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Journal of Nutritional Biochemistry 28 (2016) 147–154

Journal of Nutritional Biochemistry

The combination of conjugated linoleic acid (CLA) and extra virgin olive oil increases mitochondrial and body metabolism and prevents CLA-associated insulin resistance and liver hypertrophy in C57Bl/6 mice

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Received 20 May 2015; received in revised form 8 October 2015; accepted 13 October 2015

Abstract

Clinical conditions associated with obesity can be improved by daily intake of conjugated linoleic acid (CLA) or extra virgin olive oil (EVOO). Here we investigated whether dietary supplementation with CLA and EVOO, either alone or in combination, changes body metabolism associated with mitochondrial energetics. Male C57Bl/6 mice were divided into one of four groups: CLA (1:1 *cis*-9, *trans*-11:*trans*-10, *cis*-12; 18:2 isomers), EVOO, CLA plus EVOO or control (linoleic acid). Each mouse received 3 g/kg body weight of the stated oil by gavage on alternating days for 60 days. Dietary supplementation with CLA, alone or in combination with EVOO: (a) reduced the white adipose tissue gain; (b) increased body VO_2 consumption, VCO_2 production and energy expenditure; (c) elevated uncoupling protein (UCP)-2 expression and UCP activity in isolated liver mitochondria. This organelle, when energized with NAD^+-linked substrates, produced high amounts of H_2O_2 without inducing oxidative damage. Dietary supplementation with EVOO alone did not change any metabolic parameter, but supplementation with CLA itself promoted insulin resistance and elevated weight, lipid content and acetyl-CoA carboxylase-1 expression in liver. Interestingly, the *in vivo* antioxidant therapy with *N*-acetylcysteine abolished the CLA-induced rise of body metabolism and liver UCP expression and activity, while the *in vitro* antioxidant treatment with catalase mitigated the CLA-dependent UCP-2 expression in hepatocytes; these findings suggest the participation of an oxidative-dependent pathway. Therefore, this study clarifies the mechanisms by which CLA induces liver UCP expression and activity, and demonstrates for the first time the beneficial effects of combined CLA and EVOO supplementation.

Keywords: Conjugated linoleic acid; Extra virgin olive oil; *N*-acetylcysteine; Uncoupling protein; Body metabolism; Mitochondria

1. Introduction

The incidence of obesity is increasing dramatically in all societies as a perplexing byproduct of the high-feeding and low-physical-activity behavior of contemporary humans. The excessive accumulation of body fat mass, which is the fifth leading risk for global deaths, leads to pathological consequences such as type 2 diabetes mellitus, cardiovascular diseases and cancer. Therefore, immediate therapeutic actions are highly sought after to slow down the escalating progress of these diseases [1].

Daily intake of either conjugated linoleic acids (CLAs, 18:2 *n*-6) or extra virgin olive oil (EVOO) is an alternative dietary therapy that has health beneficial effects by improving clinical conditions related to obesity. CLAs are positional and geometric isomers of linoleic acid (LA) with a conjugated double-bound system that are synthesized by bacteria in the ruminant gut [2] and can also be obtained by enzymatic isomerization of LA [3]. Scientists have proposed different mechanisms for the CLA antiobesity effects, such as enhancement of resting metabolic rates (energy expenditure), modulation of lipid metabolism in adipocytes and increase in fatty acid β-oxidation [4,5]. The most common effect associated with CLA intake, especially of its *trans*-10, *cis*-12 isomer, is the prevention of body fat mass accumulation in animals and humans [5,6], mediated by attenuation of the expression and activity of adipogenic transcription factors such as the peroxisome proliferator-activated receptor (PPAR)-γ, and arrest of adipocyte differentiation/development [7–9].

Although some researchers have reported that CLA increases the energy expenditure, the currently available data are still controversial due to variations in the experimental parameters of dietary supplementation studies such as the CLA dose and isomeric form, length of treatment and animal model (for review, see [10]). Up-regulation of uncoupling protein (UCP)-2 in several tissues accounts for the positive

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http://dx.doi.org/10.1016/j.jnutbio.2015.10.008
0955-2863/

effects [11–14]. The adverse effects of CLA supplementation include liver enlargement [15–17] and reduction of insulin sensitivity [18–20]. The Food and Drug Administration has approved the CLA addition to certain foods (http://www.foodnavigator-usa.com).

EVOO, one of the main components of Mediterranean diet, seems to play a role in the prevention and/or management of various chronic diseases (for review, see [21]). The main active components of EVOO are oleic acid (18:1 *n*-9, the major one), squalene and phenolic compounds such as hydroxytyrosol, tyrosol and oleuropein [22,23]. Olive oil consumption has positive effects on fat oxidation in nonobese and obese subjects, without apparent effects on energy expenditure in the obese group [24,25]. It also decreases insulin resistance and improves the grading of fatty liver and liver span in patients with nonalcoholic fatty liver disease [26]. Olive oil may induce activation of hepatic fatty acid β-oxidation genes by the transcription factor PPAR-α, and down-regulation of gluconeogenic and lipogenic genes through the inhibition of the transcription factor sterol regulatory element-binding protein I [27]. In addition, the intake of olive oil at low percentages improves insulin resistance and increases the secretion of hepatic triglycerides as very low density lipoprotein and decreases the lipolytic flux from peripheral adipose tissue to the liver in rats [28].

We have recently reported that C57BL/6 mice fed a CLA-supplemented diet [*cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers; 1:1 CLA mixture; ~3 g per kg body weight (b.w.)] on alternating days exhibit high liver mitochondrial metabolism associated with liver enlargement and increased UCP-2 mRNA expression level and UCP activity [17]. Here, we addressed whether (a) the aforementioned changes in liver mitochondrial energetics elevate the body energy parameters, (b) EVOO prevents liver hypertrophy and improves insulin resistance induced by CLA-supplemented diet and (c) dietary supplementation with EVOO and CLA, either alone or in combination, affects other morphological and functional parameters in C57BL/6 mice. The results of the present study help to clarify the mechanisms by which the stated oils induce the UCP expression and/or activity in the liver.

2. Material and Methods

2.1. Animals and experimental protocol

Five-week-old male C57BL/6J mice, each weighing approximately 15 g, were obtained from a breeding colony at the University of São Paulo, Ribeirão Preto campus. The protocols were approved by the Committee for Ethics in Use of Animals of the University (protocol no. 12.1.1538.53.9). The mice had *ad libitum* access to water and standard laboratory rodent chow (6003 Nuvilab CR1, Curitiba, PR, Brazil), which contained 40% carbohydrates, 22% protein and 4% fat; they were housed at 23°C±2°C on a 12-h light:dark cycle. After a 6-day adaptation period, the mice were randomly divided into one of four groups (n=20/group), and each mouse received 0.1 ml of oil by gavage, as described in Table 1. CLA, EVOO and corn oil were obtained from GNC Pro Performance-Clarinol (Pennsylvania, USA), Sovena Portugal (Algés, PT) and Mazola (São Paulo, BR), respectively. Each dose of oil administered corresponded to approximately 3 g/kg b.w. or 2.2% of the dietary daily intake. The body weights were measured once a week. After 60 days, the mice were euthanized, and the white adipose tissues (i.e., perirenal and epididymal) and livers were quickly removed and weighed; liver right lobe and adipose tissues were frozen at −80°C for redox state, mRNA analyses and lipid quantification (extracted by Bligh and Dyer method); the residual livers were used for the isolation of mitochondria. Ten days before euthanasia, blood samples were obtained from the tail tips of mice following an overnight fast. Then, the animals received glucose (1.5 g/kg b.w.) by gavage for the glucose tolerance test (GTT) using a blood glucose meter (Onetouch Ultra, Johnson & Johnson Company, USA). The plasma levels of triacylglycerols (TAG), total cholesterol and high-density lipoprotein cholesterol (HDL-chol) were determined using enzymatic colorimetric methods according to the instructions of the manufacturer (Labtest, Brasil). For the *N*-acetylcysteine (NAC) treatment, 5-week-old mice from CLA or control supplemented groups received NAC in the drinking water (daily intake of 0.1 g/kg b.w.). After 30 days, the mice were euthanized, as described above.

Table 1
Experimental design of dietary supplementation with CLA and/or EVOO

Day	Groups			
	Control	CLA	EVOO	CLA+EVOO
Pair	LA	CLA	LA	CLA
Odd	LA	LA	EVOO	EVOO

LA: linoleic acid (corn oil; 60% of LA); CLA: conjugated linoleic acid (*cis*-9, *trans*-11 and *trans*-10, *cis*-12; 40% of each isomer); EVOO: extra virgin olive oil (78% of oleic acid).

2.2. Indirect calorimetry

After 15 days of diet supplementation, each mouse was placed individually into a hermetic chamber connected to an indirect calorimetry system (Oxylet, Pan Lab, Spain), with *ad libitum* access to water and food. Oxygen consumption (VO_2) and CO_2 release (VCO_2) were recorded for 48 h. Respiratory quotient (RQ) was calculated as VCO_2/VO_2 ratio. The energy expenditure was calculated using the following formula: $EE=(3.85+(1.232\times RQ))\times VO_2\times 1.44$.

2.3. Isolation of mitochondria

A liver homogenate was prepared in 250 mM sucrose, 1 mM ethylene glycol-*bis*(2-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2) and 0.1% bovine serum albumin (BSA) and centrifuged as previously described [29]. The protein concentration of the mitochondrial pellet was measured using the Biuret method with BSA as a protein standard. The experiments were carried out in standard medium (30°C) containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES buffer (pH 7.2), 2 mM inorganic phosphate, 1 mM magnesium chloride and 0.1 mM EGTA.

2.4. Respiratory rates

Respiratory rates were determined by oxygen consumption, which was monitored in an Oxygraph-2k respirometer (Oroboros, Innsbruk, Austria) containing 2 ml of air-saturated respiration medium. Phosphorylating (state III) respiration was initiated by the addition of 200 nmol ADP/mg protein. Phosphorylation efficiency (ADP/O ratio) was calculated from the amount of ADP added and the amount of oxygen consumed during state III respiration.

2.5. Reactive oxygen species (ROS) generation

ROS were monitored spectrofluorimetrically using 2 μM Amplex Red in the presence of horseradish peroxidase (1 U/ml) [30] at 563/587 nm excitation/emission wavelength pairs and slit widths of 5 nm in a Model F-4500 Hitachi fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with continuous stirring.

2.6. Liver redox state

Reduced (GSH) and oxidized (GSSG) glutathione and protein carbonyl levels were assessed in the liver homogenates (50 mg/ml in cold 0.1 M Tris–HCl buffer, pH 7.4). GSH and GSSG were assessed by the fluorimetric ortho-phthalaldehyde method [31]. Protein carbonyl was assessed colorimetrically by the selective binding of 2,4-dinitrophenyl hydrazine to protein carbonyl groups [32,33].

2.7. Culture of HepG2 cells

HepG2 cells were obtained from the American Type Culture Collection, No. HB 8065. The cell line was cultured in Dulbecco's medium with 10% defined supplement fetal bovine serum plus 100 IU/ml penicillin G, 1 μg/ml amphotericin and 100 mg/ml streptomycin. The cells were seeded into six-well plates, with 1×10^5 cells/well in 2 ml of culture medium at 37°C, flushed with 5% CO_2 in air for 24 h. After this period, the cells were incubated in DMEM containing albumin (1%) with 20 μM CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12; 45% of each isomer), 30 μM ciprofibrate or not (control), and in the presence or absence of 5.5 IU/ml catalase. After the 24-h incubation period, the cells were rinsed with buffered saline (PBS, pH 7.4) and then used for analysis of UCP2 mRNA expression.

2.8. Analysis of mRNA expression

Total RNA was isolated using the Trizol reagent (Invitrogen, Grand Island, NY, USA). For real-time polymerase chain reaction (PCR) analysis, RNA was reverse transcribed using Promega master mix (Madison, WI, USA). During PCRs, quantification of mRNA transcripts was determined using SYBR-green fluorescent dye (ABI) and primers human UCP-2: TCCTGAAGCCAACCTCATG and GGCAGAGTTCATGTATCTCGTC; human RPL: CTCTTCCTTTCTCCGCCATC and TCCAGTTTTCATCCGAATCCAC; UCP2: CGGTA-CACTTTCCCTCTGGATAC[FAM]G and GCGCACTAGCCCTTGACTCTC; PPARα: CATTTCCCTTTGTGTGGCTG and ATCTGGATGTTGGCTCTGC; ACC1: AAGGCTATGTGAAG-GATGTGG and CTGTCTGAAGAGGTTAGGGAAG; β-actin: CACTTTCTACAATGAGCTGCG and CTGGATGGCTACGTACATGG and RPL: CAAAATCGCCCTATTCCTCA and CCCAGCTTCTTGCTCCT (Sigma-Aldrich). Relative expression of mRNAs was determined after normalization with RPL or β-actin using the ΔΔCt method [34]. Quantitative PCR was performed using Eppendorf Realplex4 Mastercycler Instrument (Eppendorf).