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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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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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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).

3. Results

First, we assessed whether dietary supplementation with CLA and EVOO, either alone or in combination, affects the body metabolism or weight gain in mice. Compared with the control group, mice fed CLA- or CLA+EVOO-supplemented diet exhibited higher levels of VO_2, VCO_2 and EE (Fig. 1). In these groups, the RQ values were not significantly different, thus indicating a similar carbohydrate oxidation level. On the other hand, EVOO supplementation did not change any of the parameters of mice body metabolism. Moreover, CLA and/or EVOO supplementation did not alter the mice body weight gain within a 60-day period (control: 9.4±0.4 g, CLA: 8.5±1.1 g, EVOO: 8.7±1.8 g, EVOO+CLA: 9.2±1.3 g, mean±S.E.M.; $P>.05$; $n=5–6$). These results demonstrate that CLA supplementation, regardless of the presence of EVOO, increases the body metabolism but does not reduce the body weight gain in mice. Factors such as changes in food intake and rear/movement pattern probably influenced this effect.

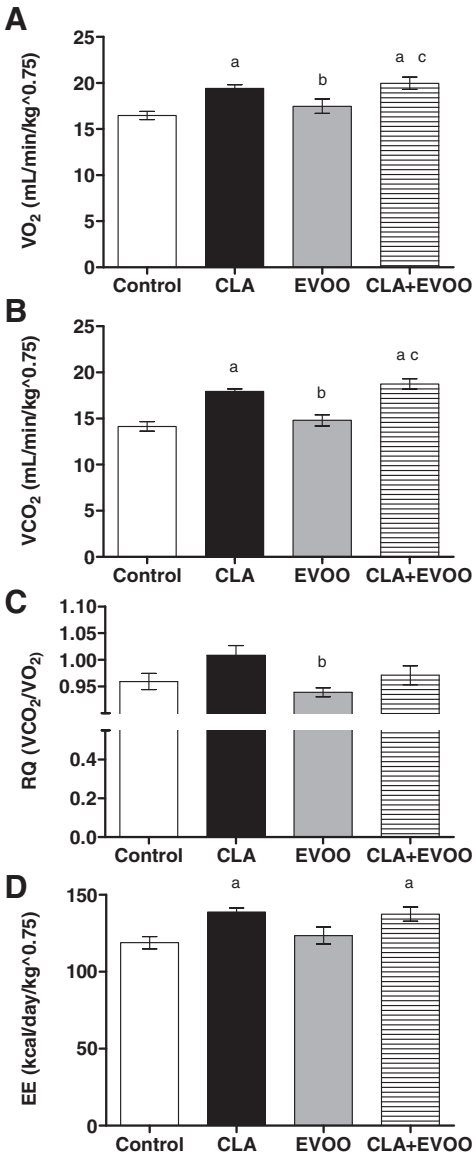

Fig. 1. Calorimetric parameters of mice fed a diet supplemented with CLA and/or EVOO. (A) Volume of oxygen consumed (VO_2). (B) Volume of carbon dioxide produced (VCO_2). (C) Respiratory quotient (RQ). (D) Energy expenditure (EE). Mean±S.E.M. $^aP<.05$ vs. control; $^bP<.05$ vs. CLA; $^cP<.05$ vs. EVOO ($n=8–11$).

Next, we assessed whether dietary supplementation with CLA and/or EVOO alters the blood glucose levels and lipid metabolism. Analysis of the kinetics (Fig. 2A) and the area under the curve (Fig. 2B) of the GTT revealed that the glucose uptake was slower in mice fed CLA-supplemented diet, and faster in mice fed EVOO- or CLA+EVOO-supplemented diet, as compared with the control group. The latter groups also exhibited significantly reduced fasting levels of circulating cholesterol, increased levels of HDL-chol and decreased levels of cholesterol contained in other lipoproteins (non-HDL) when compared with mice fed the CLA-supplemented diet (Table 2). The circulating TAG levels were similar among the groups studied. These results demonstrate that EVOO supplementation prevents insulin resistance induced by CLA supplementation and improves the plasma cholesterol profile.

At the end of the weight gain period, the animals were sacrificed, and their visceral white adipose tissue (WAT) and liver were weighed. Although body weight gain did not change significantly among the groups studied, mice fed CLA- and CLA+EVOO-supplemented diet exhibited reduced WAT weight (Fig. 3A) when compared with control mice; mice fed CLA+EVOO-supplemented diet displayed higher WAT weight than mice fed CLA-supplemented diet. Compared with the control group, only mice fed CLA-supplemented diet exhibited increased liver weight (Fig. 3B), total lipids, TAG (not statistically different) and cholesterol contents (Table 3), and elevated levels of acetyl-CoA carboxylase-1 (ACC1) mRNA expression (Fig. 4). ACC catalyzes the synthesis of malonyl-CoA, which is both an intermediate in fatty acid synthesis and an allosteric inhibitor of fatty acid oxidation. ACC1 gene expression in liver is positively regulated by high glucose and insulin [35,36], and is related to insulin resistance [37]. Liver lipid contents and ACC1 expression in mice fed CLA+EVOO-supplemented diet were similar to those found in control mice. These results demonstrate that the addition of EVOO to CLA-supplemented diet partially reverses the CLA-induced hypertrophic and hypotrophic effects in the liver and adipose tissue, respectively, which in liver can be related to improved lipid synthesis and storage.

To examine whether the high body metabolism induced by CLA is related to an increase in mitochondrial metabolism, we monitored O_2 consumption by mitochondria isolated from liver of mice fed CLA-

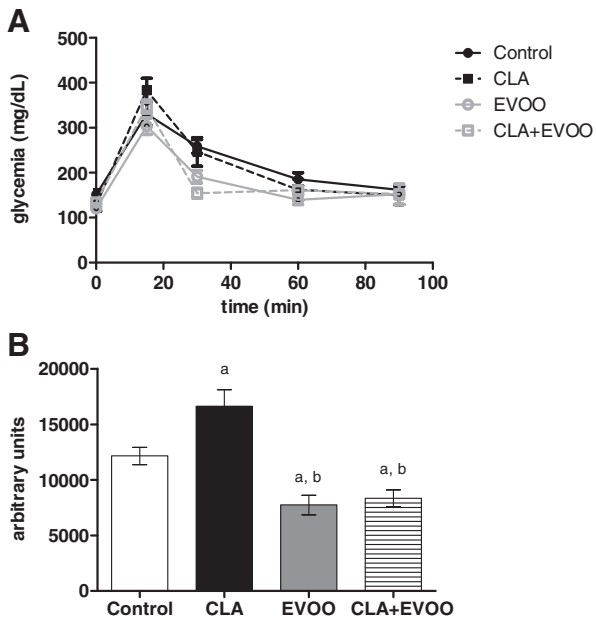

Fig. 2. Kinetic profile (A) and area under the curve (B) of the GTT in mice fed a diet supplemented with CLA and/or EVOO. Mean±S.E.M. $^bP<.05$ vs. CLA ($n=3–4$).

Table 2
Levels of circulating TAG, cholesterol, HDL-chol and non-HDL-chol in mice fed a diet supplemented with CLA and/or EVOO

	TAG	Cholesterol	HDL-chol	Non-HDL
Control	100.9±25.5	118.7±16.3	45.3±2.9	73.4±16.1
CLA	103.5±35.0	125.1±38.2	45.3±2.7	85.3±37.5
EVOO	119.0±51.1	101.7±16.0[b]	56.9±12.2[a,b]	45.6±9.1[a,b]
CLA+EVOO	97.2±33.6	94.6±7.6[b]	57.7±11.5[a,b]	38.4±12.1[a,b]

Values in mg/dL. Mean±S.E.M.
[a] $P<.05$ vs. control.
[b] $P<.05$ vs. CLA ($n=4–9$).

Table 3
Levels of TAG, cholesterol and total lipids in livers from mice fed a diet supplemented with CLA and/or EVOO

	TAG	Cholesterol	total lipids
Control	46.4±7.8	1.41±0.17	155.0±7.6
CLA	65.2±10.8	2.77±0.57[a]	213.9±30.8[a]
EVOO	43.2±8.0	2.37±0.008	143.8±10.7[b]
CLA+EVOO	45.8±5.4	1.57±0.17[b]	161.3±8.1[b]

Values in mg/g wet liver weight. Mean±S.E.M.
[a] $P<.05$ vs. control.
[b] $P<.05$ vs. CLA ($n=4–6$).

and/or EVOO-supplemented diet. In fatty-acid-free mitochondria (isolated in the presence of 0.1% BSA), the four groups studied exhibited similar respiration rates for both phosphorylating (state III, Fig. 5A) and resting (state IV, Fig. 5B) states, respiratory control ratio (RCR=state III/IV state, Fig. 5C) and phosphorylation efficiency (ADP/O, Fig. 5D). In the presence of LA, a substrate for UCP activity, mitochondria from all groups presented, in general, a slight decrease in the state III and an increase in the state IV respiration rates, resulting in lower RCR and ADP/O ratios. However, mitochondria from mice fed CLA- or CLA+EVOO-supplemented diet displayed significantly higher RCR (ΔRCR, Fig. 5E) and ADP/O (ΔADP/O, Fig. 5F) variations in the absence or presence of LA as compared with the other groups. It indicates a weakness of the coupling between respiration and phosphorylation, as well as denotes an increased UCP activity in mice fed CLA- or CLA+EVOO-supplemented diet.

To confirm that UCPs were involved in the hepatic mitochondrial metabolism uncoupling, we measured the UCP2 mRNA expression level, the most abundant liver UCP isoform. The UCP2 expression in mice fed CLA-supplemented diet was significantly greater than the UCP2 expression in the other three mice groups. The UCP2 expression in mice fed EVOO-supplemented diet was similar to that in the control group (Fig. 6). These results agree with our previous report [17] and further indicate that EVOO supplementation partially reverses the elevated UCP2 expression induced by CLA, but not the enhanced mitochondrial and body metabolism.

The literature has evidenced that relatively low ROS levels can act as intracellular signaling molecules (for review, [38]) and that the UCP expression and activity in hepatocytes treated with lipids depend on the increase in ROS production [39,40]. To assess whether ROS participate in the modulation of liver UCP expression by CLA alone or in combination with EVOO, we first measured the mitochondrial H_2O_2 generation. When energized with complex I substrates, liver mitochondria from mice fed CLA- and CLA+EVOO-supplemented diet produced more H_2O_2 compared with the other groups (Fig. 7A). However, when energized with succinate, liver mitochondria from mice fed control diet and CLA- and CLA+EVOO-supplemented diet produced similar amounts of H_2O_2 (Fig. 7B). Compared with the control group, liver mitochondria from mice fed EVOO-supplemented diet exhibited significantly diminished H_2O_2 generation regardless of the energy substrate used. Despite the increased H_2O_2 levels, the concentration of reduced and oxidized glutathione (expressed as the GSH/GSSG ratio) and oxidized proteins (carbonyl groups) did not significantly differ among the four groups studied (Table 4), which indicates that CLA and EVOO supplementation, alone or in combination, does not induce liver oxidative stress.

Mice fed CLA-supplemented diet were treated with NAC, an effective precursor of cysteine for the tissue GSH synthesis. This *in vivo* antioxidant treatment mitigated the rise in the liver UCP2 mRNA expression induced by CLA supplementation (Fig. 8A). NAC treatment prevented the drop in ADP/O caused by LA (ΔADP/O, Fig. 8B), without reducing the high H_2O_2 generation supported by complex I substrates (Fig. 8C) in isolated liver mitochondria. As consequence, body metabolism was not increased in NAC-treated mice fed CLA-supplemented diet (Fig. 8D). Of note, the *in vivo* NAC treatment did not disrupt the effect of CLA on the liver (Fig. 8E) and WAT (Fig. 8F).

Finally, hepatocytes were treated with CLA or ciprofibrate (a PPAR-α ligand) in the absence or presence of catalase, an antioxidant enzyme. Both CLA- and ciprofibrate-treated cells presented elevated UCP-2 mRNA expression levels as compared with the control group (Fig. 9). Co-incubation with the antioxidant abolished the rise in UCP-2 mRNA expression in CLA- or ciprofibrate-treated hepatocytes, thus

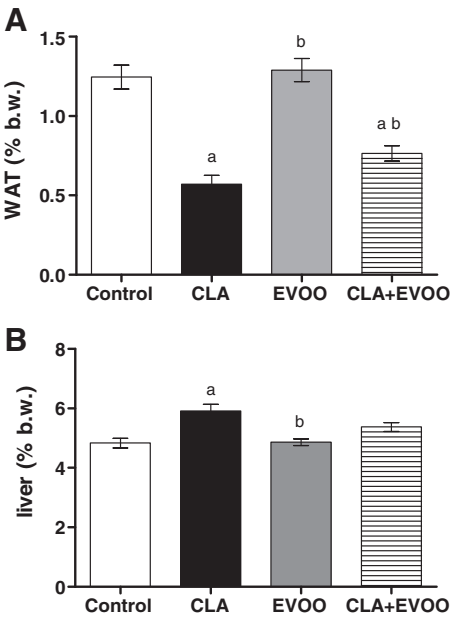

Fig. 3. White adipose tissues (A) and liver (B) as a percentage of body weight in mice fed a diet supplemented with CLA and/or EVOO. Mean±S.E.M. [a]$P<.05$ vs. control; [b]$P<.05$ vs. CLA ($n=6–9$).

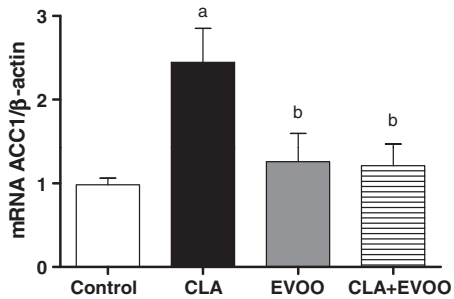

Fig. 4. Liver ACC1 mRNA expression level in mice fed a diet supplemented with CLA and/or EVOO. β-Actin gene was used for normalization. Mean±S.E.M. [a]$P<.05$ vs. control; [b]$P<.05$ vs. CLA ($n=4–6$).

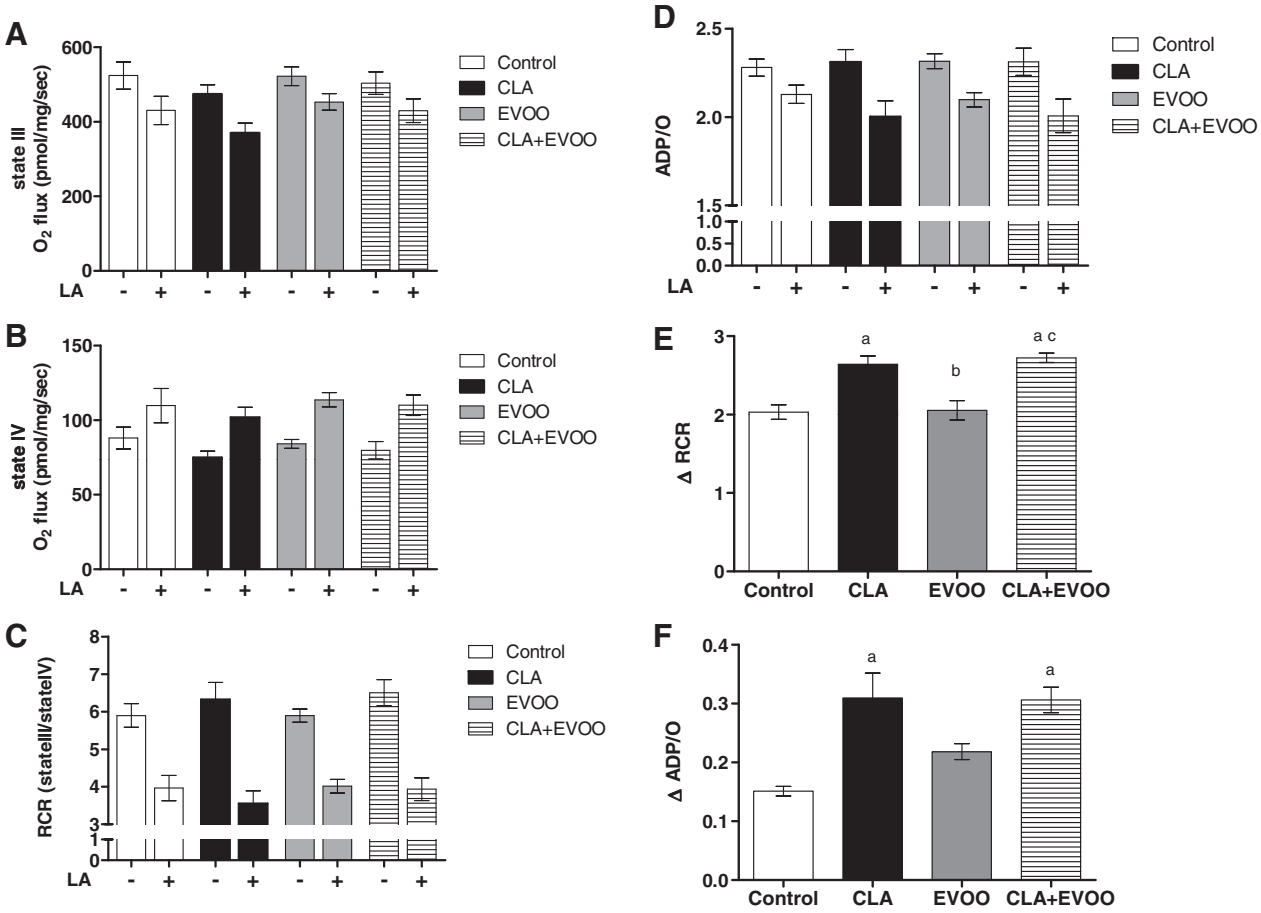

Fig. 5. State III (A) and state IV (B) respiration rates, RCR (C) and ADP/O ratios (D) of liver mitochondria isolated from mice fed a diet supplemented with CLA and/or EVOO. Mitochondria were energized with glutamate/malate (5 mM) without or with addition of 2 μM LA to the reaction medium. RCR (E) and ADP/O ratio (F) variations (Δ) in the absence or presence of LA calculated from the data contained in C and D, respectively. Mean±S.E.M. $^{a}P<.05$ vs. control; $^{b}P<.05$ vs. CLA; $^{c}P<.05$ vs. EVOO (n=4–6).

demonstrating that this process depends on the occurrence of an oxidative stress condition.

4. Discussion

The increase in adaptive thermogenesis associated with UCP up-regulation enhances energy expenditure in animals [41]. We have recently demonstrated that feeding mice a low-fat (4%) diet supplemented with ~2% CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12; 45% of each isomer) on alternating days reduces adiposity, increases the lipid flow to the liver and elevates the UCP-2 expression and UCP activity in this organ [17]. Here, we demonstrated that this pattern of CLA supplementation also enhances the body metabolism, in agreement with literature data [13,42,43]. Brown adipose tissue is probably not associated to this effect since this organ has presented 55% mass reduction with our CLA supplementation [17], as shown by Takahashi and coworkers [44], who also found reductions in the gene expression of UCP-1 and UCP-3. Such rise in body metabolism was associated with increased liver UCP-2 expression and UCP activity in liver and was dependent on ROS signaling molecules generated by mitochondria. On the other hand, EVOO supplementation, at the same concentration used for CLA, did not alter body metabolism and fat gain, UCP-2 expression and UCP activity. These findings suggest that EVOO supplementation improves clinical conditions related to obesity by acting through other mechanisms [45]. EVOO, when supplemented together with CLA, maintained the metabolic effects of CLA, i.e., the high body and mitochondrial metabolism, and mitigated some adverse effects of CLA supplementation, such as liver enlargement and insulin resistance. Therefore, this study demonstrates for the first time the beneficial effects of the combined EVOO and CLA supplementation.

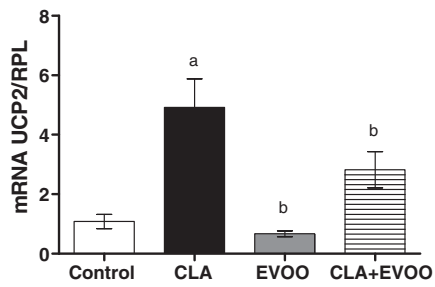

Fig. 6. Liver UCP2 mRNA expression level in mice fed a diet supplemented with CLA and/or EVOO. RPL gene was used for normalization. Mean±S.E.M. $^{a}P<.05$ vs. control; $^{b}P<.05$ vs. CLA (n=4–6).

The five homologous UCP isoforms (UCP1–UCP5) are widely distributed among mammalian organs [46]. These proteins are located at the inner mitochondrial membrane where, in the presence of free fatty acids, they dissipate the proton electrochemical gradient built up by the respiratory chain, thereby diverting energy from adenosine triphosphate synthesis to heat production [47,48]. A slight decrease in the proton electrochemical gradient stimulates electron transfer through the respiratory chain and, consequently, increases carbohydrate, fatty acid and/or amino acid oxidation. PPAR-α controls the

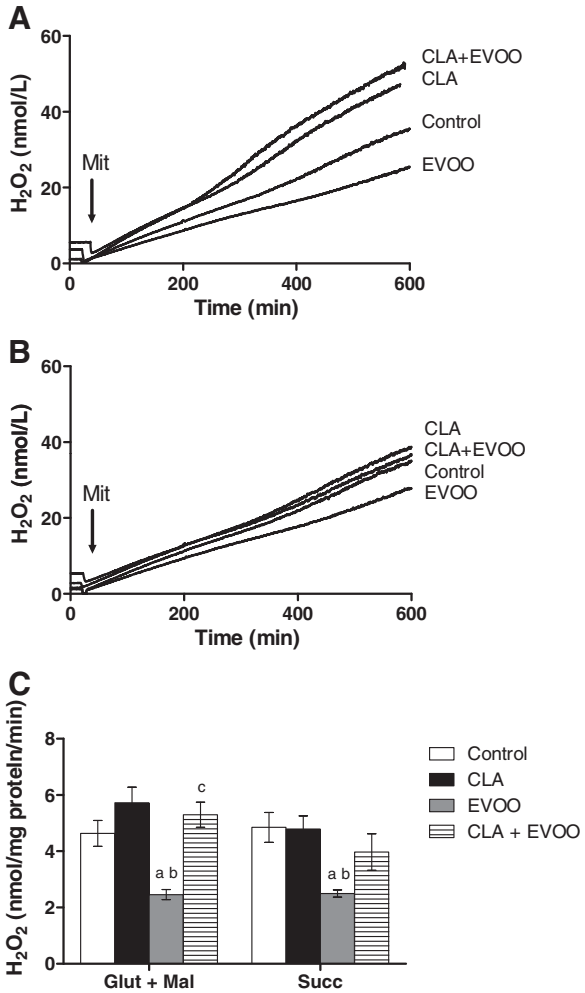

Fig. 7. H_2O_2 generation in liver mitochondria from mice fed a diet supplemented with CLA and/or EVOO. Mitochondria (Mit, 1 mg/ml) were added to the standard reaction medium described in the "Materials and methods" section in the presence of 5 mM glutamate (Glut) and malate (Mal) (A) or 5 mM succinate (Succ) (B). Traces are representative of four to six experiments with different mitochondrial preparations. (C) Mean±S.E.M. [a]$P<.05$ vs. control; [b]$P<.05$ vs. CLA; [c]$P<.05$ vs. EVOO.

UCP-2 gene transcription [49] and a range of fatty acid metabolism genes [50] in the liver. PPAR-α binds oleic acid and *cis*-9, *trans*-11-CLA more strongly than it binds *trans*-10, *cis*-12-CLA, with dissociation constants in the nM range in a liver cell line [51]. Despite this, oleic acid is rapidly assimilated into neutral and polar lipids in primary hepatocytes and seems to neither activate the PPAR-α receptor [52] nor regulate the expression of their target genes [53]. In contrast, CLA positively modulates hepatic metabolism *in vivo* and in rat hepatoma cell line [54]. Although CLA specifically activates the PPAR-α-dependent gene expression, its effects on body composition and expression of liver mRNA encoding UCPs and mitochondrial fatty acid oxidizing enzymes seem to be independent of PPAR-α activation, as revealed by studies on PPAR-α-null mice [13]. Polyunsaturated fatty acid regulation of hepatic gene transcription involves at least two distinct pathways: a PPAR-α-dependent and a PPAR-α-independent pathway [13,53]. Zhang and collaborators have proposed that the up-regulation of UCP-2 is controlled by a PPAR-α-mediated pathway and a PPARα-independent oxidative stress pathway, as found in mesencephalic cells. In this cell model, a selective PPAR-α agonist activates PPAR-α, induces ROS generation and up-regulates UCP-2 expression, while a PPAR-α antagonist and PPAR-α knockdown inhibit PPAR-α activity but only partially decreases UCP-2 up-regulation. The last treatments, in combination with antioxidants, completely suppress UCP-2 up-regulation [55]. In agreement, we found that CLA supplementation up-regulated the liver UCP-2 expression by increasing mitochondrial H_2O_2 generation. As the oxidant species did not cause oxidative stress in the liver, they probably work as signaling molecules (for review, see [56]). Antioxidant therapy interrupted the redox signaling pathway in the cytosol and prevented UCP-2 up-regulation by CLA, as demonstrated here by the *in vivo* NAC treatment, which improves the cytosolic antioxidant capacity in mice [57], and by the *in vitro* incubation of hepatocytes with catalase, which decomposes H_2O_2. On the other hand, EVOO therapy partially reduced both mitochondrial H_2O_2 generation and UCP-2 overexpression in the liver of CLA-supplemented mice; this is an additional evidence of the connection between these events. EVOO supplementation itself strongly reduced mitochondrial H_2O_2 generation, suggesting that this oil acts as a mitochondria-targeted antioxidant. Data on the antioxidant effect of EVOO supplementation on liver mitochondria are scarce in the literature (see [58] for review). Some researchers have suggested that polyphenolic compounds that exist in EVOO mediate the antioxidant activity [59,60].

We have demonstrated here and in a previous study [17] that intermittent feeding with CLA during 60 days markedly increases the liver size and slightly elevates the TAG content. Although daily diet supplementation with 1%–3% CLA or *trans*-10, *cis*-12-CLA isomer alone decreases the body fat content, it promotes the development of fatty liver and insulin resistance in mice [20]. Even in the absence of obesity, insulin resistance leads to hepatic steatosis [61]. The mechanisms underlying the association between these clinical conditions involve increased hepatic *de novo* lipogenesis and induced lipolysis of adipocytes, with flux of free fatty acids to the liver and skeletal muscle [62]. In fact, CLA exerts its antiobesity effects primarily through the suppression of adipogenesis and the stimulation of lipolysis and apoptosis in WAT [20]. The CLA actions in the WAT, in turn, augment the availability of triglycerides containing lipoproteins that can undergo hydrolysis by the action of lipoprotein lipase in other tissues. The excess of free fatty acids released is taken up directly and causes accumulation of lipids in skeletal muscle, liver and β-cells, which disrupts the normal metabolic and secretory functions of these tissues and cells [63].

Mechanistically, CLA significantly reduces the transcript levels of genes coding for proteins involved in glucose and fatty acid import or biosynthesis, including glucose transporter 4 and PPAR-γ in WAT [44,64]. The *trans*-10, *cis*-12-CLA isomer leads to adipocyte inflammation and induces tumor necrosis factor-α and interleukin-6 gene expression [65], associated with the activation of the nuclear factor κB (NF-κB) pathway [66]. In the present study, it is noteworthy that dietary supplementation with CLA+EVOO improved circulating glucose metabolism and liver hypertrophy. The anti-inflammatory properties of hydroxytyrosol, the major EVOO polyphenolic compound, probably mediate the aforementioned beneficial effects of CLA+EVOO supplementation. Hydroxytyrosol prevents obesity, hyperglycemia, hyperlipidemia and insulin resistance induced by high-fat diet in mice [67]. *In vitro*, this phenolic compound suppresses NF-κB activation and controls proinflammatory genes in cancer cell lines [68,69] and in Aβ-induced toxicity in neuroblastoma N2a cells [70].

Table 4
Liver redox state in mice fed a diet supplemented with CLA and/or EVOO

	GSH/GSSG	Carbonyl groups (nmol·mg protein^{-1})
Control	4.7±0.4	1.0±0.2
CLA	5.3±0.6	0.8±0.2
EVOO	4.2±1.2	1.1±0.5
CLA+EVOO	5.8±0.4[c]	1.4±0.2

Mean±S.E.M.
[c] $P<.05$ vs. EVOO (n=4–5).

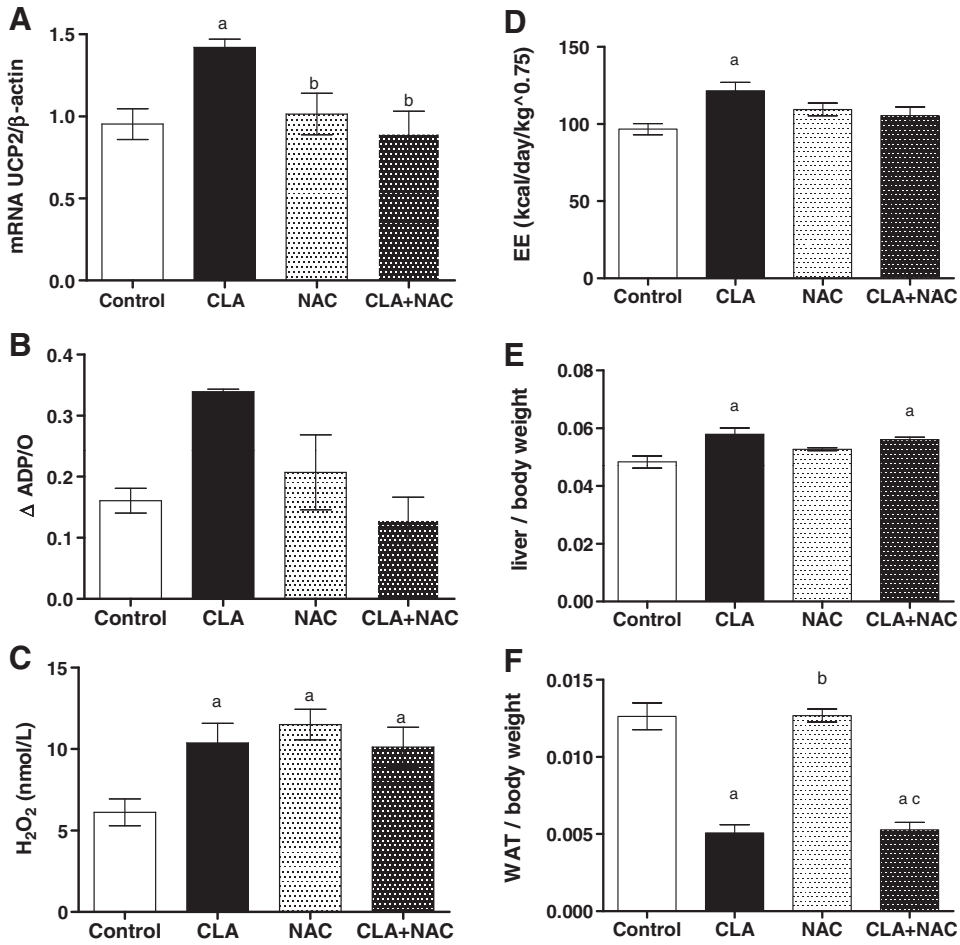

Fig. 8. Overall effects of NAC treatment in mice fed CLA-supplemented diet. (A) Liver UCP2 mRNA expression level. (B) ΔADP/O ratio in the presence or absence of LA. (C) H_2O_2 generation in liver mitochondria energized with glutamate/malate. (D) Body energy expenditure (EE). (E) Liver weight. (F) WAT weight. (Experimental conditions of the assays were described in the legends of previous figures.) Mean±S.E.M. $^{a}P<.05$ vs. control; $^{b}P<.05$ vs. CLA. $^{c}P<.05$ vs. NAC. ($n=5$).

In conclusion, CLA dietary supplementation increases body metabolism associated with UCP overexpression and/or activity in liver mitochondria by acting through an oxidative-dependent pathway, but without inducing oxidative damage. We propose that H_2O_2 generated in mitochondria directly signals UCP-2 up-regulation. EVOO protects from insulin resistance and liver enlargement without suppressing CLA action on mitochondrial and body metabolism. Therefore, the combination of CLA and EVOO is a potential novel dietary supplement to prevent body fat gain.

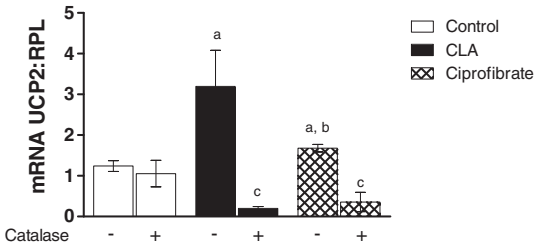

Fig. 9. UCP2 mRNA expression level in hepatocytes treated with 20 μM CLA, 30 μM ciprofibrate or etanol (control) in the presence (+) or absence (−) of 5.5 IU/ml catalase. This assay was conducted in DMEM, as described in the "Materials and methods" section. RPL gene was used for normalization. Data are expressed as the mean±S.E.M. of four independent experiments. $^{a}P<.05$ vs. control; $^{b}P<.05$ vs. CLA; $^{c}P<.05$ vs. the same condition without catalase.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Acknowledgments

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