Conjugated linoleic acid and hepatic lipogenesis in mouse: role of the mitochondrial citrate carrier

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Abstract Conjugated linoleic acid (CLA) is able to reduce adiposity by affecting lipid metabolism. In particular, CLA administration to mice reduces body fat mass with a concomitant lipid accumulation in the liver. We investigated the effects of CLA on the activity of the mitochondrial citrate carrier (CIC), which is implicated in hepatic lipogenesis. The transport activity of the CIC, measured both in intact mitochondria and in the proteoliposomes, progressively increased with the duration of CLA feeding. An increase in the CIC activity of ∼1.7-fold was found in 16 week CLA-treated mice with respect to control animals. A kinetic analysis showed a 1.6-fold increase in the V_{max} of citrate transport but no change in the K_m value. Western blot experiments revealed an increase of ∼1.7-fold in the expression of CIC after CLA treatment. A strict correlation between the increase in CIC activity and the stimulation of the cytosolic lipogenic enzymes was also found. These data indicate that the CIC may play a role in the onset of hepatic steatosis in CLA-fed mice by supplying the carbon source for de novo fatty acid synthesis.—Ferramosca, A., V. Savy, L. Conte, S. Colombo, A. W. C. Einerhand, and V. Zara. **Conjugated linoleic acid and hepatic lipogenesis in mouse: role of the mitochondrial citrate carrier.** ***J. Lipid Res.*** **2006.** 47: **1994–2003.**

Supplementary key words tricarboxylate carrier • lipogenic enzymes • hepatic steatosis

Conjugated linoleic acid (CLA) is a family of positional and geometric dienoic isomers of linoleic acid (1). These compounds are naturally occurring molecules that are present in several dairy products (2). In fact, they are formed from linoleic and α-linolenic acid in the intestine of ruminant animals, thus appearing in milk, in milk derivatives, and in the meat of these animals. The main CLA isomer in natural products is the *cis*-9,*trans*-11-octadecadienoic acid, even if the majority of studies implying the use of these molecules as food supplements have been carried out with a 1:1 mixture of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers. It has been largely demonstrated that CLA has positive effects in cancer (3), cardiovascular diseases (4), diabetes, and obesity (5). In particular, mice fed a CLA-enriched diet for just a few weeks showed a consistent decrease in fat mass and a concomitant increase in lean body mass (6–9). It has also been demonstrated that the fat-lowering effect is attributable to the *trans*-10,*cis*-12 isomer (10, 11). Furthermore, the extent of the decrease in adiposity was different in the various organisms tested (5). The adipose tissue reduction found in mice was accompanied by significant liver enlargement caused by an accumulation of lipids in this organ (8, 9, 11–13). The mouse model also showed a decrease in insulin sensitivity, as indicated by the hyperinsulinemia consequent to CLA feeding (8, 9, 11). On the contrary, in an experimental model of diabetic rats, a clear decrease in insulin resistance and an improvement in glycemic parameters was found after CLA administration (14). Furthermore, it has been found that the effect of CLA on insulin resistance was transient in both lep^{db}/lep^{db} and lep^{ob}/lep^{ob} mice (15, 16).

Therefore, the implications concerning the use of CLA as a dietary supplement appear intriguing both for the species-specific effects (17) and for the molecular mechanisms underlying the changes in body composition and in the hematic parameters. In particular, very little is known about the molecular mechanisms leading to fat deposition in liver. The de novo fatty acid synthesis, as suggested by some authors (18, 19), may play a role in the onset of hepatic steatosis. Hepatic lipogenesis implies a complex series of reactions occurring in part in the mitochondrial matrix and in part in the cytosol. The functional connection between these two different cellular compartments is made possible by the presence of a mitochondrial

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Abbreviations: ACC, acetyl-coenzyme A carboxylase; 1,2,3-BTA, 1,2,3-benzenetricarboxylate; CIC, mitochondrial tricarboxylate or citrate carrier; CLA, conjugated linoleic acid; CPT, carnitine palmitoyltransferase.

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transport protein, the tricarboxylate or citrate carrier (CIC) (20, 21). This integral protein of the inner mitochondrial membrane, belonging to the large family of mitochondrial metabolite carriers (22), catalyzes the efflux of citrate from the mitochondrial matrix toward the cytosol, thus playing an important role in intermediary metabolism. In fact, the transported citrate generates in the cytosol acetyl-CoA, which is the primer for de novo fatty acid and cholesterol biosyntheses (23). The CIC has been purified to homogeneity from different organisms (24–26), functionally characterized (27–29), and cloned in rat liver and *Saccharomyces cerevisiae* (30, 31). Detailed studies have been carried out on the topology of the CIC and on the role of individual amino acid residues in the mechanism of transport (32–34). Despite the fact that a great deal of data are currently available on the structural and functional properties of this carrier protein, comparatively little is known about the regulation of its transport activity. Furthermore, very little is known about the integration of its function with that of the cytosolic lipogenic enzymes, such as acetyl-coenzyme A carboxylase (ACC) and FAS, to which the CIC physiologically supplies substrates. The data currently available indicate that the transport activity of the CIC is downregulated during starvation (35) and in type 1 diabetes (36), whereas a significant increase in its activity has been reported in hyperthyroidism (37). Interestingly, differential effects of dietary fatty acids on the function of the CIC have been reported (38–40).

Therefore, we have undertaken an extensive study with the aim of investigating a possible modification of mouse CIC activity consequent to CLA administration. In mice fed a diet enriched with 1% CLA, composed mainly of a 1:1 mixture of *cis*-9,*trans*-11- and *trans*-10,*cis*-12-octadecadienoic acids, a significant increase in the activity of the CIC was found. A strict covariance of the CIC activity and that of the cytosolic lipogenic enzymes was detected in the liver of treated mice. Hepatic lipogenesis increased progressively with the duration of CLA feeding. The molecular mechanisms underlying the activation of the CIC were also investigated.

EXPERIMENTAL PROCEDURES

Materials

The Bio-Rad protein assay kit and hydroxyapatite were purchased from Bio-Rad. Amberlite XAD-2, Dowex AG1-X8, Pipes, Triton X-100, Triton X-114, Sephadex G-75, 1,2,3-benzenetricarboxylate (1,2,3-BTA), cardiolipin, acetyl-CoA, phosphoenolpyruvate, ATP, NADH, NADPH, pyruvate kinase, lactate dehydrogenase, malonyl-CoA, 5,5′-dithio-bis(2-nitrobenzoic acid), carnitine, and palmitoyl-CoA were from Sigma. TRIzol reagent was from Invitrogen. Clarinol™ G-80, a 1:1 mixture of the *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 isomers of CLA, was a generous gift of Lipid Nutrition, a division of Loders Croklaan. [1,5-^{14}C]citrate was from Amersham, and egg yolk phospholipids were from Fluka. Kits for the assay of triglycerides, total cholesterol, and phospholipids were purchased from Futura System, whereas that for the assay of FFAs was purchased from Roche. All other reagents were of analytical grade.

Animals

Male ICR mice, obtained from Harlan at 5 weeks of age, were housed individually in animal cages at a temperature of 22 ± 1°C. Animals were fed ad libitum either with a standard diet (control) or with the same diet containing 1% CLA (CLA-treated). The composition of both diets, freshly prepared each week and stored frozen until used, is reported in **Table 1**. Body weight, liver weight, and food intake were recorded throughout the study, ranging from 2 to 16 weeks of dietary treatment. Experiments were carried out in accordance with local and national guidelines regarding animal experiments.

Citrate transport in mouse liver mitochondria

Mouse liver mitochondria were prepared by following standard procedures. The assay of citrate transport in mitochondria was carried out as reported previously (20, 38). Briefly, freshly isolated mouse liver mitochondria were resuspended in 100 mM KCl, 20 mM HEPES, 1 mM EGTA, and 2 μg/ml rotenone, pH 7.0, at a concentration of ~5 mg protein/ml and loaded with L-malate as described previously (35). The assay of citrate transport was initiated by the addition of 0.5 mM [^{14}C]citrate to malate-loaded mitochondria incubated at 9°C and terminated by adding 12.5 mM 1,2,3-BTA. Mouse liver mitochondria were then centrifuged at 18,000 *g* for 10 min at 2°C, washed once, and extracted with 20% $HClO_4$. The mixture was centrifuged, and the radioactivity present in the supernatant was counted by liquid scintillation.

Reconstitution of the citrate transport into liposomes

Mouse liver mitochondria were frozen immediately after preparation and stored at −80°C. Aliquots of 10–15 mg of proteins were thawed and solubilized with a buffer containing 3% Triton X-100 (w/v), 20 mM Na_2SO_4, 1 mM EDTA, and 10 mM PIPES, pH 7.0, at a final concentration of ~10 mg protein/ml. After incubation for 10 min at 2°C, the mixture was centrifuged at 25,000 *g* for 20 min at 2°C, and the supernatant was referred to as the mitochondrial extract. A total of 600 μl of this extract (corresponding to ~6–7 mg of protein), supplemented with 2 mg/ml

TABLE 1. Composition of diets

Ingredient	Standard Diet	Standard Diet + 1% CLA
Proteins	17.86	17.86
Lipids	10.41	10.41
Fatty acids		
16:0	1.32	1.25
18:0	0.29	0.28
18:1 ω9	5.09	4.43
18:2 ω6	3.20	3.16
18:3 ω3	0.31	0.30
Other fatty acids	0.16	0.16
CLA *cis*-9,*trans*-11	—	0.37
CLA *trans*-10,*cis*-12	—	0.38
Other CLA isomers	—	0.04
Carbohydrates	54.40	54.40
Sugars	4.66	4.66
Mineral + vitamin mix	3.33	3.33
Crude fiber	3.61	3.61
Ash	5.73	5.73
Calories per 100 g	401	401

CLA, conjugated linoleic acid. Values shown are percentages. Standard diet was supplemented with 1% Clarinol™ G-80, a 1:1 mixture of the two main isomers (*cis*-9,*trans*-11- and *trans*-10,*cis*-12-octadecadienoic acids). Both diets had the same energy content, which was calculated using 4 kcal/g for proteins and carbohydrates and 9 kcal/g for lipids.

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cardiolipin, was applied to a cold hydroxyapatite column (Pasteur pipette containing 600 mg of dry material) and eluted with a buffer containing 0.5% Triton X-100 and 5 mM citrate/NaOH, pH 7.0. The first 600 μl of the eluate was pooled and used for the reconstitution experiments carried out by cyclic removal of the detergent with a hydrophobic column (41). The initial mixture used for the reconstitution experiments contained 50 μl of hydroxyapatite eluate, 90 μl of 10% Triton X-114, 20 μl of 20 mg/ml cardiolipin, 100 μl of 10% phospholipids in the form of sonicated liposomes, 70 μl of 100 mM PIPES (pH 7.0), and 35 μl of 200 mM citrate in a final volume of 700 μl. This mixture was carefully vortexed and then passed 15 times through the same Amberlite XAD-2 column. The external citrate was removed by gel filtration on a Sephadex G-75 column. The first 600 μl of the turbid eluate were collected and distributed in reaction vessels (180 μl). Transport was initiated by the addition of 0.5 mM [^{14}C]citrate (unless indicated otherwise) to reconstituted proteoliposomes incubated at 25°C and stopped after the indicated times by adding 20 mM 1,2,3-BTA. The radioactivity external to proteoliposomes was removed from each sample by chromatography on Dowex AG1-X8 columns. The proteoliposomes were eluted with 1 ml of 50 mM NaCl, and their radioactivity was measured by scintillation counting.

Assay of lipogenic enzymes and of total carnitine palmitoyltransferase

The enzymatic activities of ACC and FAS were determined in mouse liver cytosol. This was obtained by centrifuging the post-mitochondrial supernatant at 20,000 *g* for 20 min. The pellet was discarded and the supernatant was then centrifuged at 105,000 *g* for 1 h. On the resulting cytosol, the ACC activity was measured using a NADH-linked assay as described (42). FAS activity was determined as a decrease in the NADPH absorption at 340 nm (43). Total carnitine palmitoyltransferase (CPT) activity was determined spectrophotometrically at 412 nm in freshly isolated mouse liver mitochondria using 5,5′-dithio-bis(2-nitrobenzoic acid) essentially as described (44).

Assay of lipids

For the determination of body fat, animals were eviscerated and then dehydrated in an oven at 150°C for 5–7 days until a constant mass was achieved. The dried carcass was homogenized, and lipids were extracted from 1 g aliquots with a 1:1 mixture of chloroform and methanol as described previously (45). The extracts were dried and weighed. For the determination of hepatic lipids, total lipids were extracted from mouse liver using

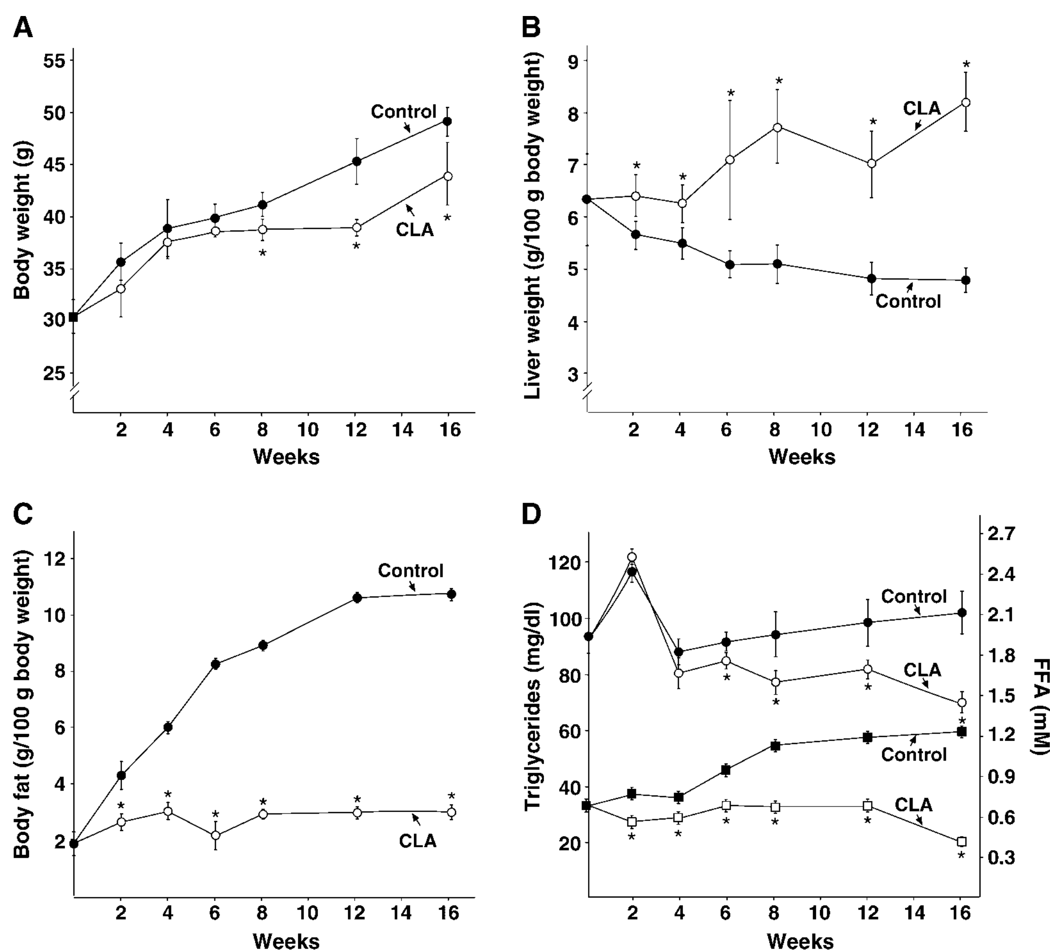

Fig. 1. Effects of dietary treatments on body weight (A), liver weight (B), body fat (C), and plasma lipid levels (D). Body weight, liver weight, and body fat are shown for male mice fed a standard diet (closed circles) (control) or a conjugated linoleic acid (CLA)-supplemented diet (open circles) for the times indicated. Plasma levels of triglycerides (circles) or FFAs (squares) are shown for control mice (closed symbols) and CLA-treated animals (open symbols). Each point represents the mean ± SEM for five animals. All parameters were subjected to the *t*-test (* $P < 0.05$).

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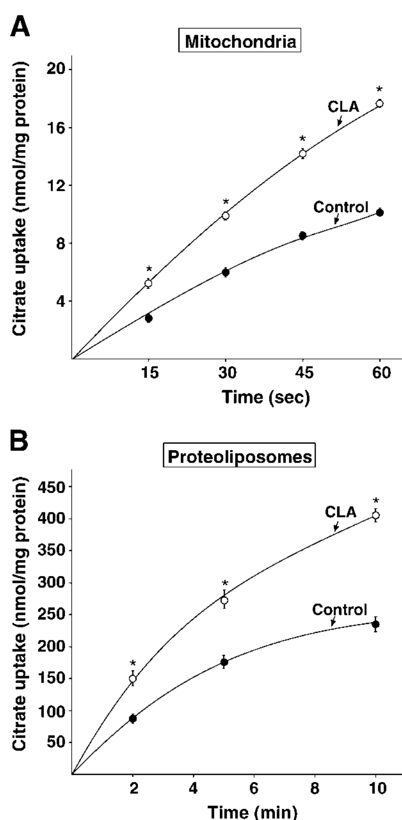

Fig. 2. Transport of citrate into mouse liver mitochondria (A) and proteoliposomes (B). A: Malate-loaded mitochondria, isolated from mice fed for 16 weeks with a standard diet (closed circles) (control) or with the same diet supplemented with 1% CLA (open circles), were incubated with 0.5 mM [^{14}C]citrate for the indicated times. B: Proteoliposomes, reconstituted with the purified mitochondrial tricarboxylate or citrate carrier (CIC) from CLA-treated mice (open circles) and control mice (closed circles) and containing 10 mM citrate, were incubated with 0.5 mM [^{14}C]citrate for the indicated times. The data reported represent means ± SEM for five animals after 16 weeks of dietary treatment. All parameters were subjected to the *t*-test (* $P < 0.05$).

chloroform and methanol (1:1). The extracts were dried under nitrogen flow and resuspended in a suitable volume of 0.1% Triton X-100 before carrying out the individual assay of triglycerides, cholesterol, and phospholipids using commercial kits. For the determination of plasma lipids, control and treated mice were starved overnight before euthanasia. Blood was collected and centrifuged to separate plasma. Plasma triglycerides and FFA levels were measured using commercial kits.

Northern blot analysis

Total RNA from mouse liver was extracted using TRIzol reagent essentially as described previously (46). The extracted RNA was separated onto 1% formaldehyde-agarose gels under denaturing conditions and transferred to Hybond nylon membranes. The RNA blots were hybridized with a probe corresponding to the full-length mouse citrate carrier cDNA. The hybridization signals were normalized on the basis of 18S rRNA levels. After autoradiography, the intensity of the bands was determined by densitometric analysis.

Other methods

Protein was determined by the Bradford method (47) or by the Lowry method modified for the presence of Triton (48). PAGE of trichloroacetic acid-precipitated samples was performed in the presence of 0.1% SDS according to standard procedures. The mitochondrial proteins, separated by SDS-PAGE, were then transferred to nitrocellulose membranes. For immunodecoration, antisera directed against the C terminus of the rat liver citrate carrier and against mammalian porin were used.

Statistical analysis

Experimental data represent means ± SEM. Student's *t*-test was performed to detect significant differences between the control and CLA-treated animals. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effects of CLA on animal growth, tissue weights, and plasma lipid levels

Male mice were divided into two groups and fed a standard diet (control) or the same diet supplemented with 1% CLA for different times. CLA, as reported in Table 1, was added as a 1:1 mixture of the two main isomers, the *cis*-9,*trans*-11- and *trans*-10,*cis*-12-octadecadienoic acids. Whereas food intake during the course of the study was practically identical in both groups of animals (data not shown), a decrease in body weight was clearly observed in

TABLE 2. CIC transport activity

Week	Mitochondria: Control	Mitochondria: CLA	Mitochondria: Percentage Increase	Proteoliposomes: Control	Proteoliposomes: CLA	Proteoliposomes: Percentage Increase
	nmol·min^{-1}·mg protein^{-1}			*nmol·10 min^{-1}·mg protein^{-1}*		
0	11.3 ± 0.8	—	—	235.0 ± 9.2	—	—
2	11.2 ± 0.3	12.0 ± 0.3[a]	7.1	240.1 ± 16.8	269.1 ± 8.9[a]	12.1
4	11.7 ± 0.4	13.2 ± 0.6[a]	12.8	228.0 ± 13.9	269.0 ± 2.8[a]	18.0
6	11.2 ± 0.2	13.9 ± 0.3[a]	24.1	238.1 ± 17.9	293.4 ± 5.3[a]	23.2
8	11.2 ± 0.1	15.5 ± 0.4[a]	38.4	231.9 ± 8.0	319.8 ± 16.8[a]	37.9
12	11.3 ± 0.3	17.2 ± 0.1[a]	51.3	230.5 ± 15.4	359.0 ± 11.4[a]	55.8
16	10.3 ± 0.1	17.5 ± 0.6[a]	69.9	231.0 ± 10.2	401.2 ± 8.1[a]	73.7

CIC, mitochondrial tricarboxylate or citrate carrier. The transport activity of the CIC in mouse liver mitochondria and in the reconstituted system (proteoliposomes) was measured at the times indicated. The values reported represent means ± SEM (n = 4).
[a] $P < 0.05$ by the *t*-test.

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CLA-fed mice (**Fig. 1A**). The weight loss became significant from 8 weeks onward of CLA administration. As widely reported in the literature (8, 9, 11–13), a strong and significant increase in liver weight was found in mice treated with 1% CLA with respect to control animals (Fig. 1B). After 16 weeks of CLA administration, a 1.8-fold increase in liver weight was found in treated animals. In contrast, the CLA diet compared with the control diet greatly reduced body fat accumulation over time (Fig. 1C). Furthermore, plasma levels of both triglycerides and FFA were lower in CLA-fed mice with respect to control animals (Fig. 1D).

Citrate transport into liver mitochondria and proteoliposomes

The transport activity of the CIC was measured in mouse liver mitochondria. **Figure 2A** shows citrate uptake by mitochondria freshly isolated from mice fed for 16 weeks with a standard diet (control) or the same diet supplemented with 1% CLA. In these experiments, equal amounts of liver mitochondrial protein from both types of mice were used. The CIC activity was significantly higher in the mitochondria of CLA-fed mice with respect to control animals, which showed a transport activity very similar to that found previously in standard diet-fed animals (35, 38). In the first part of the substrate uptake [i.e., during the linear range of transport of citrate into mitochondria (approximately the first 30 s)], a 1.7-fold increase in CIC activity was consistently found.

The functional reconstitution of the solubilized carrier into liposomes represents an alternative method for the

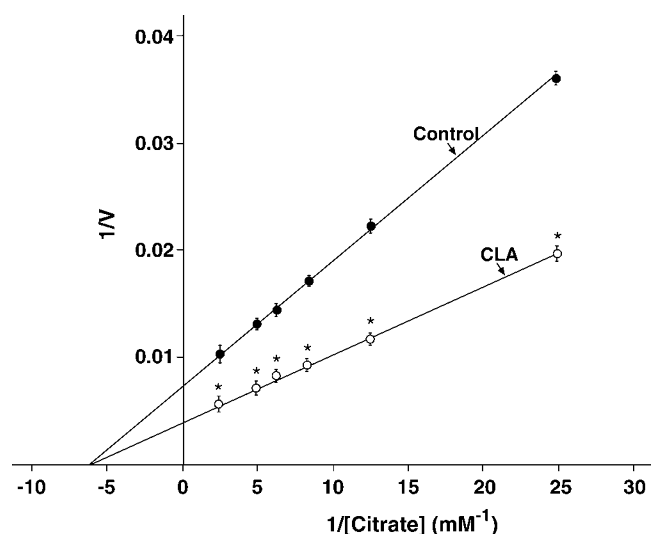

Fig. 3. Dependence of the rate of citrate uptake on substrate concentration in proteoliposomes reconstituted with the CIC purified from CLA-treated and control mice. Proteoliposomes were reconstituted with the CIC as described in Experimental Procedures. [^{14}C]citrate was added at the concentrations indicated. The citrate-citrate exchange was stopped 1 min after the addition of the radiolabeled substrate by 20 mM 1,2,3-benzenetricarboxylate. V is expressed as nmol·min^{-1}·mg protein^{-1}. Each point represents the mean ± SEM for five animals. All parameters were subjected to the *t*-test (* $P < 0.05$).

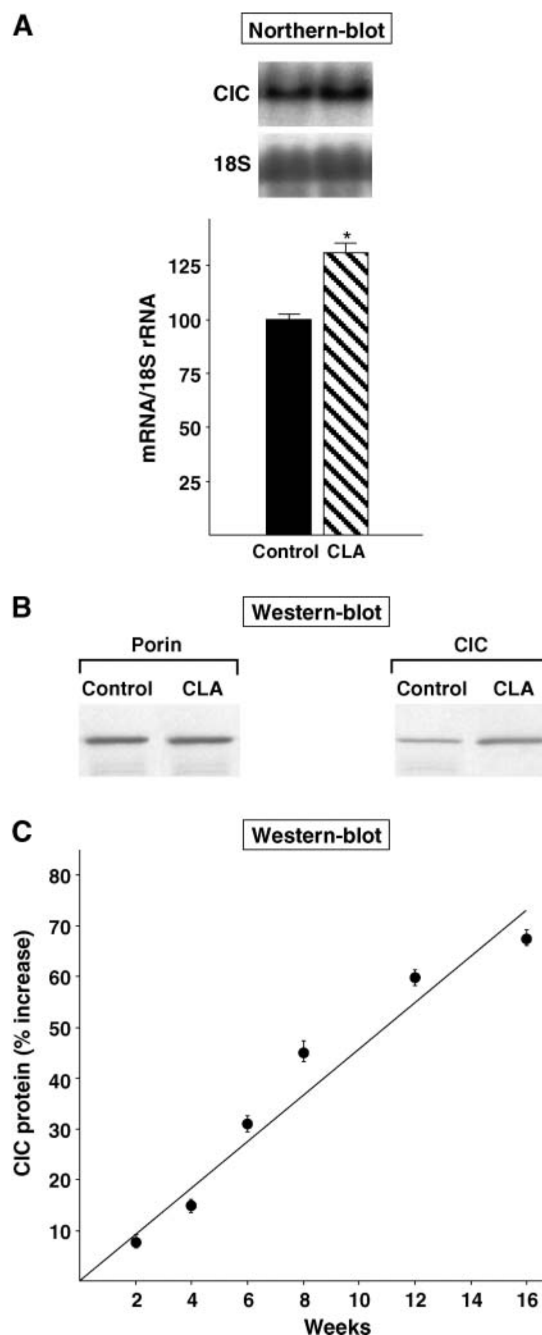

Fig. 4. Effect of CLA on mRNA and protein levels of the CIC. A: Total RNA from livers of mice fed for 16 weeks with a standard diet (control) or with the same diet supplemented with 1% CLA was hybridized with mouse CIC cDNA. Variations with respect to the control animals were calculated after correcting for loading differences on the basis of 18S rRNA levels. The values reported in the graph represent means ± SEM (* $P < 0.05$). B: Liver mitochondrial proteins from control and CLA-fed mice (16 weeks of dietary treatment) were separated by SDS-PAGE, transferred to nitrocellulose, and then immunodecorated with antiserum against either a C-terminal peptide of the rat CIC or the mammalian porin. C: Time dependence of the percentage increase in CIC protein levels in CLA-fed mice with respect to control animals. The values reported in the graph represent means ± SEM (n = 3).

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TABLE 3. ACC and FAS activities

Week	ACC Activity			FAS Activity		
	Control	CLA	Percentage Increase	Control	CLA	Percentage Increase
		$nmol \cdot min^{-1} \cdot mg\ protein^{-1}$			$nmol \cdot min^{-1} \cdot mg\ protein^{-1}$	
0	31.4 ± 0.7	—	—	32.4 ± 1.2	—	—
2	32.5 ± 0.4	34.4 ± 0.5[a]	5.8	31.9 ± 1.6	34.4 ± 0.4[a]	7.8
4	34.7 ± 1.0	42.7 ± 0.3[a]	23.0	36.4 ± 0.6	43.3 ± 1.0[a]	18.9
6	36.2 ± 0.8	47.6 ± 1.3[a]	31.5	37.6 ± 2.1	49.1 ± 1.2[a]	30.5
8	37.2 ± 1.5	53.3 ± 0.9[a]	43.3	37.9 ± 2.7	54.4 ± 0.6[a]	43.5
12	35.7 ± 0.8	56.4 ± 0.6[a]	58.0	38.6 ± 2.3	62.2 ± 1.6[a]	61.1
16	36.4 ± 0.6	62.5 ± 0.5[a]	71.7	38.2 ± 1.5	65.7 ± 1.2[a]	72.0

ACC, acetyl-coenzyme A carboxylase. The activities of ACC and FAS were measured in the cytosol of mouse hepatocytes at the times indicated. The values are expressed as nmol NADH (ACC) or NADPH (FAS) oxidized$\cdot min^{-1} \cdot$mg $protein^{-1}$ and represent means ± SEM (n = 4).

[a] $P < 0.05$, difference from the control group.

assay of CIC activity. This experimental system offers several advantages with respect to the assay carried out in intact mitochondria. The advantages include more defined internal and external substrate concentrations, the absence of possible protein effectors, and a well-characterized lipid composition of the liposomal membranes. On the contrary, the lipid composition of mitochondria is more variable because it is influenced by the fatty acid composition of the diet. To overcome all of the problems present in the intact organelles and possibly capable of interfering with the CIC assay, we reconstituted the partially purified CIC into liposomes. To this end, we followed a well-established procedure that has been shown to result in the effective recovery of carrier proteins from mitochondria (41). As shown in Fig. 2B, the reconstituted CIC activity of CLA-fed mice was significantly higher than that of control animals. Interestingly, in both experimental systems (i.e., intact mitochondria and proteoliposomes), we found an almost identical increase in CIC activity consequent to CLA feeding.

The time dependence of the CLA effect on CIC activity is reported in **Table 2**. The transport activity of this mitochondrial carrier increased linearly with the duration of CLA feeding both in intact mitochondria and in the proteoliposomal system, apparently not reaching a maximum even after 16 weeks of treatment.

TABLE 4. Total CPT activity

Week	CPT Activity		
	Control	CLA	Percentage Increase
		$nmol \cdot min^{-1} \cdot mg\ protein^{-1}$	
0	5.8 ± 0.2	—	—
2	5.1 ± 0.2	6.6 ± 0.1[a]	29.4
4	5.5 ± 0.2	7.3 ± 0.2[a]	32.7
6	5.3 ± 0.3	7.9 ± 0.3[a]	49.1
8	4.8 ± 0.1	9.2 ± 0.3[a]	91.7
12	4.9 ± 0.1	6.5 ± 0.5[a]	32.6
16	4.9 ± 0.3	6.6 ± 0.2[a]	34.7

CPT, carnitine palmitoyltransferase. Total CPT activity was measured in freshly isolated mouse liver mitochondria at the times indicated. The values are expressed as nmol 5,5′-dithio-bis(2-nitrobenzoic acid) reduced$\cdot min^{-1} \cdot$mg $protein^{-1}$. Data are means ± SEM (n = 4).

[a] $P < 0.05$, difference from the control group.

To search for possible changes in the kinetic parameters of the CIC, we measured the rate of citrate-citrate exchange in proteoliposomes at different external citrate concentrations. As shown in **Fig. 3**, V_{max} was strongly increased in mice fed with 1% CLA for 16 weeks (208 nmol$\cdot min^{-1} \cdot$mg $protein^{-1}$) with respect to control animals (130 nmol$\cdot min^{-1} \cdot$mg $protein^{-1}$). An increase of 1.6-fold in V_{max} values was reproducibly found in treated animals. On the contrary, as shown in Fig. 3, the K_m values were unchanged in CLA-fed mice with respect to control animals. This finding indicates that the affinity of the CIC for its substrate is the same in both kinds of animals, independent of the dietary treatment.

We conclude that CLA is able to greatly increase the transport activity of the CIC both in intact liver mitochondria and in the reconstituted system. No change in the affinity of the carrier protein for its substrate was detected. The increase in carrier activity was linearly dependent on the duration of CLA administration to mice.

Effect of the CLA-enriched diet on CIC mRNA and on protein levels in mouse hepatocytes

To investigate whether the CLA-enriched diet was able to increase the level of CIC in mouse mitochondria, we carried out Northern and Western blot experiments (**Fig. 4**). Figure 4A shows an increase of 1.3-fold in CIC transcript after 16 weeks of CLA administration to mice. Such increases in CIC mRNA appeared not dependent on the time of CLA feeding, being already evident at shorter times of treatment (data not shown). In the Western blot experiments, we found a significant increase in the level of the carrier protein in the mitochondrial membranes of CLA-fed mice (1.7-fold increase after 16 weeks) (Fig. 4B). On the contrary, the level of porin, a protein of the outer mitochondrial membrane, was unchanged in both types of mitochondria (Fig. 4B). Interestingly, as shown in Fig. 4C, the levels of the CIC protein in the mitochondrial membranes increased linearly with the duration of CLA administration to mice.

We conclude that CLA administration to mice stimulates the synthesis and the membrane insertion of CIC in liver mitochondria. The consequent increase in citrate efflux from mitochondria supplies the cytosol with the carbon source for hepatic lipogenesis.

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Hepatic de novo fatty acid synthesis

The activities of ACC and FAS, the enzymes involved in the de novo fatty acid synthesis, were measured in the cytosol of mouse hepatocytes. **Table 3** shows that both enzymatic activities increased in CLA-fed animals with respect to control mice. It is interesting that the extent of the increase was not only almost identical for both enzymes but was also very similar to that reported previously for the CIC. Furthermore, also in this case, the stimulating effect of CLA on hepatic lipogenesis was linearly dependent on the duration of treatment.

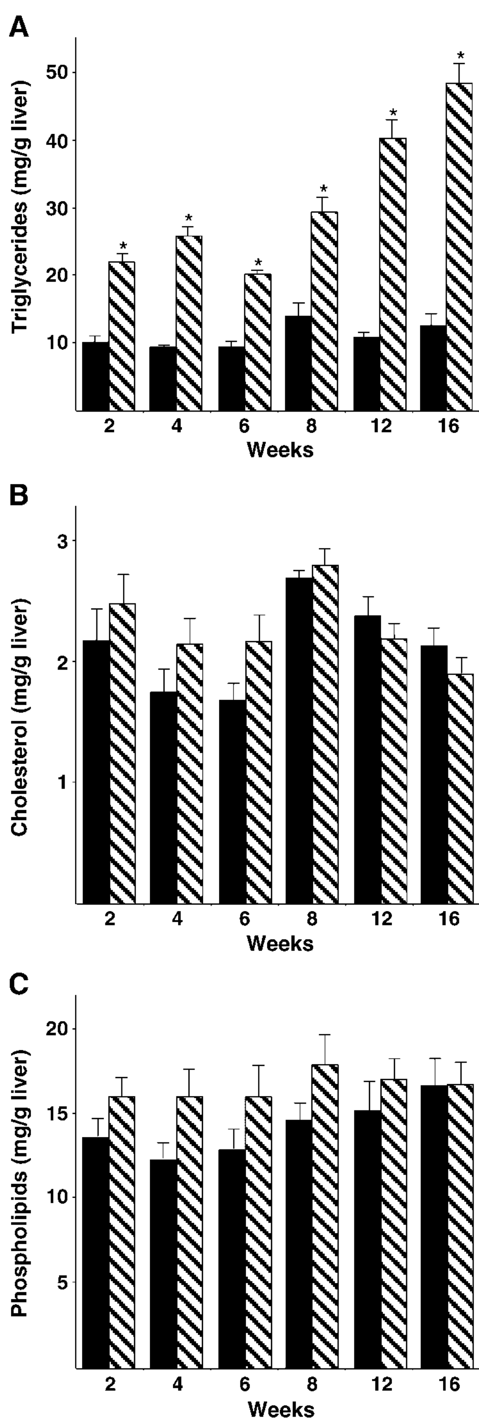

Fig. 5. Lipid analysis of livers from control and CLA-fed mice. The levels of liver triglycerides (A), cholesterol (B), and phospholipids (C) were determined at the times indicated. Triglycerides, total cholesterol, and phospholipids were measured using commercial kits on the lipid fraction extracted from frozen livers as described in Experimental Procedures. Black bars, control mice; striped bars, CLA-fed mice. Each point represents the mean ± SEM for three liver samples. All of the values were subjected to the *t*-test (* $P < 0.05$).

We conclude that CLA greatly increases de novo fatty acid synthesis in mouse hepatocytes and that there is a strict coordination between the efflux of citrate from the mitochondrial matrix and its use in the liver cytosol for lipogenesis.

Hepatic fatty acid oxidation

In liver, both the synthesis and oxidation of fatty acids occur. Therefore, we investigated the effect of CLA on the activity of CPT, which is the rate-limiting step for hepatic fatty acid oxidation. **Table 4** shows that total CPT activity was increased significantly in liver mitochondria of CLA-fed mice. Interestingly, we found a progressive increase in CPT activity over time until the 8th week of treatment, followed by a drastic reduction in the extent of enzyme activation over a longer period (12–16 weeks).

Liver lipid composition in CLA-treated animals

The data reported in Fig. 1B show a significant increase in the liver weight of CLA-fed mice. Therefore, we investigated the liver lipid content of these animals to detect a possible increase in specific lipid classes. To this end, we determined the levels of triglycerides, total cholesterol, and phospholipids. **Figure 5A** shows that the CLA-enriched diet strongly increased the level of triglycerides in mouse liver and that this effect was already evident after 2 weeks of CLA treatment. In fact, at this time, the triglyceride content of liver was more than doubled compared with that of control mice. Furthermore, it is interesting that the level of triglycerides in CLA-fed mice remained approximately twice that found in control animals until the 8th week of treatment. In the 12th and 16th weeks, the level of triglycerides in treated animals became four times those found in control animals. Despite the dramatic effect on the level of triglycerides (Fig. 5A), no significant effect of CLA on the level of total cholesterol (Fig. 5B) and phospholipids (Fig. 5C) was found in the hepatocytes of treated mice at any time during the treatment.

Therefore, we conclude that CLA administration to mice induces a massive accumulation of triglycerides in liver.

DISCUSSION

It is well known that CLA is able to reduce body fat in several animal species (5). The mouse, one of the most CLA-responsive species, shows a massive lipodystrophy associated with significant liver enlargement. This latter event can depend on several factors, such as the inhibition of lipid uptake by adipose tissue (6) leading to the hepatic accumulation of fat of dietary origin, the altered secretion of liver lipoprotein (49), or reduced hepatic fatty acid

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oxidation (50). In addition, as suggested by other authors (18, 19), de novo fatty acid synthesis may represent a further factor responsible for hepatic steatosis. Therefore, this study was designed to investigate a possible effect of CLA on the CIC, which plays a key role in hepatic lipogenesis for several reasons: *i*) citrate is formed in the mitochondrial matrix mainly from dietary carbohydrates and is exported by the CIC to the cytosol, where citrate generates acetyl-CoA, the substrate for ACC, the first enzymatic step of de novo fatty acid synthesis (**Fig. 6**); *ii*) citrate exported to the cytosol is not only the substrate for lipogenesis but is also the positive allosteric modulator of ACC (Fig. 6); and *iii*) the activity of the CIC is modulated by dietary and hormonal factors.

The results obtained indicate that the CIC represents one of the major targets of CLA in liver. The overexpression of this transport protein, induced by CLA feeding, and the consequent increase in citrate efflux from mitochondria supply the cytosol with the carbon units, in the form of acetyl-CoA, necessary for hepatic lipogenesis. The activity of the CIC increased progressively over time, almost doubling after 16 weeks of CLA feeding. A progressive increase was also found in the activities of the cytosolic lipogenic enzymes. Interestingly, a strong correlation in the extent of the increase in CIC activity and in that of the lipogenic enzymes was clearly evident during the course of the study. Because of the pivotal role played by the CIC in de novo fatty acid synthesis, it is tempting to speculate that a specific suppression of this carrier protein should lead to a concomitant reduction in hepatic lipogenesis.

It is clear on the basis of this and previous studies (38–40) that CIC activity is significantly influenced by dietary fatty acids, being strongly reduced in animals fed a diet supplemented with linoleic or linolenic acid. This is the first time that some polyunsaturated fatty acids, such as CLA, have been able to strongly increase CIC transport activity. Why is this? Several studies have demonstrated that dietary CLA is able to incorporate into phospholipids (12, 13), thus raising the possibility of a modification in the lipid microenvironment of the membrane-embedded CIC and, consequently, in its transport activity. On the basis of our results, obtained both in mitochondria and in the proteoliposomal system, we can exclude this possibility. On the other hand, we also found increases in both the immunoreactive protein and the relative mRNA in CLA-treated animals. This suggests a specific effect of CLA on the synthesis of CIC. Moreover, it is worth mentioning that the extent of the increase in CIC transport activity (and in the protein immunodetected in the mitochondrial membranes) was higher than that expected on the basis of the mRNA levels. CLA, therefore, besides increasing the transcription of the CIC gene, is probably able to influence mRNA stability and/or translation efficiency.

In this study, the increase in the level of hepatic triglycerides was already evident and statistically significant in mice fed CLA for 2–4 weeks (Fig. 5A), whereas, at the same time, the increases in CIC and in the lipogenic enzyme activities were very low (Tables 2, 3). During the same period, a consistent increase in CPT activity, indicating stimulated hepatic fatty acid oxidation, was observed (Table 4). This phenomenon has already been reported in other studies (18, 50), even if the actual fatty acid oxidation in liver may be less evident than that measured in vitro because of the inhibition of CPT by malonyl-CoA, an intermediate of hepatic lipogenesis. Furthermore, in previous investigations (49), hepatic lipoprotein secretion was

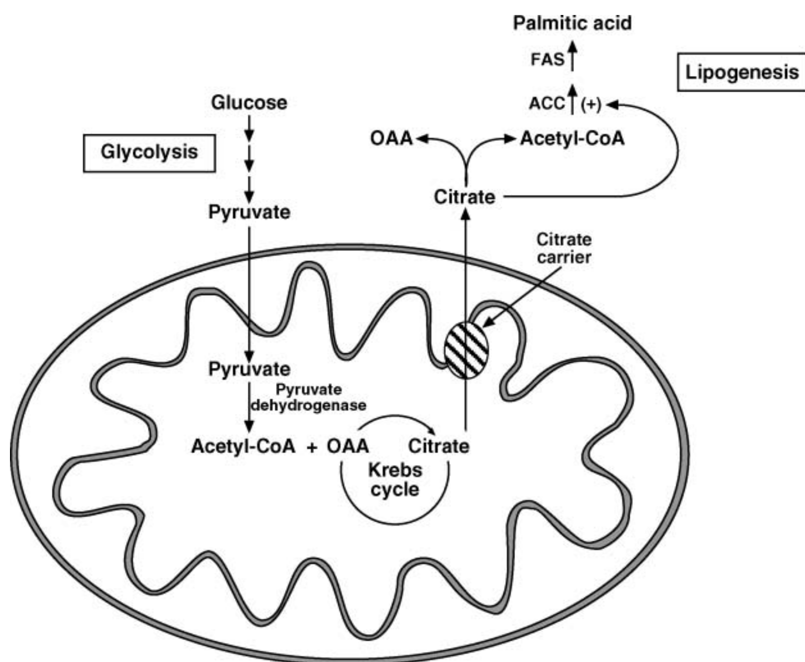

Fig. 6. CIC and cytosolic hepatic lipogenesis. Schematic model depicting the key role of the CIC in hepatic lipogenesis. OAA, oxaloacetate.

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not reduced in CLA-treated mice, which, concomitantly, showed decreases in the plasma levels of triglycerides and FFAs similar to those found in this study (Fig. 1D). In addition, CLA feeding induces specific genes in liver that are implicated in cellular fatty acid uptake and trafficking (11, 19). Therefore, the general picture concerning the metabolic changes occurring in the liver of CLA-fed mice, and possibly responsible for the hepatic accumulation of triglycerides, is quite complex. In this study, we demonstrated that hepatic lipogenesis, capable of converting dietary carbohydrates into fat, increases progressively with the duration of CLA feeding, thus representing one of the factors leading to hepatic steatosis. This is the first time that such a clear time dependence of the hepatic lipogenic program of mice under CLA treatment has been reported. However, the reason for this phenomenon is currently unknown. The monitoring, over time, of further parameters is certainly necessary to obtain more insights into the metabolic changes, and their interconnection, occurring in CLA-fed mice.

The comprehension of the biochemical mechanisms of CLA action helps in the correct formulation of dietary treatments and in the appropriate administration of food supplements, which are all directed to a safe reduction of adiposity and body weight.

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