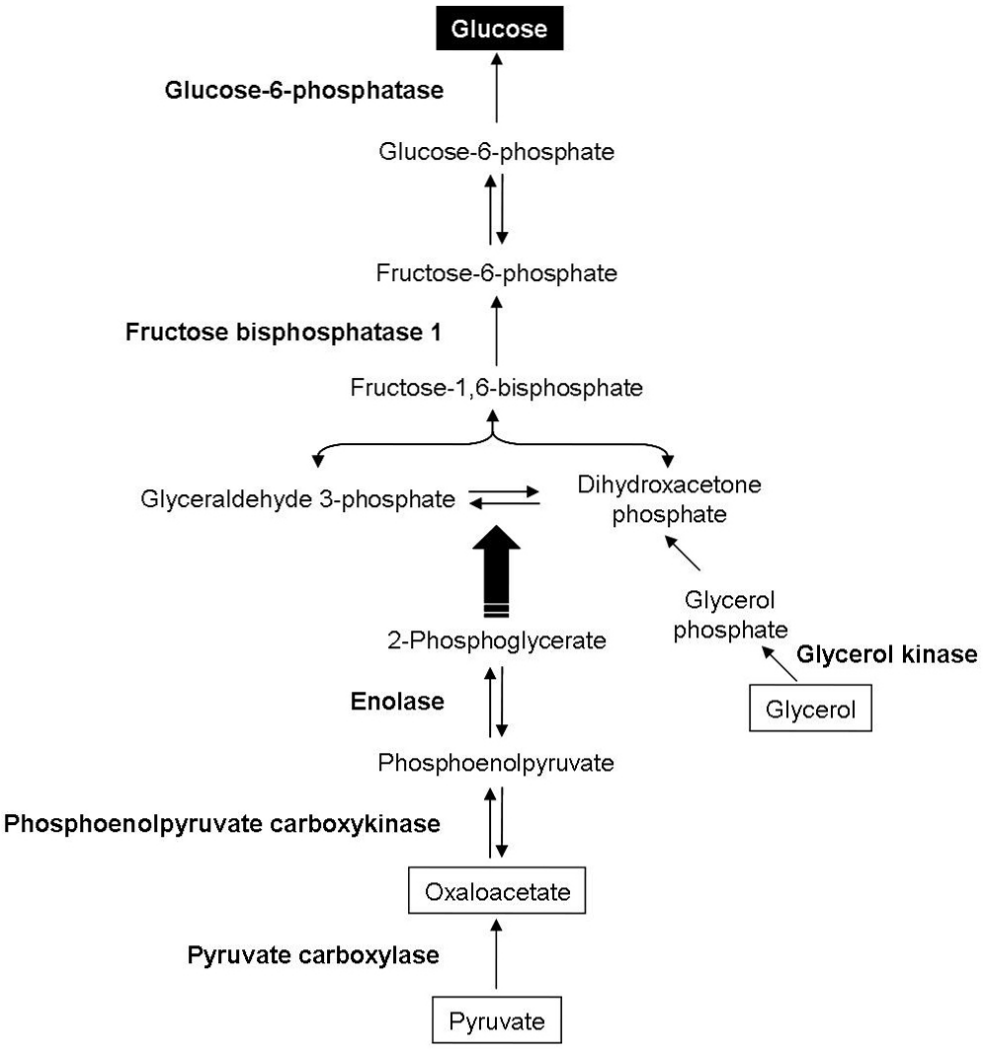

Figure 4. Schematic representation of the gluconeogenesis pathway indicating the enzymes whose mRNA levels were analyzed by real-time PCR (in bold). The main gluconeogenic substrates are boxed: pyruvate, oxaloacetate and glycerol (produced from the hydrolysis of triglycerides enters the pathway after conversion to dihydroxyacetone phosphate).

or c9,t11-CLA diets (Figure 6B). In contrast, the c9,t11-CLA diet substantially decreased the lipid area compared to control. We observed a strong positive correlation between the mRNA levels of NR4A receptors and the hepatic content of fat (Figure **7**). Hepatic mRNA levels of SREBP-1, PPARα and fatty acid synthase (FAS) were not significantly modified by experimental diets (data not shown), but t10, c12-CLA-enriched diet significantly increased mRNA of FAT/CD36 (Figure 8A). Interestingly, FAT/CD36 mRNA expression was positively correlated with the expression of NR4A receptors in liver (Figure 8B).

5. DISCUSSION

We have previously demonstrated that CLA isomers differentially affect lipid and glucose metabolism and exert opposite effects on the development of atherosclerosis in ApoE-deficient mice (26, 27). Here we show that the reported increase in plasma glucose levels produced by t10,c12-CLA in this animal model is associated to the selective up-regulation of the hepatic expression of NR4A receptors as well as to the induction of NR4A target genes involved in gluconeogenesis. To our knowledge, this is the first paper showing that the hyperglycemic effects of a nutrient (t10,c12-CLA) could be mediated, at least in part, by a isomer-specific effect of CLA on hepatic expression of NR4A receptors.

In our experimental approach we observed an increase in plasma glucose levels upon consumption of t10,c12-CLA in agreement with previous studies in mice (17, 22). Because NR4A receptors have recently emerged as key transcriptional regulators in the hepatic control of glucose metabolism we sought to investigate the expression profile of these receptors and key genes associated to gluconeogenesis. Indeed, Pei *et al.* (33) demonstrated that inhibition of NR4A receptors activity in the liver lowered blood glucose in physiologic conditions and diabetes, while

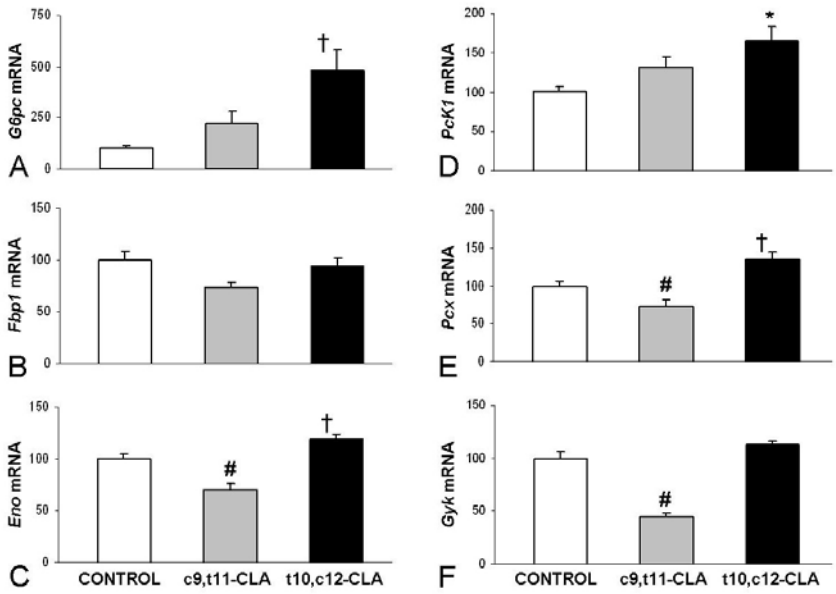

Figure 5. Effect of CLA-enriched diets on the hepatic expression of key genes involved gluconeogenesis. ApoE-deficient mice fed control (white bars; n= 10;), c9,t11-CLA- (shaded bars; n= 10) or t10,c12-CLA-(black bars; n= 9) enriched diets for 12 wk were fasted for 18 h and expression of genes involved in gluconeogenesis was analyzed by real-time PCR. Genes analyzed were: glucose-6-phosphatase (*G6pc*) (A), fructose-1,6-bisphosphatase (*Fbp1*) (B), enolase (*Eno*) (C), phosphoenolpyruvate carboxykinase 1 (*Pck1*) (D), pyruvate carboxylase (*Pcx*) (E), and glycerol kinase (*Gyk*) (F). Data (relative to controls) are shown as mean ± SEM. Statistical analysis was carried out by ANOVA. $P < 0.05$: * versus control; † versus control and c9,t11-CLA-enriched diet; # versus control and t10,c12-CLA-enriched diet.

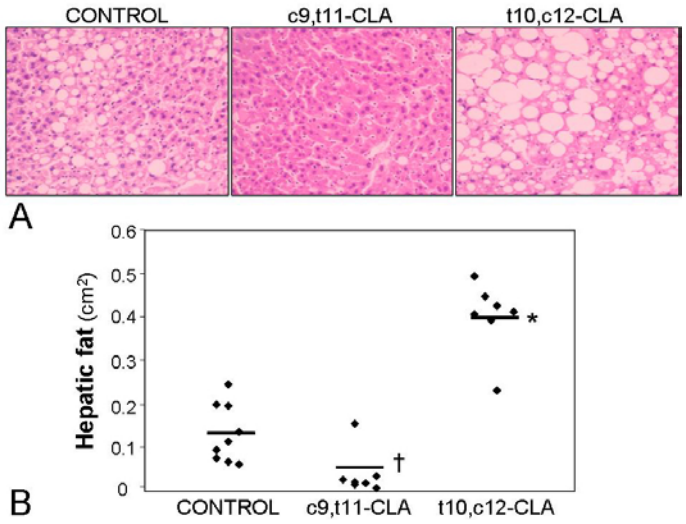

Figure 6. Effect of CLA-enriched diets on fat accumulation in the liver. (A) Representative micrographs of liver sections stained with hematoxylin and eosin from ApoE-deficient mice fed with control (n = 9), c9,t11-CLA (n = 7) or t10,c12-CLA diets (n = 7). (B) Result of morphometric quantification of fat content of liver sections from mice of the three experimental groups. Each symbol represents one animal, bars represent means. Statistical analysis was carried out by ANOVA. $P < 0.05$: * versus control and c9,t11-CLA -enriched diet; † versus control and t10,c12-CLA-enriched diet.

over-expression of all three NR4A receptors regulates the expression of gluconeogenic enzymes in a NBRE–dependent manner, indicating a direct modulation of the gluconeogenesis pathway by these transcription factors. NR4A receptors have also been implicated in glucose transport system and could be involved in insulin resistance mechanisms (45). Since acute changes in plasma NEFA lead to acute changes in gluconeogenesis (46) and some reports *in vitro* in cell culture have shown that NR4A receptors can be induced by NEFA (47), we hypothesized

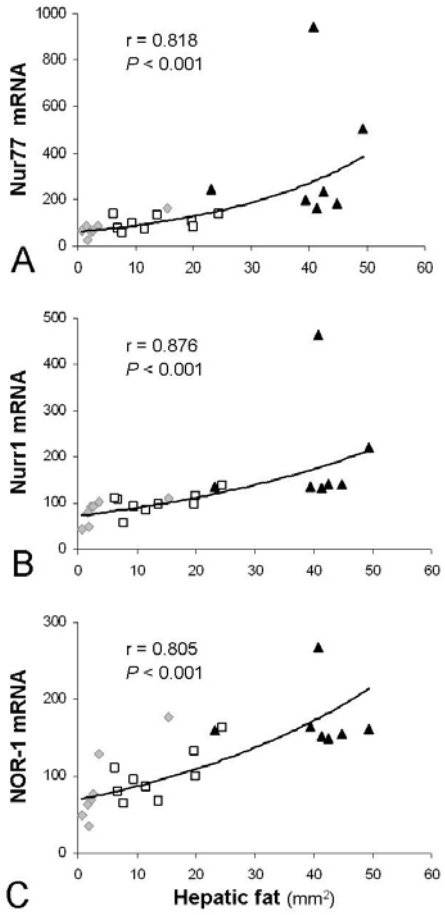

Figure 7. Hepatic expression of NR4A receptors correlates with fat accumulation in the liver. Results of Spearman correlation test between hepatic fat content (area in mm^2) and mRNA levels of NR4A receptors (arbitrary units) in the liver (Nur77 [A], Nurr1 [B] and NOR-1 [C]) in ApoE-deficient mice consuming control (squares), c9, t11-CLA (diamonds) and t10,c12-CLA (triangles) enriched diets.

that the chronic elevation of plasma NEFA levels by t10,c12-CLA could promote the expression of these transcription factors *in vivo*, showing their importance in metabolism. To test this hypothesis we evaluated the expression of NR4A receptors (Nur77, Nurr1 and NOR-1) in liver. Our data clearly show that all three NR4A receptors, in particular NR4A1 (Nur77), were up-regulated in the liver of animals receiving the t10,c12-CLA-enriched diet for 12 week, whereas control or c9,t11-CLA-enriched diets had not effect. In addition, this induction was accompanied by the hepatic overexpression of key enzymes involved in the gluconeogenic pathway including glucose-6-phosphatase, enolase, phosphoenolpyruvate carboxykinase and pyruvate carboxylase. By contrast, c9,t11-CLA down-regulated enolase, pyruvate carboxylase and glycerol kinase. Therefore, CLA exert an isomer-specific effect on the hepatic gluconeogenesis pathway consistent with their dissimilar effect on plasma glucose concentrations.

In mice t10,c12-CLA induce a dysfunctional cross-talk between adipose tissue and liver that results in metabolic disorders such dyslipidemia (13, 48, 49), hyperinsulinemia and reduced tissue response to insulin (23, 24, 26, 31) and hepatic steatosis (13, 22, 23, 48). In our study the increase in insulin levels observed in the t10,c12-CLA group did not reached statistical significance; however, this trend simultaneous to an increase in fasting plasma concentrations of glucose, triglycerides and NEFA suggest that this isomer favors an insulin-resistant state. t10,c12-CLA significantly induced liver steatosis, in agreement with previous studies in this model (13, 17, 23, 27, 48). This has been associated with the up-regulation of PPARγ and SREBP-1c and their responsive genes involved in fatty acid uptake and lipogenesis pathway such as FAS and the fatty acid transporter FAT/CD36 (23, 49). In our study CLA experimental diets had no effect on hepatic expression of SREBP-1 and FAS ("lipogenic genes"). However, t10,c12-CLA diet significantly increased hepatic expression of FAT/CD36, in agreement with previous studies (49), suggesting induction of fatty acid uptake. FAT/CD36 is a multifunctional membrane protein participating in uptake of long-chain fatty acids and modified LDL, playing an important role in lipid homeostasis. Interestingly, hepatic expression of NR4A

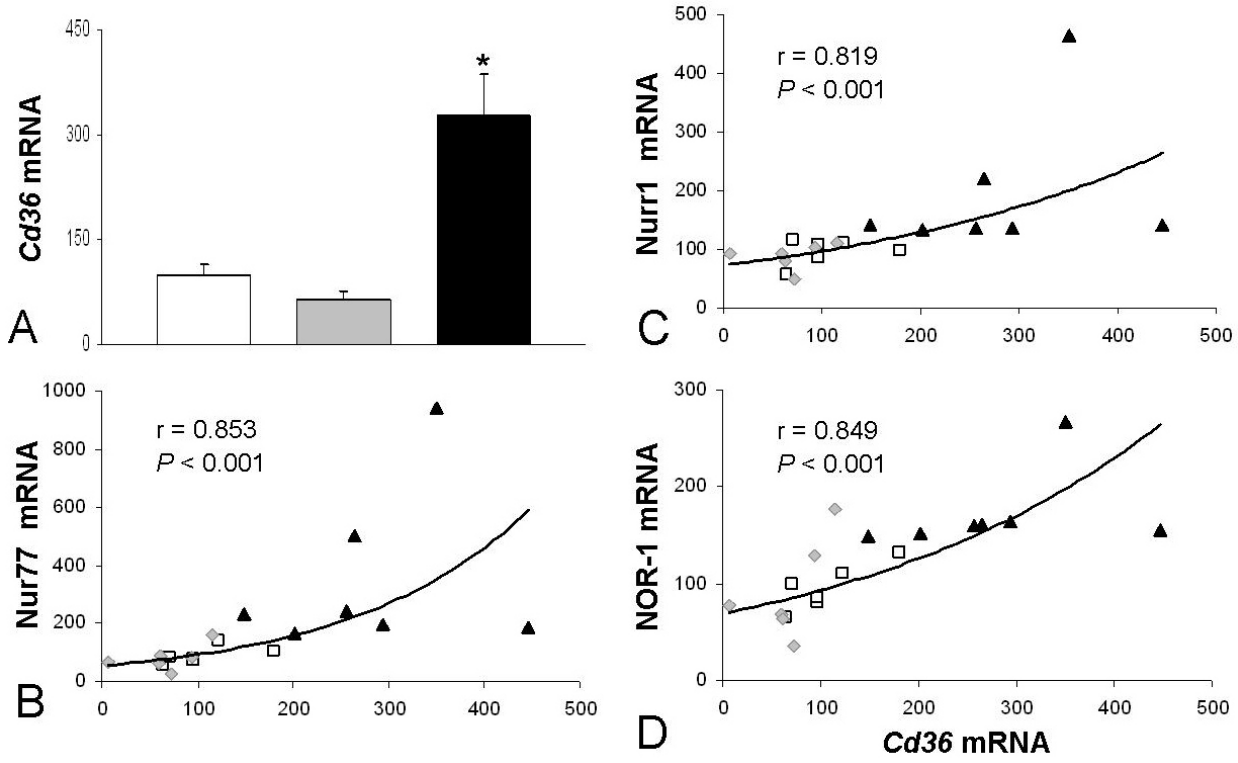

Figure 8. Effect of CLA-enriched diets on hepatic FAT/CD36 expression. (A) Hepatic expression of FAT/CD36 (relative to controls) in fasted ApoE-deficient mice fed control (n= 10; white bars), c9,t11-CLA- (n= 10; shaded bars) or t10,c12-CLA- (n= 9; black bars) enriched diets for 12 wk was analyzed by real-time PCR. Data are shown as mean ± SEM. Statistical analysis was carried out by ANOVA. P <0.01: * versus control and c9,t11-CLA-enriched diet. (B-D) Results of Spearman correlation test between expression of FAT/CD36 and mRNA levels of NR4A receptors (arbitrary units) in the liver (Nur77 [B], Nurr1 [C] and NOR-1 [D]) in ApoE-deficient mice consuming control (squares), c9, t11-CLA (diamonds) and t10,c12-CLA (triangles) enriched diets.

receptors positively correlates with both FAT/CD36 expression levels and the hepatic content of fat. In accordance with this, recently Maxwell *et al.* (50) have shown that inhibition of Nur77 in skeletal muscle cells strongly reduces FAT/CD36, expression suggesting a positive regulation of this fatty acid translocase by Nur77. However, information on NR4A target genes in liver is limited and the relative contribution of NR4A receptors, that exhibit dissimilar ability to heterodimerize between them and with RXR, to the hepatic metabolism it still poorly understood.

In summary, in ApoE-deficient mice the hyperglycemic and hyperlipidemic effect induced by t10,c12-CLA is associated to the up-regulation of the hepatic expression of NR4A receptors and their downstream gluconeogenic target genes. Furthermore, NR4A receptors could also mediate hepatic lipid accumulation through the up-regulation of FAT/CD36. The impact of NR4A receptors and their regulation in metabolic disorders in humans deserves to be further evaluated.

6. ACKNOWLEDGEMENTS

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Abbreviations ApoE: apolipoprotein E; CLA: conjugated linoleic acids; Eno: enolase; FAS: fatty acid synthase; FAT: fatty acid transporter; Fbp1: fructose-1,6-bisphosphatase; G6pc: glucose-6-phosphatase; Gyk: glycerol kinase; NBRE: NGFI-B response element; NFκB: nuclear factor kappaB; NGFI-B: nerve growth factor-induced clone B; NOR-1: neuron-derived orphan receptor-1; NR4A: nuclear receptor subfamily 4 group A; Pck1: phosphoenolpyruvate carboxykinase 1; Pcx: pyruvate carboxylase; PUFA: polyunsaturated fatty acids; SREBP: sterol regulatory element binding protein

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Send correspondence to: Jose Martinez-Gonzalez, Centro de Investigacion Cardiovascular, Hospital de la Santa Creu i Sant Pau (pabellon Nº 11), Sant Antoni Maria Claret 167, 08025 Barcelona (Spain), Tel: 34-93-5565896, Fax: 34-93-5565559, E-mail: jmartinez@csic-iccc.org

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