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Effects of *trans*-10,*cis*-12 CLA on liver size and fatty acid oxidation under energy restriction conditions in hamsters

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ABSTRACT

Objective: Little evidence exists concerning the effects of *trans*-10,*cis*-12 conjugated linoleic acid (CLA) under energy restriction. Thus, the effects of this CLA isomer on adipose tissue size, liver composition, as well as on expression and activity of carnitine-palmitoyl transferase I (CPT-I) and acyl CoA oxidase (ACO), in hamsters fed an energy-restricted diet were analyzed.
Methods: Hamsters were fed a high-fat diet for 7 wk and then subjected to 25% energy-restricted diets supplemented with 0.5% linoleic acid or 0.5% *trans*-10,*cis*-12 CLA for 3 wk. Serum insulin, free-triiodothyronine and non-esterified fatty acid levels, liver triacylglycerol, protein and water contents, and CPT-I, ACO, and Peroxisome proliferator-activated receptor alpha (PPARα) expressions and enzyme activities were assessed.
Results: Energy restriction reduced liver size, serum levels of insulin, free-triiodothyronine, and non-esterified fatty acid and increased CPT-I activity. Liver composition was not modified. No differences were found between both restricted groups, with the exception of CPT-I and ACO oxidative enzyme activities, which were greater in hamsters fed the CLA diet.
Conclusions: Energy restriction does not cause *trans*-10,*cis*-12 CLA to induce liver hyperplasia. Although this CLA isomer increases liver CPT-I and ACO activities, this effect does not result in reduced hepatic triacylglyerol content or decreased adipose tissue size. Consequently, this CLA isomer seems not to be a useful tool for inclusion in body weight loss strategies followed during obesity treatment.

Introduction

Numerous studies have demonstrated that the *trans*-10,*cis*-12 isomer of conjugated linoleic acid (CLA) reduces adiposity by decreasing fat deposition in rodents [1]. This effect has been observed in all the rodent species analyzed, but differences in the magnitude of body fat reduction have been identified (mice > hamsters > rats) [1]. It has been proposed that this CLA body-fat lowering action is due to multiple mechanisms, some of which, such as decreased lipoprotein lipase activity, decreased proliferation and differentiation of preadipocytes, and increased apoptosis, take place in the white adipose tissue [1,2].

Moreover, the liver, which plays an important role in body lipid metabolism, was shown to be affected by CLA feeding and, in turn, hepatic effects of CLA are involved in its regulation of body fat accumulation [1,2]. In many studies CLA feeding has resulted in increased fatty acid oxidation in mitochondria and microsomes in either isolated adipocytes or liver [3–6]. This means that CLA directs fatty acids toward oxidative pathways rather than to their storage as triacylglycerols in adipose tissue.

The vast majority of published studies performed in rodents has been carried out by adding CLA to either normal fat or high-fat diets, showing that *trans*-10,*cis*-12 CLA blocks body fat gain [1]. In contrast, very little information is available concerning CLA effects and its mechanisms of action under energy restriction conditions. Establishing if CLA is also effective in this dietary situation is very important to determine the extent to which CLA supplements could be useful in enhancing body fat loss promoted by dietary treatment of obesity based on energy restriction.

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Thus, the present work aimed to analyze the effects of *trans*-10,*cis*-12 CLA on adipose tissue size, liver composition, and hepatic fatty acid oxidation under restricted feeding in hamsters. For this purpose, the expression and activity of two rate-limiting oxidative enzymes, carnitine-palmitoyl transferase I (CPT-I) and acyl CoA oxidase (ACO), were assessed. The expression of PPARα, the transcriptional factor that regulates these enzymes, was also measured.

The results obtained in the present study will help to clarify whether CLA supplements could be useful not only in preventing overweight and/or obesity, but also in enhancing the loss of previously stored body fat promoted by energy-restricted diets. Nevertheless, more human studies are needed in this field of research.

Materials and methods

Animals, diets, and experimental design

The experiment was conducted with 24 male Syrian Golden hamsters (9-wk-old; initial body weight 82.6 ± 1.4 g) purchased from Harlan Ibérica (Barcelona, Spain) and took place in accordance with the Institution's guide for the care and use of laboratory animals. Animals were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy) and placed in an air-conditioned room (22 ± 2°C) with a 12-h light-dark cycle. After a 6-d adaptation period, the hamsters were fed a semipurified high-fat diet for 7 wk to increase their body fat content. This diet consisted of 200 g/kg casein (Sigma, St. Louis, MO, USA), 4 g/kg L-methionine (Sigma), 220 g/kg wheat starch (Vencasser, Bilbao, Spain), 320 g/kg sucrose (local markets), 150 g/kg palm oil (Agra-Unilever, Leioa, Spain), 5 g/kg sunflower oil (local markets), 46 g/kg cellulose (Vencasser), and 4 g/kg choline-HCl (Sigma). Vitamin (11 g/kg) and mineral (40 g/kg) mixes were formulated according to AIN-93 guidelines [7] and supplied by ICN Pharmaceuticals (Costa Mesa, CA, USA).

After this experimental period, eight animals were sacrificed (HF group). The remaining hamsters ($n = 16$) were divided into two weight-matched groups and subjected to energy restriction for 3 wk. To calculate the appropriate quantity of diet to be offered to the restricted groups, a third weight-matched group (reference group) was used to establish its spontaneous food intake. A 25% energy reduction was applied to the mean food intake of this group. Semipurified standard diet for restricted groups consisted of 200 g/kg casein, 4 g/kg L-methionine, 390 g/kg wheat starch, 230 g/kg sucrose, 65 g/kg sunflower oil, 46 g/kg cellulose, 4 g/kg choline-HCl, 11 g/kg vitamin mix, and 40 g/kg mineral mix. This diet was supplemented with 5 g/kg *trans*-10,*cis*-12 CLA (Natural Lipids Ltd., Hovdebygda, Norway) in the Restricted CLA group (RCLA group) and 5 g/kg linoleic acid, provided by sunflower oil, in the Restricted control group (RC group). All the experimental diets were freshly prepared once a week, gassed with nitrogen, and stored at 0°C to 4°C to avoid rancidity. Body weight and food intake were measured daily.

At the end of each experimental period (fattening and restriction), the hamsters were sacrificed after 12 h of fasting under anesthesia (chloral hydrate) by exsanguination. Adipose tissues from epididymal, perirenal, and subcutaneous regions and liver were dissected and weighed and then immediately frozen. Serum was obtained from blood samples after centrifugation (1000 *g* for 10 min at 4 °C). All samples were stored at −80°C until analysis.

Analysis of liver triacylglycerol, protein, and water contents

Total lipids were extracted from liver following the Folch method [8] and the lipid extract was dissolved in isopropanol. Triacylglycerols were measured in this extract by using a commercial kit (BioSystems, Barcelona, Spain). The quantification of protein content was carried out by spectrophotometry using the Lowry method [9], and water content was measured gravimetrically by drying liver samples at 105 °C until constant weight.

Enzyme activities in liver

CPT-I and ACO activities were assessed in the mitochondrial/peroxisomal fraction. Liver samples of 0.75 g were homogenized in 3 vol (wt/vol) of buffer pH 7.4 containing 0.25 mol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L Tris–HCl. Homogenates were centrifuged (700 *g* for 10 min at 4°C) and the supernatant fluid was again centrifuged (12°000 *g* for 15 min at 4°C). Pellets were resuspended in 70 mmol/L sucrose, 220 mmol/L mannitol, 1 mmol/L EDTA, 2 mmol/L HEPES buffer, pH 7.4. CPT-I activity was assayed by using the Bieber et al. method [10] and ACO following Lazarow method [11]. The pellet protein content was determined using the Bradford method [12]. CPT-I activity was expressed as nmol CoA formed/min/mg protein, and ACO activity was expressed as nmol NADH formed/min/mg protein.

For acetyl coenzyme A carboxylase (ACC, E.C. 6.4.1.2) activity measurement, liver samples of 0.5 g were homogenized in 5 mL of buffer pH 7.6, containing 150 mM KCl, 1 mM $MgCl_2$, 10 mM *N*-acetyl-cysteine, and 0.5 mM dithiothreitol. After centrifugation at 100°000 *g* for 40 min at 4 °C, the supernatant was obtained. Enzyme activity was measured from the acetyl-CoA-dependent incorporation of $^{14}HCO_3^-$ into acid-stable product [13,14]. Soluble protein in the supernatant was determined using the Bradford method [12]. Results were expressed as nmol bicarbonate incorporated/min/mg protein.

Extraction and analysis of RNA and quantification by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from 100 mg of liver using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were then treated with DNA-free Kit, DNase Treatment, and Removal Reagents (Ambion; The RNA Company, Applied Biosystems, Foster City, CA, USA) to remove any contamination with genomic DNA. The yield and quality of the RNA were assessed by measuring absorbance at 260, 270, 280, and 310 nm and by electrophoresis on 1.3% agarose gels. Total RNA (1.5 μg) of each sample was reverse-transcribed to first-strand complementary DNA (cDNA) using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

Relative CPT-I, ACO, and PPARα mRNA levels were quantified using real-time PCR with an iCycler–MyiQ Real Time PCR Detection System (BioRad). β-Actin mRNA levels were similarly measured and served as the reference gene. With the upstream and downstream primers (300 nM each), 0.1 μL of each cDNA was added to PCR reagent mixture, SYBR Green Master Mix (Applied Biosystems). Specific primers were designed (GeneBank: CPT-I, AY762566; ACO, NM_030721; PPARα, AJ555631; β-Actin, AJ312092) and synthesized commercially (Tib Molbiol, Berlin, Germany), and the sequences were as follows:

CPT-I:
5'-GCA GAG GAC GGG CAT TGT A-3' (forward)
5'-TGT AGC CTG GTG GGT TTG G-3' (reverse)

ACO:
5'-GCT GGC CGT GTC CAT AGC-3' (forward)
5'-TTA TCC GTG GGT CCA AAC TGA-3' (reverse)

PPARα:
5'-GAG AAA GCA AAA CTG AAA GCA GAG A-3' (forward)
5'-GAA GGG CGG GTT ATT GCT G-3' (reverse)

β -Actin:
5'-ACG AGG CCC AGA GCA AGA G-3' (forward)
5'-GGT GTG GTG CCA GAT CTT CTC-3' (reverse).

The PCR parameters were as follows: initial 2 min at 50°C, denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and combined annealing and extension at 60°C for 1 min. All sample mRNA levels were normalized to the values of β-actin and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [15].

Serum insulin and non-esterified fatty acid concentrations

Commercial kits were used to analyze serum insulin (EZRMI 13K; Linco, St. Charles, MO, USA) and fT3 (EiAsy Triiodothyronine–Free; Diagnostics Biochem Canada Inc., London, Ontario, Canada) by ELISA, and non-esterified fatty acids (NEFAs) (Roche Diagnostics, Mannheim, Germany) by spectrophometry.

Statistical analysis

Results are presented as mean ± SEM. Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Repeated measures of ANOVA statistical analysis was applied to analyze body weight loss time courses. The rest of parameters were analyzed by using one-way ANOVA followed by Student-Newman-Keuls post-hoc test. Correlations were analyzed by Pearson's test. Statistical significance was assessed at the $P < 0.05$ level.