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Moderate doses of conjugated linoleic acid isomers mix contribute to lowering body fat content maintaining insulin sensitivity and a noninflammatory pattern in adipose tissue in mice☆

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Abstract

Conjugated linoleic acid (CLA) modulates body composition, especially by reducing adipose tissue. However, despite the increasing knowledge about CLA's beneficial effects on obesity management, the mechanism of action is not yet fully understood. Furthermore, in some human studies fat loss is accompanied by impairment in insulin sensitivity, especially when using the *trans*-10,*cis*-12 isomer. The aim of this work was to study the effects of moderate doses of CLA on body fat deposition, cytokine profile and inflammatory markers in mice. Mice were orally treated with a mixture of CLA isomers, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 (50:50), for 35 days with doses of CLA1 (0.15 g CLA/kg body weight) and CLA2 (0.5 g CLA/kg body weight). CLA had discrete effects on body weight but caused a clear reduction in fat mass (retroperitoneal and mesenteric as the most sensitive depots), although no other tissue weights were affected. Glucose and insulin were not altered by CLA treatment, and maintenance of glucose homeostasis was observed even under insulin overload. The study of gene expression (Emr1, MCP-1, IL-6, TNFα, PPARγ2 and iNOS) either in adipocytes and/or in the stromal vascular fraction indicated that CLA does not lead to the infiltration of macrophages in adipose tissue or to the induction of expression of pro-inflammatory cytokines. The use of a mixture of both isomers, as well as moderate doses of CLA, is able to induce a reduction of fat gain without an impairment of adipose tissue function while preserving insulin sensitivity.

Keywords: Conjugated linoleic acid; Insulin sensitivity; Macrophage infiltration; Mice; Adipose tissue

1. Introduction

Conjugated linoleic acid (CLA) has been tested extensively for its ability to modify body composition in humans. In particular, the mixture containing mainly the two bioactive isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA (see Ref. [1] for a review) reduces body fat at the same time as preserving lean body mass [2–4]. Supplementation with the two bioactive CLA isomers is mainly associated with beneficial effects such as stimulation of immune response [5,6] and improvement of insulin sensitivity [7] and lipid metabolism [8]. However, studies on type 2 diabetics showed conflicting results [9,10] and some studies on healthy obese subjects have found an increase in markers of lipid peroxidation [11]. Furthermore, when supplemented with the isomers separately, particularly at high concentration of either the *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA, increased insulin resistance in obese subjects by these single isomers has been reported [12–14], whereas the mixture of both bioactive isomers did not affect the insulin sensitivity of subjects with the metabolic syndrome [12] and improved it in sedentary men [7]. Additionally, supplementation with CLA triggers a fat loss which is associated with insulin resistance, robust hyperinsulinemia and massive steatosis

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[15,16] in sensitive animal models such as mice. Antiadipogenic effects of CLA and liver steatosis have been attributed to the *trans*-10,*cis*-12 CLA isomer [16,17]. Furthermore, proinflammatory cytokine concentrations were recently found to increase in white adipose tissue of mice treated with *trans*-10,*cis*-12 CLA associated with a recruitment of macrophages in the adipose tissue [18].

Reported adverse effects of CLA in mice, which are the more sensitive species, are accompanied by important losses of fat, thus driving an excess of fatty acids to the liver and fatty acids also competing with the flux of glucose to other peripheral tissues, a situation that, together with selective effects of CLA on lypolisis and lipogenesis, may give the impression of loss of insulin sensitivity and, perhaps, a proinflammatory state. However, this is not the situation seen in humans, in whom very modest effect on fat loss has been demonstrated while preserving muscle mass. The aim of this study was to analyze the effects of a supplementation with moderate doses of bioactive isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA in mice fed with a standard-fat diet, an animal model particularly sensitive to the potential adverse effects of CLA. Our interest was to use the bioactive compounds usually tested in humans and a range of doses comparable to the doses assayed in humans. In addition, it was our interest to test the potential infiltration of macrophages in adipose tissue and to analyze the profile of expression of cytokines.

2. Methods and materials

2.1. Animals

Male mice (C57BL/6J) from Charles River (Barcelona, Spain) were housed in groups of four in plastic cages, acclimated to 22°C with a 12-h light/12-h dark cycle. Animals were fed *ad libitum* with a standard diet (D12450B) (Research Diets Inc, New Brunswick, Canada) which contains 10% calorie content as fat (25 g/100 g soybean oil and 20 g/100 g lard), 70% calorie content as carbohydrate (315 g/100 g corn starch, 35 g/100 g maltrodextrin 10, 350 g/100 g sucrose and 50 g/100 g cellulose) and the remaining 20% as protein (casein and L-cystine). Food intake and body weight were recorded every 3 days during the experiment. Fresh food was provided to the mice biweekly. At 30 days of treatment, animals were starved for 3 h and tail blood samples were obtained to perform plasma determinations and were then submitted to the insulin tolerance test (ITT). One week after, the animals were sacrificed under feeding conditions.

All experimental procedures were performed according to both national and institutional guidelines for animal care and use.

2.2. Conjugated linoleic acid treatment

The CLA used was Tonalin TG 80 derived from safflower oil (kindly provided by Cognis). Tonalin is composed of triglycerides containing approximately 80% CLA, with a 50:50 ratio of the active CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12.

Mice weighing 20±0.2 g were randomly assigned to three experimental oral treatments: sunflower oil (control), CLA1 or CLA2 for 37 days. Two different doses of CLA were assessed in this study: CLA1 (0.15 g CLA/kg body weight) and CLA2 (0.50 g CLA/kg body weight), taking as reference the weight of the animals at the beginning of the experiment. Therefore, animals received a daily amount of Tonalin equivalent to 3 mg CLA/animal in the CLA1 group and 10 mg/animal in the CLA2 group. An adequate amount of commercial sunflower oil was given to the animals to achieve isocaloric load between groups.

2.3. Insulin tolerance test

Insulin tolerance test was carried out after 30 days of oral treatment. Glucose concentration was determined from tail blood samples. Then, recombinant human insulin (Humulin R; Eli Lilly, Spain), previously diluted in 0.9% saline, was intraperitoneally injected (0.8 U/kg body weight). Subsequent blood samples were taken from the tail tip, and glucose was directly measured at 15, 30, 60, 90 and 120 min postinjection using an Accu Check Sensor (Roche Diagnostics, Barcelona, Spain).

2.4. Sacrifice and tissue sampling

Mice were anesthetized by intraperitoneal injection of a mixture of xilacine (10 mg/kg body weight) and ketamine (100 mg/kg body weight) and blood collected by cardiac puncture. Liver, pancreas, gastrocnemius, stomach, brown and white adipose depots were rapidly removed, weighed, rinsed with saline containing 0.1% diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid and stored at −70°C. Blood collected by cardiac puncture with heparinized syringe and needle (0.2% heparin diluted with saline; Sigma) was centrifuged at 2500 rpm for 10 min at 4°C and plasma obtained was stored at −70°C for later analysis.

2.5. Plasma analysis

Adiponectin and insulin plasma concentrations were measured using a rat/mouse adiponectin enzyme-linked immunosorbent assay (ELISA) kit (Phoenix Europe GmbH, Karlsruhe, Germany) and Insulin Mouse Ultrasensitive ELISA (DRG Instruments GmbH, Marburg, Germany), respectively. Resistin and leptin plasma concentrations were also assessed by ELISA using the following commercial kits: Mouse Resistin Quantikine ELISA Kit and Mouse Leptin Quantikine (R&D Systems, Minneapolis, MN, USA). Commercial enzymatic colorimetric kits were used for the determination of plasma nonesterified fatty acids (NEFAs; Wako Chemicals GmbH, Neuss, Germany) and circulating concentrations of triglycerides (Sigma Diagnostics, Madrid, Spain).

2.6. Isolation of mature adipocytes and stromal vascular fraction from epididymal fat depots

Fresh epididymal white adipose tissue was minced into small pieces and placed in sterile plastic tubes with Krebs-Ringer buffer containing 25 mM $NaHCO_3$, 11 mM glucose, 25 mM Hepes, pH 7.4, 2% bovine serum albumin (BSA) (Fraction V, Sigma Diagnostics) and 1.5 mg/ml collagenase type I (Gibco-Invitrogen, Prat de Llobregat, Spain). The ratio between adipose tissue mass and incubation solution was 1:4 (w/v). The tissue suspension was incubated at 37°C with gentle shaking for 45–60 min. Once digestion was completed, samples were passed through a sterile 250-μm nylon mesh (Sefar America, Inc., Depew, NY, USA). The suspension was centrifuged at 200×*g* for 10 min, the pelleted cells were collected as stromal vascular fraction (SVF) and the floating cells were considered the mature adipocyte-enriched fraction. The latter was washed twice with Krebs-Ringer-bicarbonate-Hepes-BSA buffer and centrifuged as above. The SVF was resuspended in erythrocyte lysis buffer consisting of 0.154 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, and incubated at room temperature for 10 min. The erythrocyte-depleted SVF was centrifuged at 400×*g* for 5 min, the pellet was resuspended and washed twice in Krebs-Ringer-bicarbonate-Hepes-BSA buffer and centrifuged at 400×*g* for 5 min. After washing and short centrifugation steps, both samples, the SVF and the mature adipocyte fraction, were resuspended and disrupted by adding the buffer provided by the RNA extraction kit used afterwards (see below) and kept at −70°C.

2.7. Real-time quantitative polymerase chain reaction analysis

Total RNA was extracted using an RNAeasy Mini Kit from Qiagen (Barcelona, Spain). RNA was quantified using the NanoDrop Spectrophotometer ND-1000. Real-time polymerase chain reaction (real-time PCR) was used to measure mRNA expression levels of target genes. Aliquots of 0.5 μg of total RNA (in a final volume of 10 μl) were denatured at 90°C for 1 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 42°C for 60 min, with a final step of 5 min at 99°C in a Perkin-Elmer 9700 Thermal Cycler (PerkinElmer, Wellesley, MA, USA). Real-time PCR was completed using the LightCycler System with SYBR Green I (Roche Diagnostic GmbH, Mannheim, Germany). Primer sequences were as follows: *Adiponectin* forward, 5′-GCTCAGGATGCTACTGTTG-3′; *adiponectin* reverse, 5′- TCTCACCCTTAGGACCAAG-3′; *leptin* forward, 5′-TTGTCACCAGGATCAATGACATTT-3′; *leptin* reverse, 5′- GACAAACTCAGAATGGGGT-GAAG-3′; serum retinol-binding protein 4 (*RBP4)* forward, 5′-ACTGGGGTGTAGCCTCCTTT-3′; *RBP4* reverse, 5′-GGTGTCGTAGTCCGTGTCG-3′; glucose transporter type 4 (*Glut4)* forward, 5′-GGCATGCGTTTCCAGTATGT-3′; *Glut4* reverse, 5′-GCCCCTCAGTCATTCTCATC-3′; peroxisome proliferator-activated receptor gamma 2 (*PPARγ2*) forward, 5′-GGTGAAACTCTGGGAGATTC-3′; *PPARγ2* reverse, 5′-TAATAAGGTGGAGATGCAGG-3′; monocyte chemotactic protein-1 (*MCP-1)* forward, 5′-GCTCTCT-CTTCCTCCACCAC-3′; *MCP-1* reverse, 5′-GCTTCTT-TGGGACACCTGCT-3′; epidermal growth factor module-containing mucin-like receptor 1 (*Emr1*) forward, 5′-TTTCCTCGCCTGCTTCTTC-3′; *Emr1* reverse, 5′-CCCCGTCTCTGTATTCAACC-3′; interleukin-6 (*IL-6*) forward, 5′-TGGGAAATCGTGGAAATGAG-3′; *IL-6* reverse, 5′-GAAGGACTCTGGCTTTGTCTT-3′; tumor necrosis factor alpha (*TNFα*) forward, 5′-CGTCGTAG-CAAACCACCAA-3′; *TNFα* reverse, 5′-GAGAACCTGG-GAGTAGACAAGG-3′; inducible nitric oxide synthase (*iNOS*) forward, 5′-GGCAGCTACTGGGTCAAAGA-3′; *iNOS* reverse, 5′-TCTGAGGGCTGACACAAGG-3′; *18s* forward, 5′-CGCGGTTCTATTTTGTTGGT-3′; *18s* reverse, 5′-AGTCGGCATCGTTTATGGTC-3′. All primers were obtained from Sigma. Each PCR was performed in a total volume of 8 μl, made from diluted cDNA template, forward and reverse primers (1 μM each), and SYBR Green I master mix (including Taq polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye and dNTP mix). In order to verify the purity of the products, a melting curve was produced after each run by increasing the temperature of the reaction mixtures up to 95°C, by 0.1°C/s, starting at 55°C for 10 s. 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. Relative quantification of a target gene was calculated based on efficiency and the crossing point deviation of an unknown sample vs. a control, and expressed in comparison to the reference housekeeping gene *18s* [19]. Target gene mRNA expression normalized by the internal control *18s* was expressed relative to the control group. Data were expressed using both mRNA concentration in each cellular fraction and total mRNA content.

2.8. Statistical analysis

Data are presented as means±S.E.M. Repeated measure analyses of variance was used to determine differences in body weight gain. One-factor ANOVA was used to determine the significance of the differences in tissue weights, plasmatic concentrations of metabolites, mRNA abundances and levels with different treatments. If there was a significant difference, a least significant difference (LSD) test was used to determine the particular effect that caused that difference. $P<.05$ was statistically significant, and different superscripts discriminate differences between groups. The analysis was performed using the SPSS program for Windows version 14 (SPSS, Chicago, IL, USA).

3. Results

3.1. Body and tissue weights, food intake

CLA treatment was accompanied by a decrease in total fat mass (Table 1), particularly with the higher dose of

Table 1
Tissue weights (g) in mice supplemented with CLA

	Control	CLA1	CLA2
White adipose tissues			
Epididymal	0.365±0.008[a]	0.363±0.016[a]	0.240±0.020[b]
Retroperitoneal	0.092±0.007[a]	0.051±0.004[b]	0.036±0.004[c]
Mesenteric	0.230±0.012[a]	0.195±0.012[b]	0.158±0.010[c]
Sum	0.662±0.021[a]	0.609±0.027[a]	0.433±0.029[b]
Brown adipose tissue	0.120±0.005[a]	0.123±0.005[a]	0.088±0.004[b]
Pancreas	0.136±0.013	0.149±0.009	0.152±0.007
Gastrocnemius	0.261±0.013	0.276±0.006	0.271±0.012
Liver	1.061±0.029	1.077±0.037	1.142±0.033
Stomach	0.101±0.003	0.106±0.005	0.109±0.003

Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 9–12 mice. Means in a row without a common letter differ, $P<.05$ (one-factor ANOVA followed by LSD test).

CLA (35% lower adipose tissue weights in CLA2 vs. control group; $P<.001$). Although minor effects were seen on the rate of body weight change during the treatment, body weight gain was lower in CLA-treated groups (Fig. 1) (repeated measure analyses of variance: $P<.05$, effect of treatment), which was associated with smaller fat accumulation. Mesenteric and epididymal fat pads were respectively 31% and 34% lower in CLA2 animals vs. controls. Specific sensitivity was seen in the retroperitoneal fat pad, which was diminished twofold (61% lower) compared with the other depots. Treatment with CLA1 also affected the size of the retroperitoneal depot (45% lower) and to a lesser extent the mesenteric (15% lower) with respect to the controls. Neither the weights of liver, pancreas, stomach nor the gastrocnemius muscle was affected by treatment.

The dietary energy intake during the study was not significantly different between groups (286±5.1 in control, 271±30.6 in CLA1 and 280±7.1 kcal/animal in the CLA2 group).

3.2. Insulin tolerance test, homeostasis model assessment and plasmatic factors

Plasma concentrations of glucose and insulin were not statistically different between groups after 3 h of starvation (Table 2). Treatment with CLA did not alter the response to an ITT either measuring the change in plasmatic glucose concentrations (data not shown) or the area under the curve (681±37 in control, 665±44 in CLA1 and 734±39 mmol of glucose/min per liter in the CLA2 group). The homeostasis model assessment index (2.94±0.22 in control, 3.12±0.42 in CLA1 and 3.51±0.32 in the CLA2 group) was not different between groups either.

Plasma adiponectin concentrations in CLA1 were close to control values, whereas in the CLA2 group they were lower than those of control and CLA1 ($P<.001$, Table 2) under both fasting and feeding conditions. Concerning leptin concentrations, no significant effect in CLA groups was seen with respect to the control. However, leptin concentrations were lower in CLA2 with respect to the CLA1 group ($P<.01$, Table 2). Resistin concentrations were not affected by CLA treatment at the lower dose, whereas they showed a diminution in the CLA2 group. The treatment with CLA did not alter the plasma concentrations of glycerol (data not shown), NEFAs or triglycerides (Table 2).

3.3. Gene expression in adipocytes and SVF

CLA treatment showed a tendency to increase mRNA concentration in the SVF and particularly in mature

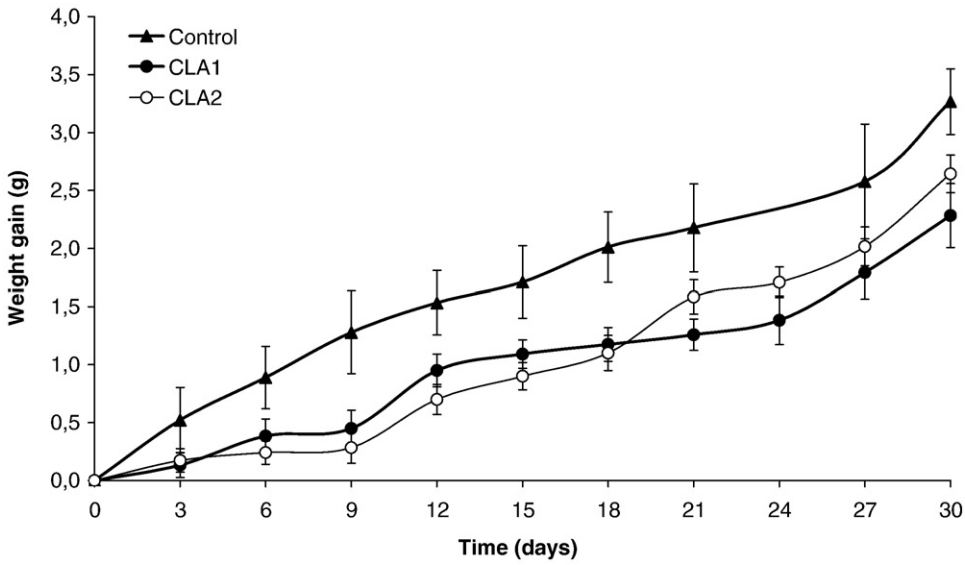

Fig. 1. Effects of CLA on body weight gain in mice. Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 9–12 mice. Repeated measures analyses of variance of decreases in body weight gain in CLA-treated animals were significant ($P<.05$). No differences between doses were found.

Table 2
Effects of CLA treatment on plasmatic concentrations of metabolites in mice

	Control	CLA1	CLA2
Fasting conditions (3h)			
Glucose (mmol/L)	6.7±0.3	6.8±0.2	7.2±0.3
Insulin (μg/L)	0.41±0.07	0.43±0.10	0.49±0.04
Adiponectin (μg/ml)	21.02±0.82^{a}	25.09±2.42^{a}	16.01±0.91^{b}
Feeding conditions			
Adiponectin (μg/ml)	22.62±0.70^{a}	23.75±1.06^{a}	16.43±0.85^{b}
Leptin (ng/ml)	4.84±0.53^{ab}	7.04±0.87^{a}	3.78±0.50^{b}
Leptin/adiponectin	0.22±0.02	0.30±0.03	0.22±0.03
Resistin (ng/ml)	23.33±0.96^{a}	25.99±1.38^{a}	19.16±0.70^{b}
Insulin (μg/L)	0.16±0.04	0.17±0.03	0.18±0.03
NEFAs (mg/dl)	16.66±3.25	13.33±2.58	13.03±2.12
Triglycerides (mg/ml)	0.79±0.09	0.70±0.08	0.67±0.06

Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 8–12 mice. Means in a row without a common letter differ, $P<.05$ (one-factor ANOVA followed by LSD test).
Samples from fasting conditions were bled from the tail tip from conscious animals. Samples from fed animals were obtained by cardiac puncture from anesthetized animals. Leptin/adiponectin ratio has been multiplied by a factor of 10^3.

Table 4
Effects of CLA supplementation on relative expression of target mRNA in mature adipocytes and SVF in mice

	Control	CLA1	CLA2
Mature adipocytes			
Adiponectin	100±4^{a}	71±9^{b}	57±3^{b}
Leptin	100±19^{a}	136±1^{a}	57±6^{b}
RBP4	100±7^{a}	126±6^{b}	87±4^{a}
Glut4	100±6^{a}	94±5^{a}	71±4^{b}
PPARγ2	100±1^{a}	80±4^{ab}	63±7^{b}
MCP-1	100±2^{a}	64±8^{b}	28±7^{c}
Stromal vascular fraction			
IL-6	100±17	98±15	72±20
TNFα	100±13	70±8	79±13
iNOS	100±15	71±4	59±11
Emr1	100±18	90±11	97±9
MCP-1	100±17	101±17	89±17
PPARγ2	100±17	72±5	68±12

Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 6–8 mice. Means in a row without a common letter differ, $P<.05$ (one-factor ANOVA followed by LSD test).

adipocytes where it attained statistical significance (Table 3). This is of special relevance because of the minor size of adipose depots in CLA-treated mice.

Mature adipocytes of animals treated with CLA showed a lower expression of most of the target mRNA determined and, particularly, at the highest treated dose — adiponectin (57%, $P<.001$), leptin (57%, $P<.038$), Glut4 (71%, $P<.01$), PPARγ2 (63%, $P<.01$) and MCP-1 (28%, $P<.001$) — with respect to the control group (Table 4). However, in the CLA1 group, only the decrease in adiponectin (29% lower, $P<.05$) and MCP-1 (36% lower, $P<.05$) attained statistical significance. The CLA1 group also showed an increase (by 26%, $P<.01$) of RBP4 expression vs. control. No effects of CLA treatment on gene expression were appreciated on the SVF, either on PPARγ2, inflammatory factors (IL-6, TNFα, iNOS), macrophage marker (Emr1) or on the main recruitment macrophage factor MCP-1 (Table 4). Taking into account the important change in adipose size by the CLA treatment and the variation in total mRNA concentration in epididymal adipose tissue, the values of mRNA gene expression were referred to the total mRNA content in the depot studied. Under this new perspective, gene expression data showed a slightly different profile than above. In mature adipocytes, only leptin increased in CLA1 (twofold, $P<.05$) and the tendency to decrease MCP-1 expression was maintained (ANOVA: $P<.05$, effect of treatment); the rest of the values were similar between CLA-treated animals and controls (Fig. 2). In SVF, no changes were observed by CLA, although a nonstatistically significant tendency in the reduction of mRNA expression of TNFα and iNOS was seen (Fig. 3).

Table 3
mRNA levels (μg/g tissue) in mature adipocytes and SVF cells isolated from epididymal adipose tissue in CLA-treated mice

	Control	CLA1	CLA2
Adipocytes	12±1^{a}	15±3^{ab}	23±4^{b}
Stromal vascular fraction	12±1	16±2	17±1

Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 6–8 mice. Means in a row without a common letter differ, $P<.05$ (one-factor ANOVA followed by LSD test).

4. Discussion

In this study, we have demonstrated that supplementation with moderate doses of an equimolar mix of *cis*-9, *trans*-11 and *trans*-10,*cis*-12 CLA in mice fed with a standard-fat diet allows for the maintenance of insulin sensitivity and does not lead to the infiltration of macrophages in adipose tissue or to the induction of expression of pro-inflammatory cytokines.

The commercial product Tonalin was selected as the source of dietary CLA because most human studies designed to assess the effects on body weight and/or composition are done using this or other available products sharing its characteristics (i.e., containing equimolar mix of both bioactive CLA isomers). Mice have been described as an animal model particularly sensitive to potential adverse effects of CLA administration [15,20], and the *trans*-10,*cis*-12 isomer seems to be the most adverse [18,21,22]. However, most animal studies have been performed with diets supplemented with 0.5–1.5% CLA, combining either a mixture of the two active isomers or only one of them (for review, see Refs. [17,23,24]). Our focus was to assess in mice the effects of the CLA used in humans but at

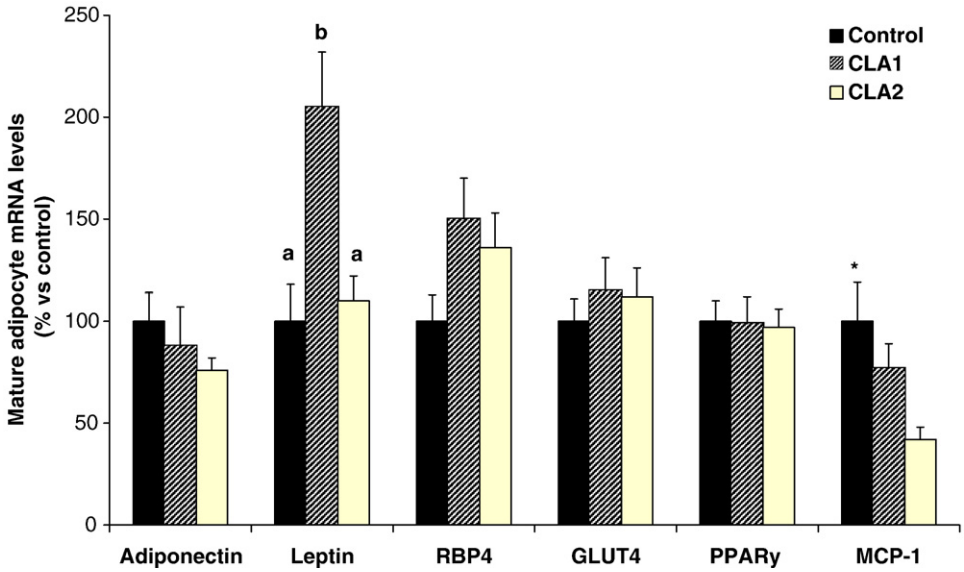

Fig. 2. Contribution of mature adipocytes isolated from epididymal fat depot to the expression of target mRNA in CLA-treated mice. Initial data, derived from equal total amount of RNA (shown in Table 4), were referred to the total mRNA content in the depot. Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. mRNA expression of the control group was designated as 100% to normalize the mRNA expression of CLA-treated groups to this value. Error bars represent means±S.E.M. (n=5–8). By one-factor ANOVA only leptin gene expression increased in CLA1 (ANOVA followed by LSD test, bars without a common letter differ, P<.05), and a significant tendency to decrease MCP-1 gene expression by treatment was seen (*). The rest of the genes were not affected by CLA treatment.

lower doses than those usually used in mice. When this amount is referred to the weight of the animals, this implies a daily dose that is around 50 times higher (1% CLA) than doses successfully used in human trials [1]. Our approach was to test a range of doses (150 and 500 mg CLA/kg per day, in CLA1 and CLA2, respectively) that are about 3–10 times (per kilogram of body weight) the 3.4 g CLA/day usually recommended for use in humans (assuming a body weight of 70 kg). In this way, taking into account that mice are the most sensitive specie [25], it is intended here to assess the efficacy of the treatment in mice, aiming to moderately reduce body fat content without showing some of the adverse effects reported. In addition, we were interested in doses of CLA below the nonobserved adverse effects levels, seeking for the

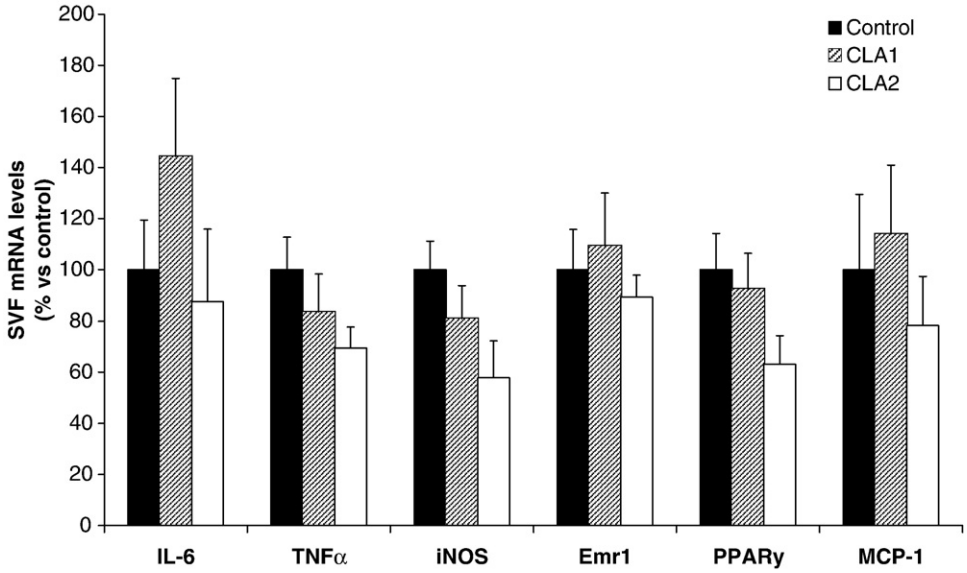

Fig. 3. Contribution of SVF isolated from epididymal fat depot to the expression of target mRNA in CLA-treated mice. Initial data, derived from equal total amount of RNA (shown in Table 4), were referred to the total mRNA content in the depot. Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. mRNA expression of the control group was designated as 100% to normalize the mRNA expression of CLA-treated groups to this value. Error bars represent means±S.E.M. (n=6–8). By one-factor ANOVA, CLA treatment did not affect gene expression.

compromise between safety and efficacy, which has been estimated to correspond to a CLA intake of about 500 mg/kg per day [20].

As expected, we found discrete effects on body weight. However, CLA caused a clear reduction in fat mass at the doses tested and no other tissue weights were affected. Contrary to other studies in mice using higher doses [21], no lipodystrophy was caused, even when the treatment was prolonged for more than 1 month, and the modest fat reduction found resembled what is seen in human trials [1]. Retroperitoneal and mesenteric tissues were the most sensitive adipose tissues, which showed decreased weight at the lower CLA dose used, while mobilization of epididymal fat pad required the higher dose of CLA. These results are in accordance with the finding that body fat reduction by CLA is also region specific in humans [3]. The referred human study included mainly women, and the CLA was more active in decreasing fat in the legs, while showing also a tendency to reduce abdominal fat when considering both sexes. Here we show that abdominal fat was the most sensitive depot to the action of CLA in male mice.

Increased inflammatory properties of adipose tissue macrophages recruited during CLA supplementation have been recently reported [18], and these cells are a considerable source of TNFα and IL-6 that can induce insulin resistance in adipocytes [26]. Adipocytes are able to synthesize and secrete the chemokine MCP-1, a recruiting factor for circulating monocytes [27], as well as nonfat cells present in adipose tissue [28,29]. These and other data are giving strong support to the fact that adipose tissue is a potent source of interleukins and other cytokines, but the majority of this release is due to nonfat cells in the adipose tissue [30]. In order to establish the specific role of both adipocytes and nonfat cells, under CLA supplementation, adipose tissue cellular fractioning and separation were performed and both types of cells were independently analyzed for expression of inflammatory factors and related markers. Epididymal fat depot was chosen as a representative adipose tissue because it has been reported that macrophage content and transcripts expression are comparable between different adipose depots in mice [31], and it is the largest among those easily distinguished anatomically, allowing then to minimize the number of animals.

Isolation of mRNA from epididymal fat revealed the presence of a higher amount in adipocytes from the CLA2 group. In PCR determinations, we performed gene amplification from samples containing an equal amount of total RNA in the different groups studied. Therefore, these results represented the gene abundance from samples containing the same amount of total RNA, but they could reflect a different number of adipocytes and this distinction can be of particular significance when CLA causes a reduction of fat depots together with an increase in total RNA content. To interpret our data in a more physiologically meaningful way, we calculated the changes in gene expression referred to the total tissue contribution and also in the conventional way, as transcript per gram of tissue, to be able to compare our data with previous references. Adiponectin and leptin gene expression in adipocytes showed a similar trend to that seen in related studies [18]. However, this profile changed — for example, leptin increase was magnified in the CLA1 group and adiponectin decrease was attenuated in CLA-treated mice — when the contribution of the depot to the whole organism was taken into account. From this angle, expression levels of Glut4 transporter and RBP4 protein in adipocytes were compatible with a status of insulin sensitivity, in close accordance with the other biomarkers studied. Our results point to the importance of taking into account not only the specific gene expression but also the total contribution of the organ of interest in CLA studies.

Feeding high doses of CLA to mice has been associated with impairment of insulin sensitivity [15,16,21,22,32], which has been attributed to a rapid and significant reduction of adipose tissue and a sharp decline in insulin-sensitizing adipokines such as leptin and adiponectin [22]. In this study, glucose and insulin were not altered by CLA treatment and maintenance of glucose homeostasis was observed even under insulin overload, suggesting that the fat loss, although moderate, was slow enough to keep up the metabolic control on insulin sensitivity.

Although higher leptin expression was found in epididymal adipocytes from CLA1 animals, the plasma concentration in CLA-treated animals was not different from control animals (although CLA2 showed lower concentration than CLA1 animals). Although we cannot rule out opposite changes in other synthesizing organs, in any case, plasma concentration was in accordance with the moderate reduction seen in fat mass [33], which was more efficient at the higher dose of CLA. Another aspect in our study concerns the circulating concentrations of inflammatory adipokines. Leptin and resistin are considered pro-inflammatory cytokines [34,35], while adiponectin seems to have an anti-inflammatory role [36] and decreases in association with insulin resistance, type 2 diabetes and visceral adiposity [37]. Therefore, the lack of change (in CLA1 group) or the reduction (in CLA2 group) in plasma resistin contributes, together with leptin, to preserving an anti-inflammatory profile in the CLA-treated mice. Concerning the role of adiponectin, recent studies have shown an improvement in glucose/insulin metabolism without increasing plasma adiponectin [38] and, in general, plasma concentrations correlate well with expression in adipose tissue [37]. In our animals, plasma adiponectin decreased only at the higher dose of CLA tested, whereas expression in epididymal adipocytes showed a tendency to decrease following a dose–response pattern without attaining statistical significance. Interestingly, the leptin/adiponectin ratio is not altered by CLA treatment at the doses tested in our study. This could suggest that the decrease of leptin (accompanying fat loss) was in concordance with lower adiponectin levels, reaching a novel set point between these two cytokines that still corresponds to the range of normality (seen by weight loss, maintenance of insulin sensitivity, lack of macrophage

infiltration and nonexpression of inflammatory markers). Our results are in close agreement with a recent study that, using ob/ob mice fed with 1.5% CLA, found a depletion of both leptin and adiponectin and this is accompanied by insulin resistance and macrophage infiltration. However, when mice received a daily injection of leptin, depletion of adiponectin is not so strong and there is neither insulin resistance nor macrophage infiltration, although they find hepatic steatosis [39]. Authors suggest that a basal level of both leptin and adiponectin may be critical to maintaining energy homeostasis, and our doses tested seem to fulfill this requirement.

A number of studies show that adipose tissue contains macrophages that participate in the inflammatory changes found in obesity and contribute to insulin resistance and hepatic steatosis [31,40–43]; as mentioned above, a recent paper has also found that nutritional supplementation with *trans*-10,*cis*-12-CLA induces local infiltration of macrophages in adipose tissue and induction of pro-inflammatory cytokines and MCP-1 expression [18]. Interestingly, mice supplemented with a *cis*-9,*trans*-11-CLA diet show a reduction in macrophage infiltration and a marked down-regulation of several inflammatory markers in adipose tissue [44]. Therefore, it was of great interest to assess the inflammatory role of potentially infiltrated macrophages in adipose tissue of mice fed with both active isomers, replicating conditions of human CLA treatments (lower doses, both isomers and in mice subjects as sensitive species), whereas to assess the specific role of each isomer per se under these conditions was out of the scope of the present study.

MCP-1 is a critical factor in the recruitment of macrophages to sites of injury and inflammation [45]. Genetic deficiency of MCP-1 and its receptor chemokine (C-C motif) receptor 2 (CCR2) reduces macrophage accumulation in adipose tissue of high fat-fed obese mice and partially protects against the development of obesity. On the other hand, mice overexpressing MCP-1 have increased numbers of macrophages in adipose tissue along with increased insulin resistance [46,47]. The expression of MCP-1 showed a decrease by CLA treatment in isolated mature adipocytes. This was in accordance with the lack of macrophage recruitment in adipose tissue, reflected by Emr1 and MCP-1 markers in SVF. Furthermore, the absence of higher pro-inflammatory cytokine expression (IL-6 and TNFα), PPARγ2 and iNOS induction in SVF was a result indicative of an absence of macrophage infiltration and inflammation in CLA-treated animals.

In summary, the use of moderate doses of the mixture of the two main active isomers may be preferable to the use of the single compounds when considering inflammatory potential and insulin tolerance. When given together as a mixture, the antagonistic role of *cis*-9,*trans*-11-CLA [44] and *trans*-10, *cis*-12-CLA [18] may compensate each other, resulting in changes in adiposity without detrimental effects. In our study, CLA treatment was able to attenuate body fat deposition in mice fed with a standard diet, without impairment of insulin sensitivity and in the absence of pro-inflammatory outcomes in adipose tissue. Conversely, an anti-inflammatory status in adipose tissue seems to be promoted by CLA which may help in the preservation of normal adipose tissue function without adverse consequences.

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