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Administration of a murine diet supplemented with conjugated linoleic acid increases the expression and activity of hepatic uncoupling proteins

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Abstract Daily intake of conjugated linoleic acid (CLA) has been shown to reduce body fat accumulation and to increase body metabolism; this latter effect has been often associated with the up-regulation of uncoupling proteins (UCPs). Here we addressed the effects of a CLA-supplemented murine diet (~2 % CLA mixture, *cis*-9, *trans*-10 and *trans*-10, *cis*-12 isomers; 45 % of each isomer on alternating days) on mitochondrial energetics, UCP2 expression/activity in the liver and other associated morphological and functional parameters, in C57BL/6 mice. Diet supplementation with CLA reduced both lipid accumulation in adipose tissues and triacylglycerol plasma levels, but did not augment hepatic lipid storage. Livers of mice fed a diet supplemented with CLA showed high UCP2 mRNA levels and the isolated hepatic mitochondria showed indications of UCP activity: in the presence of guanosine diphosphate, the higher stimulation of respiration promoted by linoleic acid in mitochondria from the CLA mice was almost completely reduced to the level of the stimulation from the control mice. Despite the increased generation of reactive oxygen species through oxi-reduction reactions involving NAD^+/NADH in the Krebs cycle, no oxidative stress was observed in the liver. In addition, in the absence of free fatty acids, basal respiration rates and the phosphorylating efficiency of mitochondria were preserved. These results indicate a beneficial and secure dose of CLA for diet supplementation in mice, which induces UCP2 overexpression and UCP activity in mitochondria while preserving the lipid composition and redox state of the liver.

Keywords Obesity · Energy metabolism · CLA · Liver · Mitochondria · UCP

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Introduction

Obesity and excessive weight gain represent the fifth leading risk for global deaths and are often implicated in cardiovascular disease, diabetes and cancer. These two conditions have been emerging among younger age groups and are threatening to reach global epidemic levels; therefore, immediate therapeutic actions are highly sought after to slow the escalating progress of these conditions (World Health Organization 2012).

Daily intake of conjugated linoleic acid (CLA) has been shown to reduce body fat accumulation in several animal

models, as well as in humans (Park et al. 1997; Azain et al. 2000; Gavino et al. 2000; Kritchevsky et al. 2000; Tsuboyama-Kasaoka et al. 2000; Cherian et al. 2002; Ostrowska et al. 1999; Terpstra 2004). The CLA family is comprised of positional and geometric isomers of linoleic acid (18:2), which lacks a methylene group separating the double bonds. They are produced by bacteria in the ruminant gut (Griinari et al. 2000), with the *cis*-9, *trans*-10 and *trans*-10, *cis*-12 isomers as the dominant forms. In humans, the main sources of CLA are from beef and dairy products (Lin et al. 1995). CLA exerts its anti-obesity effects primarily through the suppression of adipogenesis/lipogenesis and the stimulation of lipolysis and apoptosis in adipose tissue (Kennedy et al. 2010). Other related effects of CLA administration, including a decrease in energy intake (West et al. 1998; Takahashi et al. 2002) and/or an increase in energy expenditure (West et al. 1998; Takahashi et al. 2002; West et al. 2000; Ohnuki et al. 2001a), are more frequently observed in rodents than in larger mammals, but the details are not fully understood.

Enhanced energy expenditure in animals can be promoted by an increase in adaptive thermogenesis associated with the up-regulation of uncoupling proteins (UCPs). There are five homologous UCP isoforms (UCP1-UCP5) that are widely distributed among mammalian organs (Jezek and Garlid 1998). These proteins are located at the inner mitochondrial membrane where, in the presence of free fatty acids, promotes the dissipation of the proton electrochemical gradient built up by the respiratory chain, thereby diverting energy from ATP synthesis to heat production (Nicholls 1976; Garlid et al. 2000). A slight decrease in the proton electrochemical gradient stimulates electron transfer in the respiratory chain, thereby increasing substrate oxidation. This process results in the enhanced catabolism of carbohydrates, fatty acids and/or amino acids.

Despite the high thermogenic capacity of UCP1 in brown adipose tissue (BAT), most studies indicate that UCP2 is more involved than UCP1 and UCP3 (which is primarily expressed in skeletal muscle) in the CLA-promoted alterations of energy expenditure (Wang and Jones 2004). In this regard, the data reported in the literature are controversial. The up-regulation of UCP2 due to supplementation with CLA was found in *i)* white adipose tissue (WAT) from C57BL/6J mice (Tsuboyama-Kasaoka et al. 2000; Ealey et al. 2002), ob/ob mice (Roche et al. 2002) and Zuker diabetic fatty rats (Ryder et al. 2001) but not in ICR mice (Takahashi et al. 2002) and in *ii)* BAT from C57BL/6J mice (Takahashi et al. 2002; Ealey et al. 2002), ICR mice (Takahashi et al. 2002), ob/ob mice (Roche et al. 2002), Zuker diabetic fatty rats (Ryder et al. 2001) and AKR/J mice on a high-fat diet (West et al. 1998). In both WAT and BAT, hamsters showed no changes in UCP2 mRNA expression (Ribot et al. 2007). In the skeletal muscle of Sprague–Dawley rats and C57BL/6J mice, UCP2 mRNA levels were found to either increase (Takahashi et al. 2002; Choi et al. 2007) or decrease (Tsuboyama-Kasaoka et al. 2000; Ealey et al. 2002), respectively. Few studies are available regarding UCP2 regulation in the liver (Tsuboyama-Kasaoka et al. 2000; Choi et al. 2007; Tsuboyama-Kasaoka et al. 2003; Peters et al. 2001); most of these studies, which were performed in mice, indicate increased mRNA levels (Tsuboyama-Kasaoka et al. 2000; Tsuboyama-Kasaoka et al. 2003; Peters et al. 2001). These dichotomies may be due to differences in the CLA isomers, dietary CLA levels and duration of supplementation or translational/post-translational protein regulation. In this context, studies are required on the changes to mitochondrial energetics as a result of UCP activities induced by CLA diet supplementation. Therefore, in this work, we addressed the effects of a CLA-supplemented murine diet (~2 % CLA, with equal concentrations of two major isomers on alternating days) on mitochondrial energetics and UCP2 expression in the liver, as well as on other associated morphological and functional parameters. The involvement of UCPs, primarily UCP2, is proposed.

Methods and materials

Animals and experimental protocol

Five-week-old male C57BL/6J mice, each weighing approximately 20 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 (Protocols no. 10.1.794.53.0 and 10.1.794.53.0). The mice had ad libitum access to water and standard laboratory rodent chow (6003 Nuvilab CR1, Curitiba, PR, Brazil), which contained 22 % protein, 40 % carbohydrates and 4 % fat, and the mice were housed at 23± 2 °C on a 12-h light:dark cycle. After a six-day adaptation period, the mice were randomly divided into one of two groups (n=20/group). Each mouse in the control group received 0.1 mL of linoleic acid, while mice in the CLA group received 0.1 mL of conjugated linoleic acid (*cis*-9, *trans*-10 and *trans*-10, *cis*-12, 45 % of each isomer) by gavage three times a week. Each dose of CLA administered corresponded to approximately 2.2 % of the dietary daily intake (2.2 g/100 g chow). The body weights were measured once a week. After 60 days, the mice were euthanized in a CO_2 chamber in approximately 3 min. The adipose tissues (i.e., perirenal, epididymal and brown) and livers were quickly removed and weighed; the livers were used for the isolation of mitochondria. Three days before euthanasia, blood samples were obtained from the tail tips of mice following an overnight fast. The plasma levels of triacylglycerols, total cholesterol and HDL-cholesterol were

determined using enzymatic colorimetric methods according to the instructions of the manufacturer (In Vitro Diagnostica, Ltd., MG, Brazil).

Histologic evaluation

The mice were euthanized, and their livers were excised. Slices of the right lobe were fixed in 10 % formalin, washed with chilled phosphate buffered saline solution and embedded in paraffin. The specimens were then sliced and stained with hematoxylin/eosin for histological analysis, which was performed by an experienced pathologist in a blinded fashion.

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 was then centrifuged as previously described (Alberici et al. 2006). The final mitochondrial pellet was diluted to a protein concentration of 60 mg/mL and 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 (P_i); 1 mM magnesium chloride; and 0.1 mM EGTA.

Respiratory rates

Respiratory rates were determined by oxygen consumption, which was monitored using a computer-interfaced Clark-type oxygen electrode from Hansatech Instruments Ltd. (King's Lynn, Norfolk, England) equipped with magnetic stirring. 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.

Reactive oxygen species (ROS) generation

ROS were monitored spectrofluorimetrically using probes of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, 1 μM) (Garcia-Ruiz et al. 1997) or Amplex Red (2 μM) in the presence of horseradish peroxidase (HRP, 1 U/mL) (Zhou et al. 1997) at 503/529 nm and 563/587 nm excitation/emission wavelength pairs, respectively, and slit widths of 5 nm in a Model F-4500 Hitachi fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) with continuous stirring.

Lipid extraction

A slice (weighing approximately 300 mg) of each liver was separately and quickly homogenized in 1 mL of cold chloroform as previously described (Alberici et al. 2011). The liver lipids were extracted as previously described (Bligh and Dyer 1959). Each lipid extract represents a single animal.

Easy ambient sonic-spray ionization mass spectrometry

Liver lipid extracts were analyzed by easy ambient (Alberici et al. 2010) sonic-spray ionization mass spectrometry (EASI-MS) in both the negative and positive ion modes, using a single-quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) equipped with a homemade EASI source (Haddad et al. 2006; Haddad et al. 2008). The main experimental parameters were as follows (Alberici et al. 2011): the flow rate of methanol of 20 μL/min, N_2 nebulizing gas at 3 L/min, and the paper-entrance angle of ~30°. A tiny droplet (2 μL) of the liver extracts was dropped directly onto the paper surface (brown Kraft paper). Mass spectra were accumulated over 60 sec and scanned along a range of 500 to 1,000 *m/z*. When operating in the negative ion mode, the methanol solvent was doped with 0.1 % ammonium hydroxide.

Liver redox state

Reduced (GSH) and oxidized (GSSG) glutathione, protein carbonyl and lipid peroxidation 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 (Hissin and Hilf 1976), protein carbonyl was assessed colorimetrically by the selective binding of 2,4-dinitrophenyl hydrazine (DNPH) to protein carbonyl groups (Reznick and Packer 1994; Schild et al. 1997) and lipid peroxidation was assessed by the thiobarbituric reactive substances (TBARS) method (Buege and Aust 1978).

RNA isolation and cDNA synthesis

Total RNA was isolated from frozen livers by using Trizol (Invitrogen) according to the manufacturer's protocol. RNA pellets were resuspended in DEPC-treated water, and RNA quantification was performed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). An additional step of RNAse-free DNAse treatment (Promega) was carried out to eliminate any traces of DNA. The purity and integrity of the preparations were analyzed by agarose gel electrophoresis and by calculating the A_{260nm}/A_{280nm} ratio. Five micrograms of RNA were used to synthesize cDNA using oligo-

dT primers and Superscript III Reverse Transcriptase (Invitrogen).

Real time quantitative PCR

The quantification of the transcript levels was performed using an Eppendorf Realplex4 Mastercycler instrument (Eppendorf). Primers LUX (Invitrogen) and Taq-Man Universal PCR Master Mix (Applied Biosystems) were used for the reactions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used for normalization. The primer sequences used were: UCP2 - CGGTACACTTTCC CTCTGGATAC[FAM]G and GCGCACTAGCCCTT GACTCTC; GAPDH - CGGAGCAAAAGGGTCAT CATCTC[FAM]G and TGGTTCACACCCATCACAAA CAT. In all experiments, the negative controls omitting the template DNA were submitted to the same procedures to exclude any possible contamination. All reactions were carried out in triplicate. The thermal cycling conditions were: an initial step at 50 °C for 2 min; 95 °C for 10 min; and 40 cycles, with 1 cycle consisting of 15 s at 95 °C and 1 min at 60 °C. The reactions and calculations were performed according to Semighini et al. (Semighini et al. 2002) and used 2.6 Gb as the size of the *Mus musculus* genome.

Data analysis

Experiments from each preparation were performed in duplicate, and the averages were calculated. Data shown are representative of each group, and other data are the ratio averages±SEM. Statistical analysis was performed using the Mann-Whitney non-parametric test conducted using GraphPad Prism software, version 5 (GraphPad Software, USA). Values of $P<0.05$ were considered as significant.

Results

The body weight gain of mice fed a diet supplemented with conjugated linoleic acid (CLA mice) did not present significant differences from the weight gain of mice fed a diet supplemented with linoleic acid (LA, control mice) during a period of 60 days (Fig. 1a). However, the CLA mice exhibited a 13.6 % increase in liver weight (Fig. 1b) and decreases of 42.3 % and 55.8 % in the weight of white adipose tissue (WAT) and brown adipose tissue (BAT), respectively, compared to the control mice (Fig. 1c). The maintenance of body weight in these mice could be due to either increases in bone mineralization and skeletal muscle mass (Rahman et al. 2007) or body protein and water content (Park et al. 1997). Moreover, CLA supplementation in the diet reduced the fasting levels of circulating triacylglycerols (TAG) and did not significantly affect the levels of cholesterol, HDL-cholesterol or non-HDL cholesterol (Table 1).

A decrease in adipose tissue weight followed by liver enlargement could indicate an offset in the storage of lipids from the adipose tissue to the liver (Park et al. 1997; Jaudszus et al. 2010), which is a characteristic of fatty liver disease (Gholam et al. 2007). Nevertheless, the hematoxylin/eosin-stained liver sections showed well-preserved hepatic architecture with no macro- or micro-vesicular steatosis in the hepatic periportals or midzones of the CLA mice (Fig. 2b) compared to the control mice (Fig. 2a). This evidence indicates that the reduced accumulation of lipids in adipose tissue of the CLA mice was not implicated in fatty liver development, as might be expected; therefore, CLA can be catabolized in the liver and muscles as a source of energy (Ohnuki et al. 2001b).

The lipid contents from the CLA mouse livers were evaluated by EASI-MS, a technique that has been shown to provide representative lipid profiles for oils, fats (Simas et al. 2010) and ex vivo complex lipid mixtures (Alberici et al. 2011). Figure 3b shows a representative EASI(+)-MS of the lipid mixture extracted from the livers of the CLA mice compared to those of the control mice (Fig. 3a). Phospholipids, mainly phosphatidylcholine (PC), and TAG were detected via their $[L+Na]^+$ forms. The highest intensity ions presented *m/z* 780, 782, 804, 806 and 808, which correspond to PC, and *m/z* 879 and 881, which correspond to the 52 carbons of TAG. The ion of *m/z* 780 (PC 34:2) was the highest abundant in the liver spectrum of the control mice,

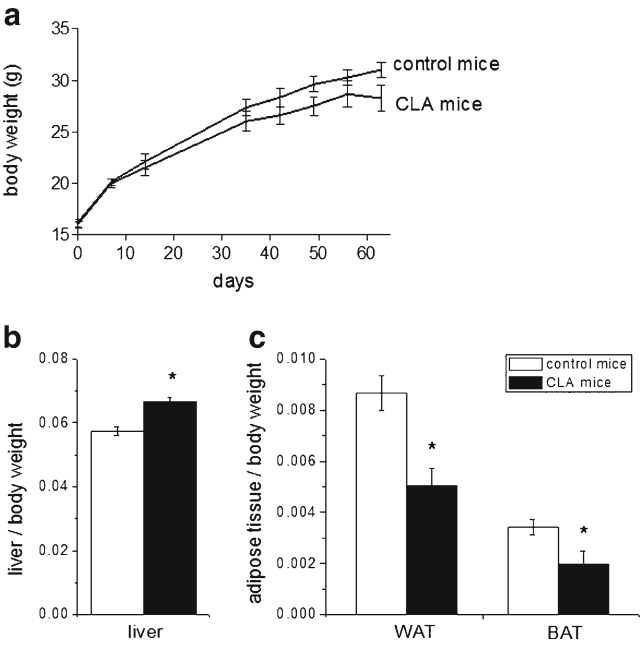

Fig. 1 Body weight (**a**), liver weight (**b**), and white (WAT) and brown (BAT) adipose tissues weight (**c**) of mice fed a diet supplemented with CLA (CLA mice) or LA (control mice). **b** and **c**: 60 days treatment. *$P<0.05$ *vs* control ($n=20–34$)

Table 1 Profile of circulating triacylglycerols (TAG), cholesterol, HDL-cholesterol (HDL-chol) and non-HDL cholesterol (non-HDL) in mice fed a diet supplemented with CLA (CLA mice) or LA (control mice)

	TAG	Cholesterol	HDL-chol	Non-HDL
Control mice	150.3±10.5	110.1±3.6	53.0±0.7	57.64±4.1
CLA mice	90.0±5.2*	116.8±3.0	51.9±2.5	65.55±3.6

*$P<0.05$ *vs* control ($n=5$)

whereas the ion of *m/z* 879 (TAG 52:3) was the highest abundant in the liver spectrum of the CLA mice, indicating a larger amount of TAG in relation to PC in the treated mice. In addition, the liver spectrum of the CLA mice presented a high abundance of the ion of *m/z* 881 (52:2), another TAG, in relation to the ion of *m/z* 780. Collectively, EASI-MS analysis indicates that a mouse diet supplemented with CLA increases the amount of TAG in relation to PC in the liver.

Figure 3d shows a representative EASI(-)-MS of the lipid mixture extracted from the livers of the CLA mice compared to those of the control mice (Fig. 3c). The phospholipid ions were detected via EASI(-)-MS in their deprotonated $[L-H]^-$ forms. The highest intensity ions presented *m/z* 766 (38:4, phosphatidylethanolamine, PE) and 885 (38:4, phosphatidylinositol, PI), and the liver spectrum of the CLA mice presented a reduced intensity of the ion of *m/z* 885 in relation to the *m/z* 766; the former corresponds to PI with 18:0 (stearic) and 20:4 (arachidonic acid) side chains. In this regard, a decrease in the PI levels promoted by CLA supplementation has been associated with anti-atherosclerotic and anti-tumorigenic effects, as well as with modulation of the immune system (Banni et al. 2001).

A slight enhancement in lipid flux through the liver could promote changes in hepatic mitochondrial metabolism, affecting both the coupling of oxidative phosphorylation and the generation of reactive oxygen species (ROS) (Schönfeld and Wojtczak 2008). Oxygen consumption was monitored in liver mitochondria, isolated in the presence of 0.1 % BSA (fat-free organelles), from both the CLA mice and the

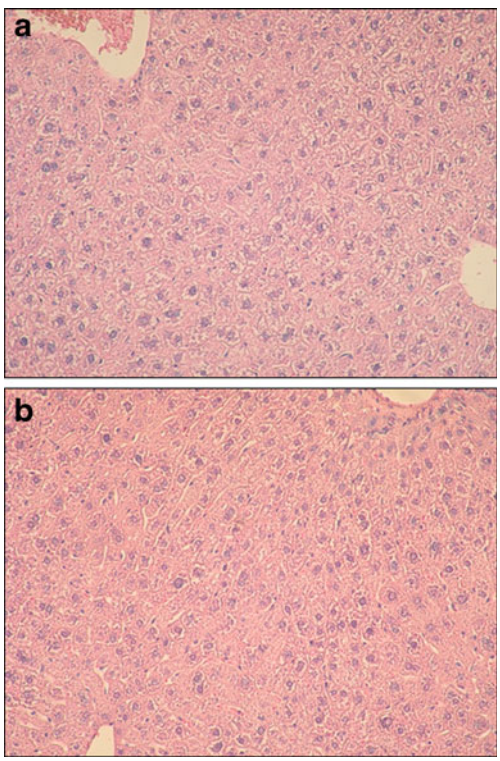

Fig. 2 Histology of right lobe of liver from control mice (**a**) and CLA mice (**b**) ($n=5$)

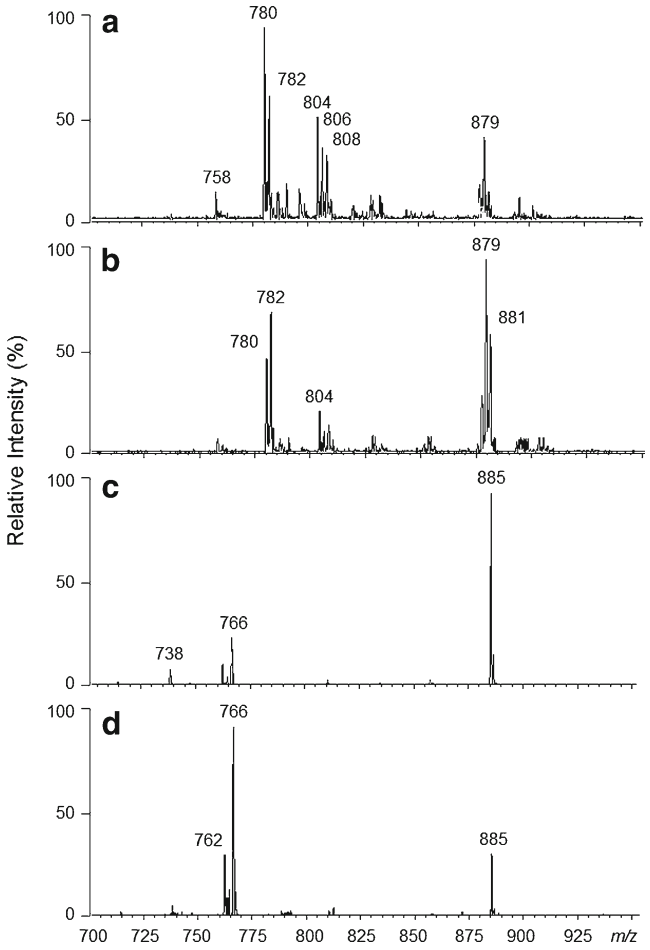

Fig. 3 Representative EASI mass spectra of liver lipid extracts from control mice (**a** and **c**) and CLA mice (**b** and **d**) in the positive (**a** and **b**) and negative (**c** and **d**) ion modes ($n=4$)

Table 2 Phosphorylating (state III) and resting (state IV) respiration rates, respiratory control ratio (RCR, state III/state IV) and phosphorylating efficiency (ADP/O) of liver mitochondria from CLA mice compared to control mice, without or with addition of 2 μM linoleic acid (LA) in the reaction medium

	State III	State IV	RCR	ADP/O
Control mice	28.8±1.6	7.1±0,3	4.0±0.1	2.08±0.02[c]
Control mice+LA	27.0±1.2	8.1±0,5	3.3±0.2	1.83±0.07[c,e]
CLA mice	27.1±1.5	7.3±0,5[a]	3.9±0.2[b]	2.01±0.08[d]
CLA mice+LA	25.3±1.9	9.1±0,8[a]	2.9±0.3[b]	1.65±0.05[d,e]

Respiration rates in nmol O_2/mg protein/min. The standard reaction medium (30 °C) contained 125 mM sucrose, 65 mM KCl, 10 mM Hepes pH 7.2, 2 mM P_i, 1 mM Mg^{2+} and 0.1 mM EGTA, in the presence of 5 mM glutamate, piruvate and malate (complex I substrate mixture). Averages ± SEM. The same symbols [a, b, c, d] and [e] are statistically different. $P<0.05$ ($n=12$)

control mice, with or without the addition of 0.2 μM linoleic acid (LA) in the reaction medium. Highly similar respiration rates, both in the phosphorylating (state III) and resting (state IV) states, as well as highly similar respiratory control ratios (RCR) and phosphorylating efficiencies (ADP/O ratio), were observed in the liver mitochondria from both CLA and the control mice in the absence of LA (Table 2). However, in the presence of LA, the mitochondria from the CLA mice showed a significant RCR decrease in association with increased resting respiration, as well as a decrease in the ADP/O ratio, thereby suggesting the action of uncoupling proteins.

Indeed, Fig. 4a shows that resting respiration of liver mitochondria from the CLA mice was 136 % stimulated by LA, an effect that was 26 % higher than for the control mice (110 %). Carboxyatractyloside (CAT), an inhibitor of adenine nucleotide translocase that promotes uncoupling by free fatty acids, reduced the stimulation induced by LA in the mitochondria from both mice groups. However, in the presence of the UCP inhibitor GDP, the higher stimulation promoted by LA in mitochondria from the CLA mice was almost completely reduced to the level of the stimulation from the control mice; this result indicates the involvement of the activity of UCP. Moreover, the LA-promoted stimulation of respiration was fully reversed in mitochondria from both mice groups via the removal of free fatty acids through the addition of BSA. In agreement with the results on mitochondrial respiration, the levels of UCP2 mRNA, which is the most abundant UCP isoform in the liver, differed significantly between the CLA and control mouse livers, as UCP2 mRNA was 2.5 times higher in CLA mouse livers (Fig. 4b).

Reactive oxygen species (ROS) were monitored in liver mitochondria isolated from the CLA mice compared to the control mice, both in the intermembrane space of organelles with Amplex Red/HRP (specific for H_2O_2, Figs. 5a–c) and in the matrix with the membrane-permeable probe H_2DCF-DA (for several ROS, Figs. 5a1–d1). In general, similar ROS generation profiles were observed in both mitochondrial compartments. When the liver mitochondria were energized with pyruvate, malate and glutamate to generate NADH through the Krebs cycle (which donates electrons to respiratory chain Complex I), the organelles from the CLA mice showed an increased rate of ROS generation compared to the control mice (Fig. 5a and a1). However, when energized with succinate to generate $FADH_2$ in the respiratory

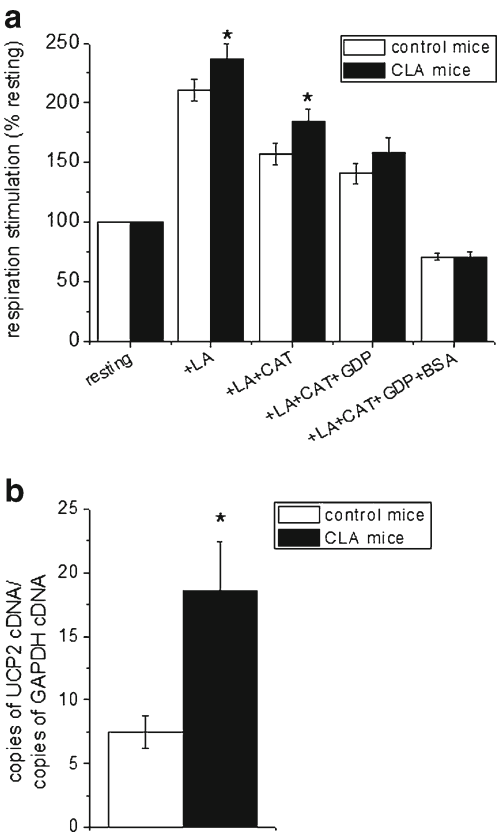

Fig. 4 **a** Effects of UCP modulators on resting respiration of liver mitochondria from control and CLA mice. Mitochondria (0.5 mg/mL) were incubated in the standard reaction medium described in Table 2, in the presence of 5 mM glutamate, piruvate and malate, and 1 μg/mL oligomycin. Linoleic acid (LA, 8 μM), carboxyatractyloside (CAT, 5 μM), GDP (1 mM) and BSA (0.01 %) were added in the reaction medium. **b** Amount of mRNA for UCP2 in liver from control and CLA mice, determined by quantitative RT-PCR and normalized to GAPDH. *$P<0.05$ *vs* control ($n=4$–7)

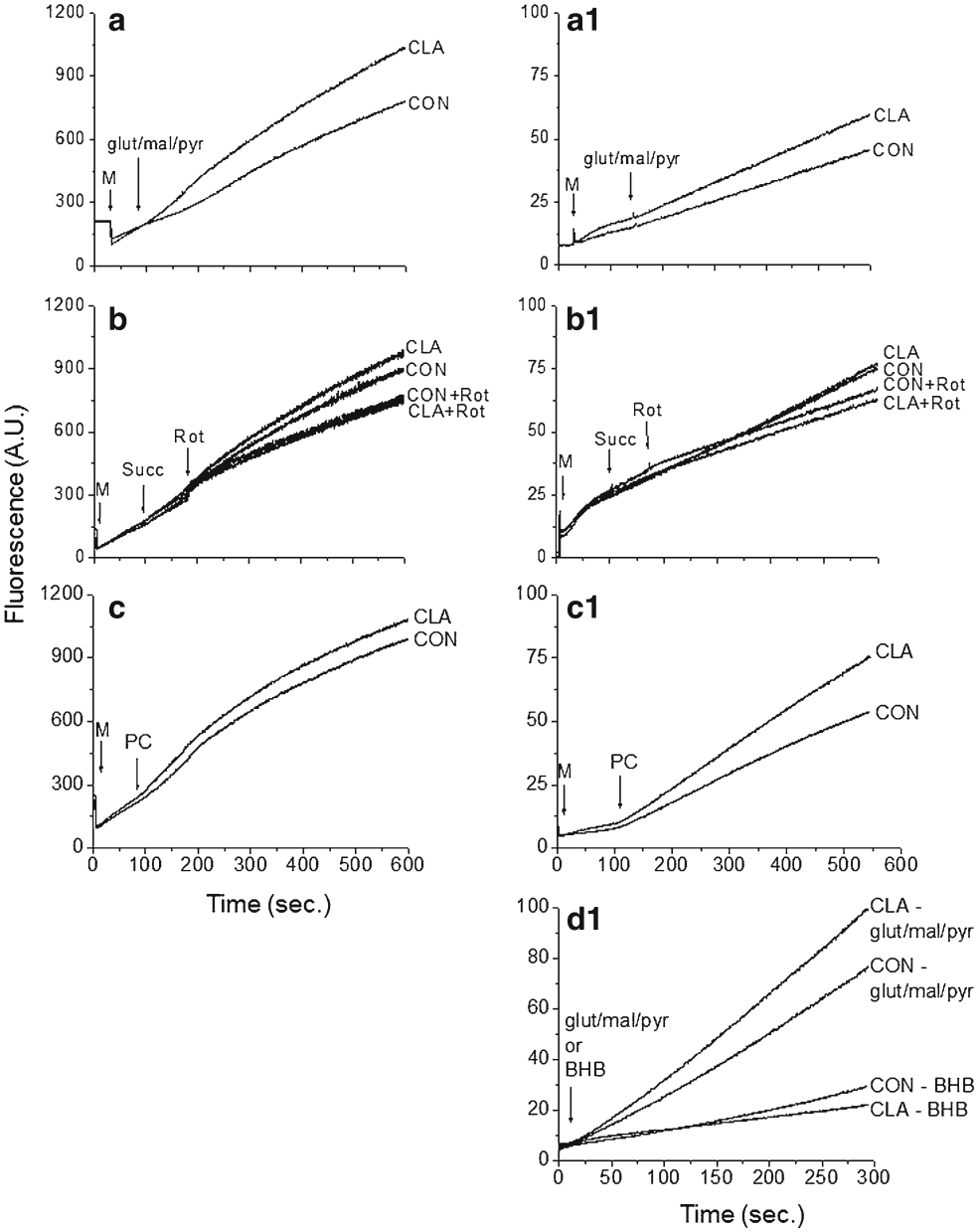

Fig. 5 ROS generation in liver mitochondria from control and CLA mice, monitored with Amplex Red (**a**–**c**) or H_2DCF-DA (**a1**–**d1**). Mitochondria (M, 1 mg/mL) were incubated in the standard reaction medium described in Table 2. Where indicated, it was added either 5 mM glutamate, malate and pyruvate (gut/mal/pyr) or 5 mM succinate (Succ) or 2 μM rotenone (Rot) or 10 μM palmitoyl-carnitine plus 2 mM malate (PC) or 5 mM β-hydroxybutyric acid (BHB). In d1, endogenous Krebs cycle intermediaries were previously exhausted by incubating mitochondria in the standard reaction medium in the presence of 1 mM ADP; after 300 sec, oligomycin was added and fluorescence monitoring was started. Traces are representative of four to seven experiments with different mitochondrial preparations

chain Complex II, the rates of ROS generation did not differ significantly in liver mitochondria from the CLA and control mice (Fig. 5b and b1). The addition of rotenone, which inhibits ROS generation by the "back flow" of electrons from Complex I to ubiquinone in succinate-energized mitochondria, reduced ROS generation in a similar manner for both mouse groups. When mitochondria were energized with a β-oxidation substrate (palmitoyl-carnitine) supplying the Krebs cycle and respiratory chain at Complex I and II, in a 4:2 ratio (NADH:$FADH_2$), the liver mitochondria from the CLA mice again generated more ROS than organelles from the control mice (Fig. 5c and c1); this effect was more prominent with H_2DCF-DA (Fig. 5c1). When NADH was directly generated at the mitochondrial matrix by β-hydroxybutyric acid (which forms acetoacetate reducing NAD^+ to NADH, thereby excluding the Krebs cycle involvement), the liver mitochondria from the CLA mice and controls showed similar ROS generation rates (Fig. 5d1). These results indicate that in the liver mitochondria of the CLA mice, ROS generation is increased in relation to the control mice and that this increase occurs during the Krebs cycle.

High rates of ROS generation may promote oxidative damage in the liver. To assess the redox state of the CLA mouse livers compared to controls, we measured the levels of reduced and oxidized glutathione (GSH/GSSG ratio), as well as the levels of oxidized proteins (carbonyl groups) and lipids (TBARS), in this tissue (Table 3**)**. The GSH/GSSG ratio, as well as levels of carbonyl and TBARS in the CLA mouse livers, did not differ significantly from the control

Table 3 Redox state of mouse livers

	GSH/GSSG	Carbonyl (nmol·mg prot^{-1})	TBARS (absorbance at 535 nm)
Control mice	4.23±0.65	6.59±0.39	9.44±0.48
CLA mice	4.47±0.61	5.50±0.43	8.65±0.63

Averages ± SEM (n=5–8)

mouse livers. Therefore, the increased ROS generation by mitochondria from the CLA mice did not cause any oxidative stress to the whole organ.

Discussion

The literature reports that daily diet supplementation with a mixture of 1–3 % CLA or the *trans*-10, *cis*-12 isomers alone decreases the body fat content in many animal models but promotes adverse effects in mice, such as the development of fatty livers and insulin resistance (Kennedy et al. 2010). In this work, we demonstrated that feeding mice a low-fat (4 %) diet supplemented with ~2 % CLA on alternating days reduces lipid accumulation in adipose tissue and, despite liver enlargement, does not augment hepatic lipid storage. Previously, CLA-induced liver enlargement in mice was reported and assigned to either hypertrophy or intracellular glycogen storage (Halade et al. 2009; DeLany and West 2000). It should be mentioned that the slight increase of TAG found in CLA mice livers was detected due to the very high sensitivity of the tool employed. Apparently, the results do not concern increased lipid storage but, rather, the increased lipid flow through the liver.

CLA is a high-affinity ligand and activator of peroxisome proliferator-activated receptors, including the liver subtype alpha receptor (PPAR-α) (Moya-Camarena et al. 1999; Konig et al. 2008; Schoonjans et al. 1996). The activation of these receptors increases the transcription of several genes, including mitochondrial and peroxisomal oxidative enzymes (Schoonjans et al. 1996), resulting in increased cellular lipid catabolism, decreased liver TAG secretion and liver hypertrophy (Fruchart et al. 1999). We found evidence of liver hypertrophy and reduced TAG plasma levels in CLA-mice, which was most likely due to PPAR-α activation. In this regard, the transcription of the *UCP2* gene was also reported to be induced by PPAR-α in the livers of mice (Rakhshandehroo et al. 2009). Accordingly, UCP2 expression and mitochondrial energetics in the liver were modified by supplementing the mouse diet with CLA: livers from CLA mice showed high levels of UCP2 mRNA, and the isolated hepatic mitochondria showed indications of UCP activity and increased ROS generation through oxi-reduction reactions involving NAD^+/NADH in the Krebs cycle. Previous reports indicate that UCPs are activated by ROS (superoxide) generated within the mitochondrial matrix (Echtay et al. 2002a; 2002b). Our results show that in mice, CLA diet supplementation alters liver metabolism by increasing ROS generation during the Krebs cycle, which may result in the activation of mitochondrial UCPs. However, this does not cause oxidative stress in the liver and, in the absence of free fatty acids, basal respiration rates and the phosphorylating efficiency of mitochondria are preserved.

The expression and up-regulation of UCP genes and proteins as a result of CLA diet supplementation was first reported in BAT, in which UCP1 overexpression associated with high mitochondrial density, increased heat generation. However, most studies have failed to demonstrate this stimulation in rats (Ealey et al. 2002; Ryder et al. 2001), hamsters (Ribot et al. 2007) and mice (Tsuboyama-Kasaoka et al. 2000; Takahashi et al. 2002; West et al. 2000; Ealey et al. 2002; DeLany and West 2000). Supplementation with CLA was also reported to up-regulate UCP3, mainly in skeletal muscle (Ealey et al. 2002; Roche et al. 2002; Ribot et al. 2007). However, in this tissue, UCP3's primary role has been proposed to be as a regulator of fatty acid utilization rather than as an uncoupler involved in thermogenesis (Samec et al. 1998). Furthermore, major evidence suggests that UCP2 is more important than the other UCP isoforms in CLA-induced alterations of energy expenditure; this protein is the most highly expressed UCP isoform and is expressed in a variety of tissues. In the present study, we demonstrated that UCP2 is expressed in the mouse liver and, for the first time that UCP2 is overexpressed and potentially activated with diet supplementation with CLA. Remarkably, a slight increase in UCP2 content and activity in most tissues could lead to higher body energy expenditure.

In conclusion, this study indicates a beneficial and secure dose of CLA for diet supplementation: in mice, a low-fat (4 %) diet plus a 2.2 % CLA mixture (*cis*-9, *trans*-10 and *trans*-10, *cis*-12 isomers; 45 % of each isomer) on alternating days induces UCP2 overexpression and potentially induces UCP2 activity in mitochondria while preserving the lipid composition and redox state of the liver. Therefore, diet supplementation with CLA may be regarded as a potential strategy for controlling obesity.

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