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Dietary Combination of Conjugated Linoleic Acid (CLA) and Pine Nut Oil Prevents CLA-Induced Fatty Liver in Mice

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Conjugated linoleic acid (CLA) strongly prevents fat accumulation in adipose tissue of mice, even if hepatic fat deposition and insulin resistance are concomitantly observed. This study investigated the possibility of maintaining the antiadiposity properties of CLA while preventing adverse effects such as liver steatosis and hyperinsulinemia. To this end, mice were divided into three groups and fed a standard diet (control) or a diet supplemented with 1% CLA (CLA) or a mixture of 1% CLA plus 7.5% pine nut oil (CLA + P). The combination of CLA + P preserved the CLA-mediated antiadiposity properties (70% fat reduction), preventing hepatic steatosis and a sharp increase in plasmatic insulin starting from the eighth week of CLA treatment. The assay of both fatty acid synthesis and oxidation in the CLA + P mice revealed a time-dependent biphasic behavior of the corresponding enzymatic activities. A sudden change in these metabolic events was indeed found at the eighth week. A strong correlation between the changes in key enzymes of lipid metabolism and in insulin levels apparently exists in CLA-fed mice. Furthermore, lower levels of lipids, in comparison to values found in CLA-fed mice, were observed in the liver and plasma of CLA + P-fed animals.

KEYWORDS: Conjugated linoleic acid; tricarboxylate carrier; citrate carrier; mitochondria; hepatic lipogenesis; lipogenic enzymes

INTRODUCTION

The acronym CLA (conjugated linoleic acid) indicates a group of positional and geometric isomers of linoleic acid (*1, 2*). These isomers are natural compounds present in low concentrations in the meat and milk of ruminant animals. The main isomer found in dairy products is *cis*-9,*trans*-11-octadecadienoic acid, but the commercially available CLA, currently used as a food supplement, contains a 1:1 mixture of this isomer and the *trans*-10,*cis*-12 isomer. Several studies have indicated that CLA has beneficial effects in the case of cancer (*3*), cardiovascular diseases (*4*), obesity, and diabetes (*5*). These beneficial effects, however, have been observed in the various species tested to different degrees (*5*) and, in some instances, they were associated with adverse effects. Mice, one of the most investigated animal species, showed a significant decrease in adipose tissue following CLA administration, but this effect was accompanied by liver steatosis and insulin resistance (*6*−*10*). Besides its capability in modulating adiposity (*11*), CLA is also able to influence the endocrine functions of the adipose tissue (*12*−*14*). It has also been demonstrated that the antiobesity effects of CLA are due to the *trans*-10,*cis*-12 isomer (*9, 15*). Despite all of these studies, a comprehensive and definitive picture illustrating the molecular mechanisms responsible for these effects is still missing.

The oil from the seeds of *Pinus koraiensis* (hereafter referred to as pine nut oil) is another dietary fat that contains, besides other fatty acids, pinolenic acid or all-*cis*-5,9,12-octadecatrienoic acid. The latter is a quite unusual polyunsaturated fatty acid (PUFA) characterized by polymethylene-interrupted double bonds (*16*). Preliminary studies carried out in model animals such as rats and mice indicated that this fat exerts some beneficial effects on lipid metabolism (*17, 18*).

In this study, we have investigated the effects of a dietary combination of CLA and pine nut oil on lipid metabolism in mice. The rationale of this study was to check whether the association between these two dietary fats would be able to maintain the beneficial effects of CLA, such as body fat reduction, while avoiding adverse effects such as liver steatosis and hyperinsulinemia. To our surprise, the association of CLA and pine nut oil not only allowed us to succeed in our purpose but also positively affected the liver and plasma lipid contents in comparison to both control and CLA-fed mice. Furthermore, the time-dependent investigation of several changes in metabolites and in enzymatic activities involved in both fatty acid synthesis and oxidation occurring in mice under this dietary

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Table 1. Composition of Diets (Percent)[a]

	control	CLA	CLA + P
proteins	17.20	17.20	17.20
lipids	13.72	13.72	13.72
fatty acids			
16:0	1.72	1.65	1.06
18:0	0.40	0.38	0.29
9−18:1	7.78	7.11	3.07
9,12−18:2	3.25	3.22	6.51
9,12,15−18:3	0.32	0.31	0.27
5,9,12−18:3			1.12
other fatty acids	0.24	0.25	0.60
CLA c9, t11		0.37	0.37
CLA t10, c12		0.38	0.38
other CLA isomers		0.04	0.04
carbohydrates	52.39	52.39	52.39
sugars	4.49	4.49	4.49
mineral + vitamin mix	3.20	3.20	3.20
crude fiber	3.48	3.48	3.48
ash	5.42	5.42	5.42
kcal/100 g	420	420	420

[a] Standard diet (Global Diet 2018S from Harlan Teklad) was supplemented with 8.5% olive oil (control), 7.5% olive oil, and 1% Clarinol G-80 (CLA) or 1% Clarinol G-80 and 7.5% PinnoThin (CLA + P). Energy content was calculated using 4 kcal/g for proteins and carbohydrates and 9 kcal/g for lipids.

treatment revealed new details of the molecular mechanisms underlying CLA action.

MATERIALS AND METHODS

Materials. Bio-Rad protein assay kit and hydroxyapatite (Bio-Gel HTP) 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, DTNB, 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 *trans*-10,*cis*-12 isomers of CLA, and PinnoThin were generous gifts of Lipid Nutrition B.V. [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 free fatty acids (FFA) was purchased from Roche. Plasma insulin concentration was analyzed with a Mercodia Ultrasensitive Mouse Insulin kit. All other reagents were of analytical grade.

Animals. Male ICR mice (210 animals in total) were obtained from Harlan at 5 weeks of age and housed individually at a temperature of 22 ± 1 °C. After 7 days of acclimatization, animals were randomly divided into three groups of 70 animals each.

The first group received a diet (Global Diet 2018S from Harlan Teklad), referred to as standard diet, supplemented with 8.5% olive oil (control group). The second group of animals were fed with the standard diet, reported above, supplemented with 7.5% olive oil and 1% CLA, mainly composed of a 1:1 mixture of *cis*-9,*trans*-11- and *trans*-10,*cis*-12-octadecadienoic acids (CLA group). The third group of mice received the standard diet, reported above, supplemented with 1% CLA and 7.5% pine oil from the seeds of *P. koraiensis* (CLA + pine nut oil group). **Table 1** reports the composition of experimental diets, including the fatty acid concentration of the lipid fraction. These diets were prepared each week and stored frozen until use. Food intake and body, liver, and adipose tissue weights were recorded throughout the study, ranging from 2 to 16 weeks of dietary treatment. At each time period 10 mice were sacrificed by decapitation. Three to five animals were used for each of the tests. Experiments were carried out in accordance with local and national guidelines regarding animal experiments.

Citrate Transport in Mice Liver Mitochondria. Mice liver mitochondria were prepared according to standard procedures. Briefly, the livers were excised rapidly and placed in a medium containing 250 mM sucrose, 10 mM Tris, and 1 mM EDTA (STE), pH 7.2, at 4 °C. Tissue samples were homogenized on ice using a Teflon Potter homogenizer. The homogenates were centrifuged at 1000*g* for 8 min, and the supernatants were centrifuged for 10 min at 8000*g* to obtain mitochondrial pellets. The resulting pellets were washed twice with STE medium and centrifuged for 10 min at 12000*g*. These freshly isolated 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 about 5 mg of protein/mL and loaded with L-malate as previously described (*19*). 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. Mitochondria were then reisolated by centrifugation at 18000*g* for 10 min at 2 °C, washed once, and extracted with 20% $HClO_4$. The mixture was centrifuged, and the radioactivity in the supernatant was counted.

Reconstitution of the Citrate Transport into Liposomes. Mice liver mitochondria were 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 about 10 mg of protein/mL. After incubation for 10 min at 2 °C, the mixture was centrifuged at 25000*g* for 20 min at 2 °C, thereby obtaining a supernatant, referred to as mitochondrial extract. Six hundred microliters of this extract (6−7 mg of protein) was supplemented with 2 mg/mL cardiolipin and chromatographed onto a cold hydroxyapatite column, essentially as described (*20*). The 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, to obtain the proteoliposomes. The external citrate was removed by gel filtration on a Sephadex G-75 column, and the proteoliposomes were then used for the transport studies. Transport was initiated by the addition of 0.5 mM [^{14}C]citrate to reconstituted proteoliposomes incubated at 25 °C and stopped after the indicated time 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, and the internal radioactivity was measured by scintillation counting.

Assay of Enzymes Involved in Fatty Acid Synthesis and Oxidation. Mice liver cytosol was obtained by centrifuging the postmitochondrial supernatant at 20000*g* for 20 min at 2 °C. The pellet was discarded, and the supernatant was then centrifuged at 105000*g* for 1 h. On the resulting cytosol the activities of the lipogenic enzymes acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS) were measured as described in ref *10*. Total carnitine palmitoyl-CoA transferase (CPT) activity was determined spectrophotometrically at 412 nm in freshly isolated mice liver and muscle mitochondria, essentially as described (*21*). CPT I activity was calculated by subtracting the CPT activity that was insensitive to 100 μM malonyl-CoA from the total CPT activity experimentally determined.

Northern Blot Analysis. Total RNA from mouse liver was extracted using TRIzol reagent essentially as described in ref *22*. The extracted RNA was separated onto 1% formaldehyde−agarose gel under denaturing conditions and transferred to Hybond nylon membrane. The RNA blots were hybridized with a probe corresponding to the full-length mice 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.

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 previously described (*23*). The extracts were dried and weighed. For the determination of liver and muscle lipids, total lipids were extracted from these tissues using chloroform and methanol (1:1). The extracts were dried under nitrogen flow and resuspended in 0.1% Triton X-100 before the lipid assay. For the determination of plasma lipids mice were starved overnight before sacrifice. The individual assays of triglycerides, cholesterol, and phospholipids were carried out using commercial kits.

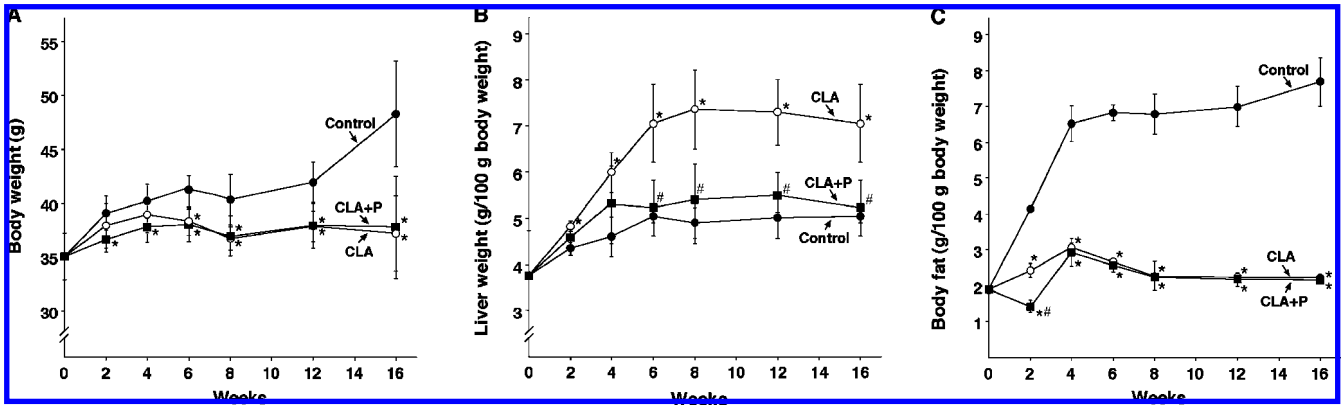

Figure 1. Effect of CLA and CLA + pine nut oil on body weight (**A**), liver weight (**B**), and body fat (**C**) of mice fed control (●), CLA (○), and CLA + pine nut oil (■, CLA + P) diets for the times indicated. Each point represents the mean ± SE for 10 animals (body weight and liver weight) or 5 animals (body fat). ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet.

Assay of Glucose and Insulin. For the determination of blood glucose and plasma insulin, mice were starved overnight before sacrifice. Blood glucose concentration was determined using reactive strips and a commercial glucometer (One Touch Basic Plus, LIFESCAN, Johnson & Johnson). Plasma insulin concentration was analyzed with commercial enzyme-linked immunosorbent assay kits.

Other Methods. Protein was determined as described (*24*) or according to the Lowry method modified for the presence of Triton (*25*). Polyacrylamide gel electrophoresis was performed in the presence of 0.1% SDS (SDS-PAGE) according to standard procedures. The mitochondrial proteins, separated by SDS-PAGE, were then transferred to nitrocellulose membrane. For the immunodecoration, antisera directed against the C terminus of the rat liver citrate carrier and against the mammalian porin were used.

Statistical Analysis. Experimental data represent the means ± SE. The data were analyzed by one-way ANOVA, and a Tukey−Kramer post hoc analysis was used to detect significant differences between the means at a level of $P < 0.05$.

RESULTS

Effect of CLA and Pine Nut Oil on Body, Liver, and Adipose Tissue Weights. Three sets of mice were fed for various periods of time, as reported under Materials and Methods, with three different diets, the compositions of which are indicated in **Table 1**. The three diets were isocaloric and differed only in the fatty acid composition of the lipid fraction. As shown in **Table 1**, the CLA diet differed from the control one essentially for the presence of 1% CLA (thus supplying to the animals 0.37% of the c9,t11 and 0.38% of the t10,c12 CLA isomers), whereas the CLA + pine nut oil diet, containing 7.5% pine nut oil in addition to 1% CLA, differed more in the fatty acid composition in comparison to the other two diets, especially for the presence of 1.12% pinolenic acid.

Food intakes were practically identical in the three groups of animals (data not shown). **Figure 1A** shows that the administration of CLA to mice induced a body weight reduction, significant from the sixth week onward, in comparison to control animals, which were fed an isocaloric diet. Interestingly, also the coadministration of CLA and pine nut oil (CLA + P) reduced the body weight of treated mice with respect to control animals, but this effect was statistically significant at all of the times tested. At the 16th week of both the CLA and the CLA + pine nut oil treatments a decrease of about 23% in body weight was found (**Figure 1A**). On the contrary, a clear increase in the liver weight was found in the CLA-treated mice in comparison to control animals (**Figure 1B**). Very interestingly, the combination of CLA and pine nut oil in the diet almost completely prevented the increase in liver weight. At longer times of CLA administration (12−16 weeks), an increase of about 1.45 times in liver weight was found in treated animals, whereas in the CLA + pine nut oil-fed mice the resulting liver weight was similar to that of control animals (**Figure 1B**). Both the CLA and CLA + pine nut oil diets strongly decreased body fat accumulation over time (**Figure 1C**). Such a decrease was impressive, the body fat representing <30% of that found in control animals after 16 weeks of CLA and CLA + pine nut oil dietary treatment. These results were further confirmed when various adipose tissues were individually analyzed (**Figure 2**). At the 16th week of the two dietary treatments (CLA and CLA + pine nut oil) the strongest decreases in comparison to control mice were found in the retroperitoneal (**Figure 2C**, 85% decrease) and in the mesenteric adipose tissue (**Figure 2D**, 77% decrease).

Altogether, these results indicate that the CLA + pine nut oil dietary mixture significantly decreases the body weight and fat accumulation in mice while efficiently preventing the liver enlargement observed with the sole administration of CLA.

Hepatic de Novo Fatty Acid Synthesis. We then investigated the role played by the de novo fatty acid synthesis in the onset of hepatic steatosis observed in CLA-fed mice. The synthesis of fatty acids occurring in liver is a complicated process that requires cooperation between mitochondrial and cytosolic enzymes. A central role in this anabolic pathway is played by the mitochondrial tricarboxylate or citrate carrier (CIC) (*26*), which catalyzes the efflux of the molecule of citrate, synthesized in the mitochondrial matrix, toward the cytosol. The transport activity of this carrier protein was therefore investigated in intact mitochondria isolated from control and treated animals. **Figure 3A** shows that in the control animals the transport activity of the mitochondrial CIC remained almost unaffected over time. On the contrary, in CLA-fed mice a time-dependent increase in the CIC activity was clearly found (**Figure 3A**). At the 16th week of CLA feeding an increase of 1.72 times in the citrate transport was reproducibly detected in comparison to control mice. In the animals fed a diet containing CLA and pine nut oil a peculiar biphasic behavior of the CIC activity was found. In fact, in these animals there was an increase in the CIC activity, which was identical to that seen in the CLA-fed animals until the sixth week of dietary treatment (**Figure 3A**). At the eighth week, however, the trend in the CIC activity started to change, thus leading, at the 12th week, to a carrier activity identical to that registered in control mice and, at the 16th week, to values even lower than the control ones (25% decrease).

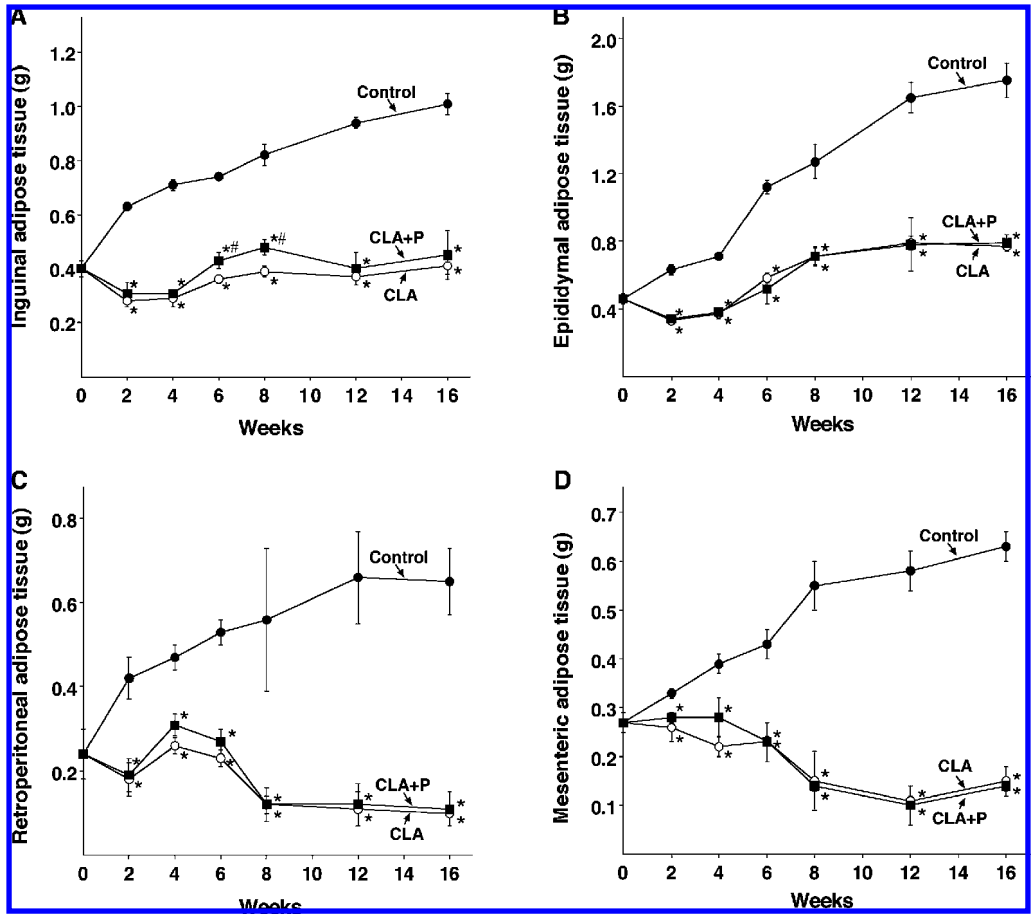

Figure 2. Effect of dietary treatments on the weight of the adipose tissue. Inguinal (**A**), epididymal (**B**), retroperitoneal (**C**), and mesenteric (**D**) adipose tissues were recorded throughout the study. Each point represents the mean ± SE for five animals. ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet.

The fatty acid composition of the diet is one of the factors capable of influencing the lipid composition of biological membranes and consequently their fluidity. Therefore, the assay of the CIC transport activity in intact mitochondria could be influenced, at least in principle, by the fluidity of the inner membrane in which this carrier protein is functionally inserted. To exclude this possibility, the CIC protein was extracted from mitochondria in an active form by using the detergent Triton X-100, then partially purified by hydroxyapatite chromatography, and eventually reconstituted into liposomes. Notably, the results obtained in the reconstituted system were similar to those previously found in intact mitochondria (**Figure 3B**). In fact, whereas in the control animals the CIC activity remained unaffected over time, a strong time-dependent increase was instead detected in CLA-fed mice. Very interestingly, the same biphasic behavior in the CIC activity, already seen in intact mitochondria, was also registered in the reconstituted system for the animals fed the CLA + pine nut oil mixture (**Figure 3B**). To get more insights into the mechanisms responsible for the changes in the CIC activity, we measured the K_m and V_{max} of citrate transport in the reconstituted system. Whereas the K_m was not affected by any type of diet at any time of treatment, the V_{max} significantly increased over time in the CLA-fed animals in comparison to the control group (**Table 2**; at the 16th week of CLA treatment a V_{max} increase of 1.73 was found). In the CLA + pine nut oil-treated mice the V_{max} increased over time, reaching values about 1.3 times higher than those found in control animals at the sixth and eighth weeks of treatment. As shown in **Table 2**, a decrease in the V_{max} of citrate transport was detected at longer times, with values lower than those of the control group at the 16th week of dietary treatment (22% decrease).

These results prompted us to investigate the amount of CIC protein in the mitochondrial membranes. **Figure 4A** shows a Western blot analysis of the mitochondrial membranes purified from the three groups of animals and immunodecorated with an antiserum against the rat liver CIC. The expression of the mitochondrial CIC in each of the three groups of mice was extremely consistent with the transport activity results previously shown (**Figure 3**; **Table 2**). The amount of porin, an outer membrane protein tested as a control, did not change at all in any group of animals or at any time of treatment. The Northern blot analysis, carried out in parallel on the same samples (**Figure 4B**), revealed an increase in the CIC mRNA starting from the fourth week of CLA treatment. This increase of about 1.3 times in comparison to the control values remained approximately constant over time. On the contrary, as shown in **Figure 4B**, the CIC mRNA of the CLA + pine nut oil-treated animals after a transient increase clearly visible at the fourth and sixth weeks of treatment suddenly decreased after longer times, reaching values comparable to those found in the control group. It must be noted, however, that the changes in the CIC mRNA (**Figure 4B**) were less evident than those found in the activity (**Figure 3**; **Table 2**) and in the amount (**Figure 4A**) of this carrier protein. These findings would suggest that the dietary treatments not only increase the transcription of the CIC gene but are probably able to influence the mRNA stability and/or translation efficiency.

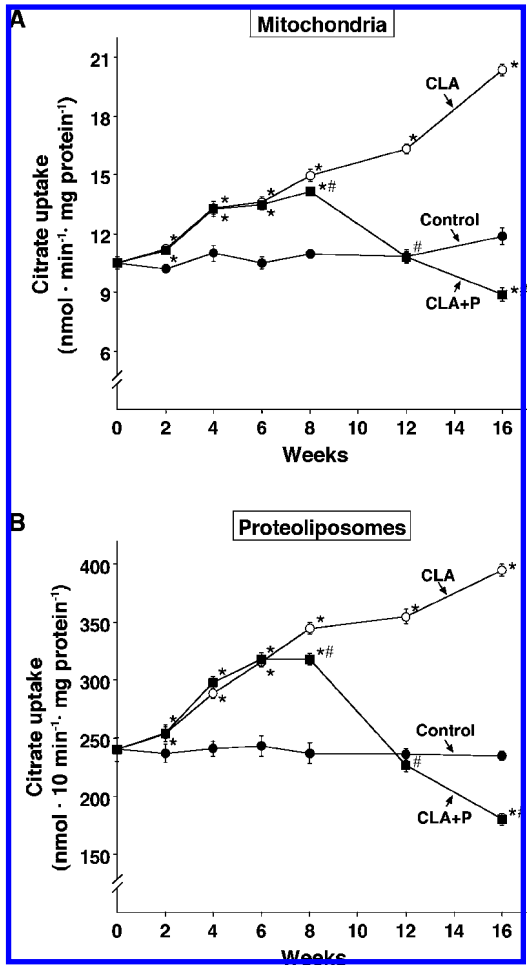

Figure 3. Transport activity of the CIC in mice fed control, CLA, and CLA + pine nut oil diets. Transport of citrate in mice liver mitochondria freshly isolated (**A**) and in the reconstituted system (proteoliposomes) (**B**) was measured at the times indicated. The values reported in the figure represent the means ± SE ($n = 5$). ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet.

Table 2. K_m and V_{max} of Citrate Transport in the Reconstituted System[a]

	K_m (mM)			V_{max} (nmol · min^{-1} · mg of protein^{-1})		
weeks	control	CLA	CLA + P	control	CLA	CLA + P
0	0.163			141.30		
2	0.164	0.164	0.166	142.54	153.47	152.11
4	0.160	0.160	0.161	142.97	169.51	169.94
6	0.157	0.157	0.163	142.81	188.26	188.25
8	0.156	0.156	0.152	143.22	198.39	190.17
12	0.160	0.160	0.166	145.49	224.56	144.83
16	0.159	0.159	0.162	145.14	250.47	113.47

[a] K_m and V_{max} values were measured in the reconstituted system at the times indicated. Proteoliposomes were reconstituted with the CIC as described under Materials and Methods. [^{14}C]Citrate, 0.04−0.40 mM, was added to proteoliposomes containing 10 mM citrate. The citrate/citrate exchange was stopped 1 min after the addition of the radiolabeled substrate by 20 mM 1,2,3-BTA. K_m and V_{max} values were calculated by linear regression.

The molecule of citrate transported outside mitochondria by the CIC is then cleaved in the cytosol, generating oxaloacetate and acetyl-CoA, the latter representing the fuel for cytosolic lipogenesis. In fact, acetyl-CoA is the substrate of acetyl-CoA carboxylase (ACC) and then of fatty acid synthetase (FAS). We therefore investigated the activity of these enzymes in the liver cytosolic fraction isolated from the three groups of animals. Whereas these activities were approximately constant over time

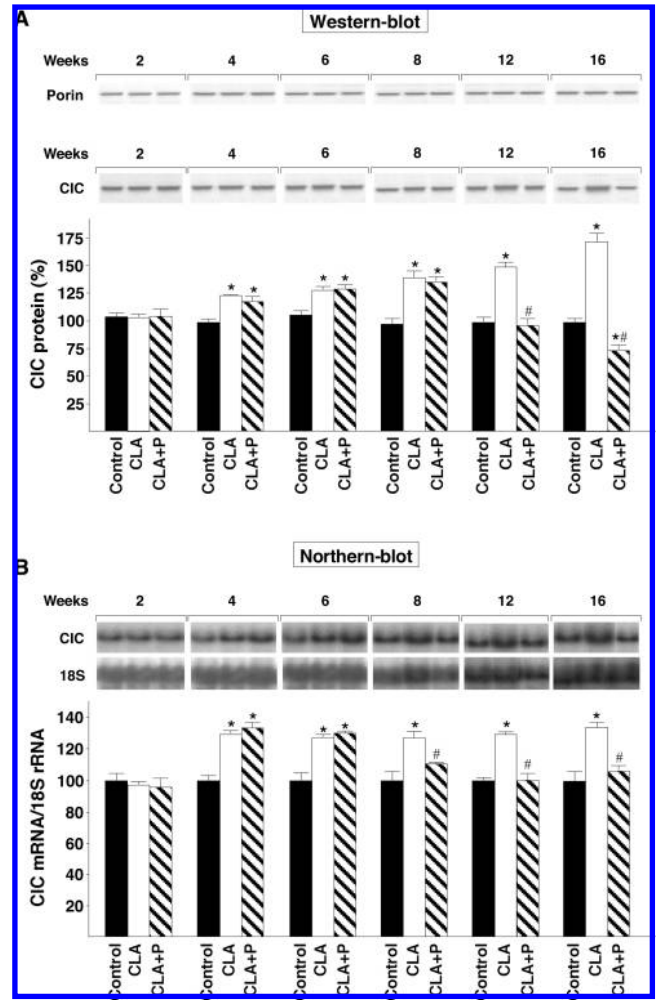

Figure 4. Effect of CLA and CLA + pine nut oil on protein levels and mRNA of the mitochondrial CIC. (**A**) Liver mitochondrial proteins from control, CLA-, or CLA + P-fed mice were separated by SDS-PAGE, transferred to nitrocellulose, and then immunodecorated with antisera against either the mammalian porin or a C-terminal peptide of the rat CIC. The values reported in the graph represent the means ± SE ($n = 3$; ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet). The amount of CIC revealed by immunodecoration at the beginning of dietary treatment was set to 100%. (**B**) Total RNA from the liver of mice fed the experimental diets was hybridized with the mouse CIC cDNA. Variations with respect to the control animals were calculated after correcting for loading differences on the basis of the 18S rRNA levels. The values reported in the graph represent the means ± SE ($n = 3$; ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet).

in the control group, a significant and time-dependent increase was clearly observed in the CLA-fed mice (**Figure 5**). An increase of about 1.75 times was indeed found for both enzyme activities at the 16th week of CLA administration. In the animals fed the CLA + pine nut oil mixture we observed an increase in the activity of both enzymes in the first 6 weeks of this dietary treatment. Such an increase was identical to that found in the CLA-fed animals. Interestingly, the time-dependent increase shown by both enzymatic activities stopped at the eighth week of dietary treatment (**Figure 5**). At the 12th week both ACC and FAS activities in the CLA + pine nut oil-treated animals were similar to those found in control animals, and at the 16th week they became lower than the control (21 and 23% decreases for ACC and FAS activities, respectively). The behavior of the

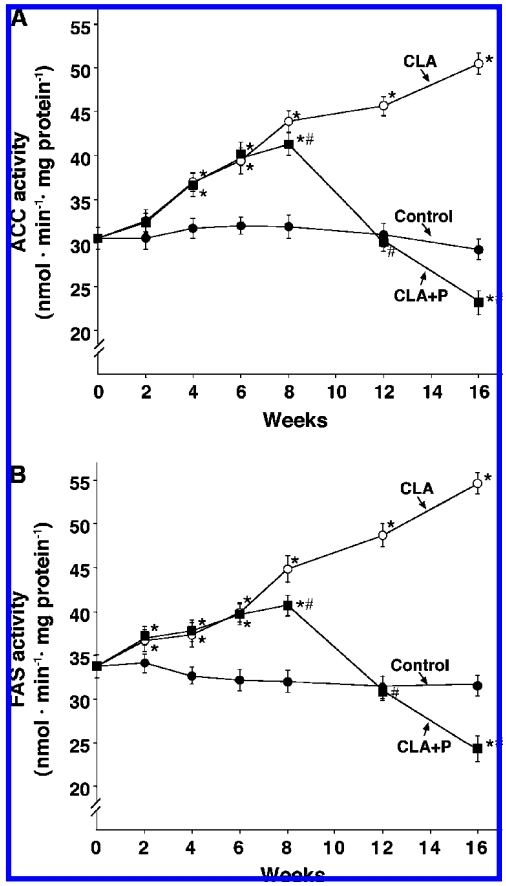

Figure 5. Effect of CLA and CLA + pine nut oil on lipogenic enzyme activities. The activities of ACC (**A**) and FAS (**B**) were measured in the cytosol of mice hepatocytes at the times indicated. The values are expressed as nanomoles of NADH (ACC) or NADPH (FAS) oxidized · min^{-1} · mg of $protein^{-1}$ and represent the means ± SE (n = 3). ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet.

lipogenic enzyme activities over time, therefore, is consistent with that of the mitochondrial CIC.

We conclude that the hepatic lipogenesis in the CLA + pine nut oil-fed animals presents a biphasic trend with a moderate increase within the first 6−8 weeks of dietary treatment, followed by a strong decrease to values lower than those of the control animals at the 16th week of feeding.

Fatty Acid Oxidation in Liver and Muscle. Liver, besides fatty acid synthesis, is also involved in fatty acid oxidation. The limiting step for this catabolic process is represented by the activity of carnitine palmitoyl-CoA transferase I (CPT I), which is responsible for the entry of fatty acids into the mitochondrial matrix, where β-oxidation of fatty acids occurs. As shown in **Figure 6A**, the CPT I activity was only moderately influenced in the control group over time. On the contrary, in the CLA-fed animals after a slight increase (about 1.3 times) in the CPT I activity found at the second and fourth weeks of dietary administration, a peak in the enzymatic activity was clearly found at the sixth week (increase of about 1.9 times in comparison to the control group). The CPT I activity then tended to decrease over longer times, remaining, however, higher in the CLA-fed animals with respect to control mice. At the 16th week of CLA administration, the CPT I activity was 1.55 times higher than that found in control mice. In the CLA + pine nut oil-treated animals the CPT I activity showed the same increase found in the CLA group until the sixth week of dietary treatment. Starting from the eighth week of dietary treatment

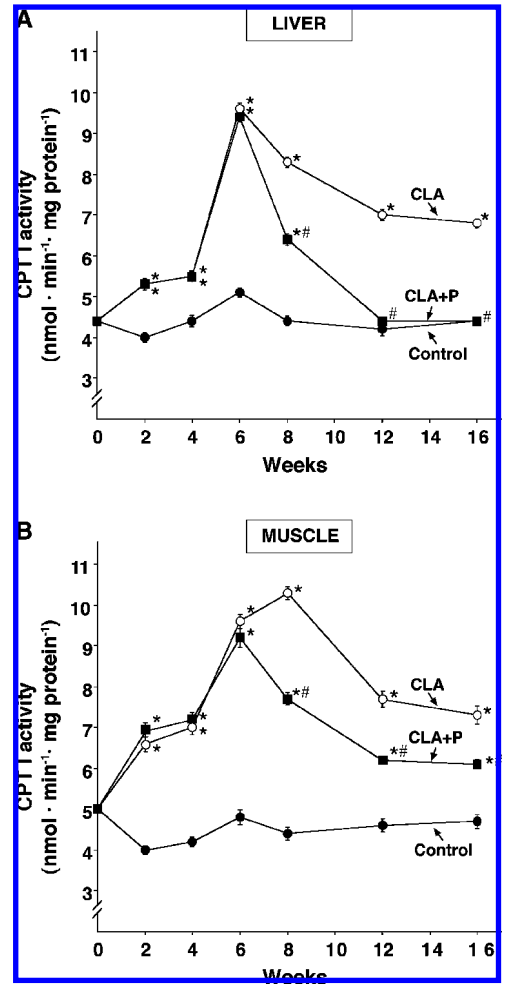

Figure 6. Effect of CLA and CLA + pine nut oil on fatty acid oxidation in liver and muscle. CPT I activity was measured in liver (**A**) and muscle (**B**) mitochondria freshly isolated from mice at the times indicated. The values are expressed as nanomoles of DTNB reduced · min^{-1} · mg of $protein^{-1}$ and were calculated as described under Materials and Methods. Data are means ± SE (n = 3). ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet.

the CPT I activity changed in different ways in the CLA and in the CLA + pine nut oil groups. In fact, the decrease in the CPT I activity was stronger in the CLA + pine nut oil group than in the CLA group, reaching, at the 12th and 16th weeks of treatment, values similar to the control ones (**Figure 6A**).

Fatty acids are also actively oxidized in muscle, and an increase in muscle metabolism has been proposed as one of the factors responsible for the fat decrease in CLA-treated animals. We therefore investigated the CPT I activity also in muscle cells (**Figure 6B**). Unlike the modest changes found in control animals, a time-dependent increase in the CPT I activity was detected in CLA-fed mice until the eighth week of treatment. At this time the increase in the CPT I activity if compared to control animals was 2.34 times, therefore higher than that previously found in liver at the sixth week (1.88 times). A decrease in the muscle CPT I activity then followed over longer times, yet this enzymatic activity remained 1.55 times higher than the control one. In the CLA + pine nut oil-fed mice the CPT I activity paralleled that of the CLA-fed animals until the sixth week of dietary treatment and then decreased as shown in **Figure 6B**. However, such a decrease did not bring the CPT I activity down to the level of the control values as observed in the liver (compare panels **B** and **A** of **Figure 6**).

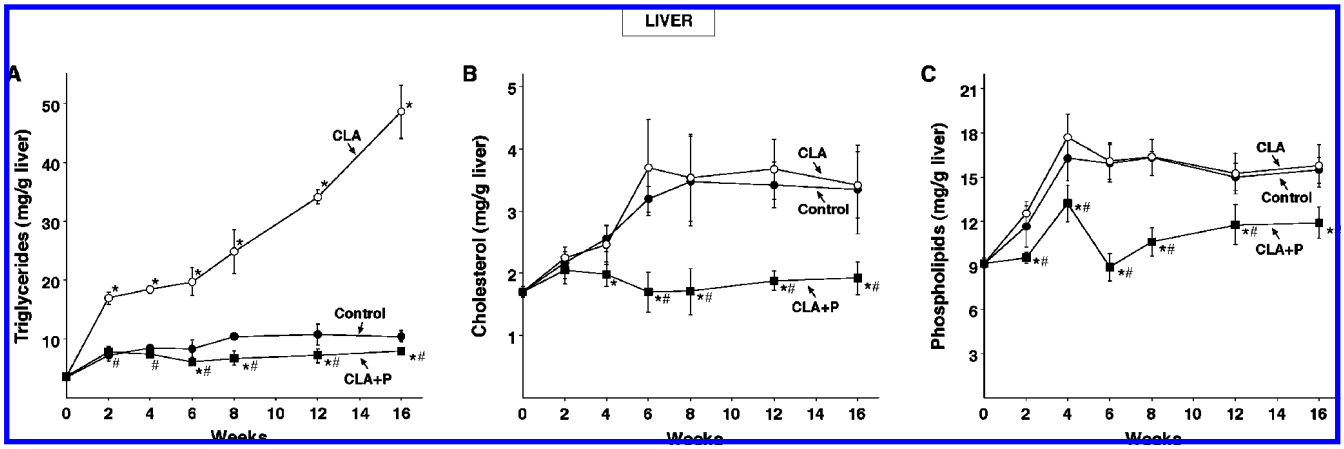

Figure 7. Lipid analysis of livers from control, CLA-, and CLA + pine nut oil-fed mice. The levels of liver triglycerides (**A**), cholesterol (**B**), and phospholipids (**C**) were determined at the times indicated. Triglycerides, total cholesterol, and phospholipids were measured using commercial kits on the lipid fraction extracted from frozen livers as described under Materials and Methods. Each point represents the mean ± SE for three liver samples. ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet.

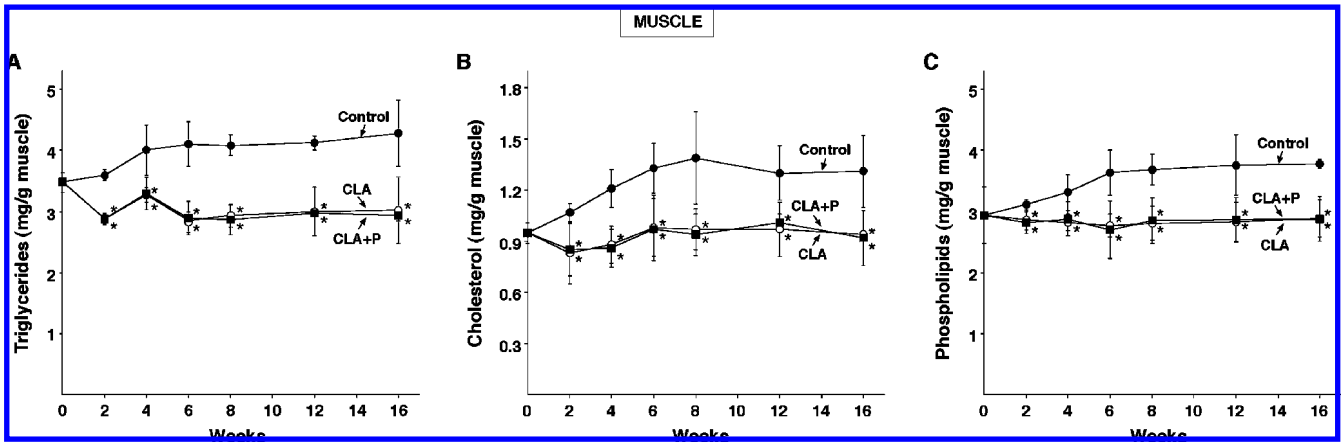

Figure 8. Lipid analysis of muscles from control, CLA-, and CLA + pine nut oil-fed mice. The levels of muscle triglycerides (**A**), cholesterol (**B**), and phospholipids (**C**) were determined at the times indicated. Each point represents the mean ± SE for three muscle samples. ∗, $P < 0.05$ versus mice fed control diet.

In the muscle cells, indeed, the CPT I activity of the CLA + pine nut oil mice was about 1.3 times higher than that of the control animals at the 12th and 16th weeks of dietary administration (**Figure 6B**).

Lipid Analysis in Liver, Muscle, and Plasma. We then analyzed the liver lipid composition in the three groups of mice (**Figure 7**). According to previous studies (*7*−*10*), the liver triglycerides sharply increased in the CLA-fed mice over time, reaching levels 4.7 times higher at the 16th week in comparison to control animals (**Figure 7A**). On the contrary, in the CLA + pine nut oil-fed mice the level of liver triglycerides was lower than the control value at any time of dietary treatment. A decrease of about 30% in the liver triglycerides was found at 8−16 weeks of this dietary treatment in comparison to control mice (**Figure 7A**). **Figure 7B** shows that in both control and CLA-fed mice there was a time-dependent increase in the level of hepatic cholesterol. On the contrary, the animals fed the CLA + pine nut oil mixture showed a clear and significant decrease in the hepatic cholesterol content (**Figure 7B**). Such a decrease was around 45−50% at longer times of dietary treatments (8−16 weeks). A comparable behavior, even if less evident, was found with the liver phospholipid content (**Figure 7C**). Whereas in the control and CLA-fed mice there was a time-dependent increase in the level of liver phospholipids, in the group of CLA + pine nut oil-fed animals we found a statistically significant decrease in this parameter (23% decrease at the 16th week). Altogether, these results indicate that the coadministration of CLA and pine nut oil not only prevents the triglyceride accumulation in liver induced by the sole CLA but also lowers other lipid parameters of the hepatic cells, such as cholesterol and phospholipids.

The same kind of analysis was then carried out in muscle cells (**Figure 8**). We found that CLA alone and the CLA + pine nut oil mixture significantly decreased the levels of triglycerides (**Figure 8A**), cholesterol (**Figure 8B**), and phospholipids (**Figure 8C**) in muscle in comparison to the values found in control animals. Reductions of about 30% for triglycerides and cholesterol and of about 20% for phospholipids were reproducibly found at longer times of dietary treatment. These results indicate that, differently from liver, CLA and CLA + pine nut oil exert the same effects on these lipid variables in muscle cells.

The assay of these parameters was then carried out in plasma (**Figure 9**). As previously found (*10*), plasma triglycerides time-dependently decreased in CLA-fed mice with respect to control animals (**Figure 9A**). Very interestingly, such a decrease was surprisingly more remarkable when the mice were fed the CLA + pine nut oil mixture (**Figure 9A**). At the 16th week of dietary treatment, the CLA-fed mice showed a 26% decrease in the level of plasma triglycerides, the decrease becoming 44% in the case of the CLA + pine nut oil-fed animals. As shown in **Figure 9B**, CLA was able to strongly decrease the plasma level

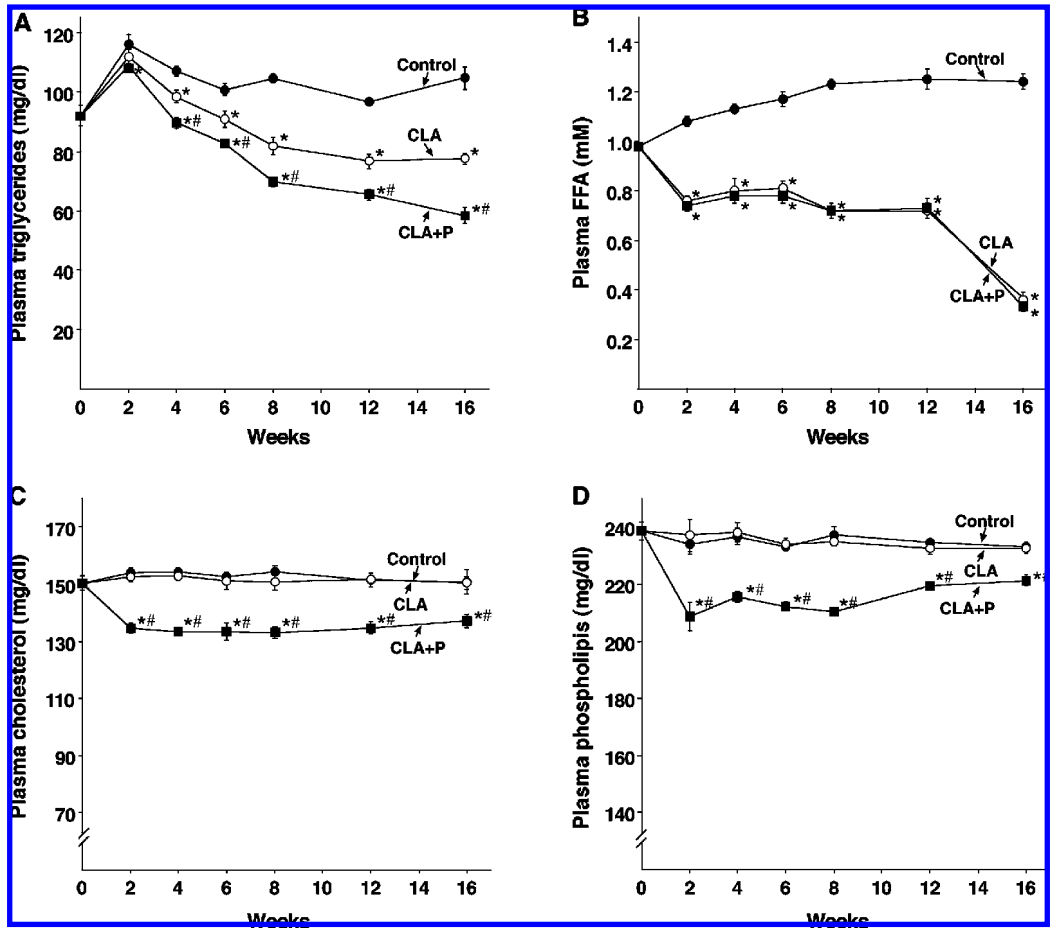

Figure 9. Effect of CLA and CLA + pine nut oil on plasma lipids. The levels of plasma triglycerides, FFA, total cholesterol, and phospholipids were determined at the times indicated, using commercial kits. The values reported in the figure represent the means ± SE ($n = 5$). ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA-supplemented diet.

of free fatty acids (FFA) with respect to control mice. Such a decrement (71% at the 16th week of dietary treatment) was of the same degree in the case of the animals fed with CLA + pine nut oil. **Figure 9C** shows that CLA did not influence at all the level of plasma cholesterol with respect to control animals. On the contrary, the coadministration of CLA and pine nut oil slightly, yet significantly, decreased the level of plasma cholesterol, which was about 15% less in the first part (2−8 weeks) and about 10% less in the final part (12−16 weeks) of this dietary treatment in comparison to control mice. A similar behavior was detected in the case of plasma phospholipids (**Figure 9D**). In fact, the CLA + pine nut oil mixture slightly decreased the level of plasma phospholipids in comparison to control and CLA-fed mice, which showed identical values over time.

Plasmatic Levels of Glucose and Insulin. The assay of the glucose levels in plasma did not reveal any significant change in the three groups of animals (**Figure 10A**). On the contrary, significant changes in the levels of plasma insulin were found as reported in **Figure 10B**. The level of plasma insulin, indeed, was approximately 3 times more in the CLA-fed mice in comparison to the control group in the first 2 months of dietary treatment. After this time, the insulin level sharply increased, becoming about 8 times more at the 12th week and about 11 times more at the 16th week of CLA dietary treatment in comparison to the values of control mice. Surprisingly, in the case of the CLA + pine nut oil coadministration the insulin levels remained approximately constant over time (about 3 times more than the control levels at any time of dietary treatment). These results indicate that diet supplementation with CLA induces insulin resistance, which is especially evident at longer times of treatment, and that this phenomenon is substantially mitigated by the coadministration of the pine nut oil.

DISCUSSION

Beneficial Effects of the CLA + Pine Nut Oil Mixture. The coadministration of CLA and pine nut oil to mice maintained all of the positive effects of CLA while preventing the undesirable effects. In fact, the mice fed with this fat mixture weighed less than the control animals at any time of dietary treatment. The body weight reduction was indistinguishable from that induced by just the CLA in the 6−16 week interval, and it was even more pronounced at the 2nd and 4th weeks of dietary treatment (**Figure 1A**). Furthermore, the inhibitions of fat deposition in the adipose tissue were identical in the CLA and CLA + pine nut oil-fed animals (**Figures 1C** and **2**). Remarkably, the CLA + pine nut oil mixture was able to efficiently prevent the liver weight increase and the liver triglyceride accumulation caused by CLA. These results indicate that the pine nut oil, when administered together with CLA, does not interfere with and/or probably contributes to the antiadiposity properties of CLA (positive effect) preventing, at the same time, the abnormal accumulation of triglycerides in the hepatic cells (adverse effect). Indeed, it must also be emphasized that the CLA + pine nut oil dietary mixture not only prevented the massive accumulation of triglycerides occurring in the liver of mice receiving the sole CLA, but that at the same time it slightly,

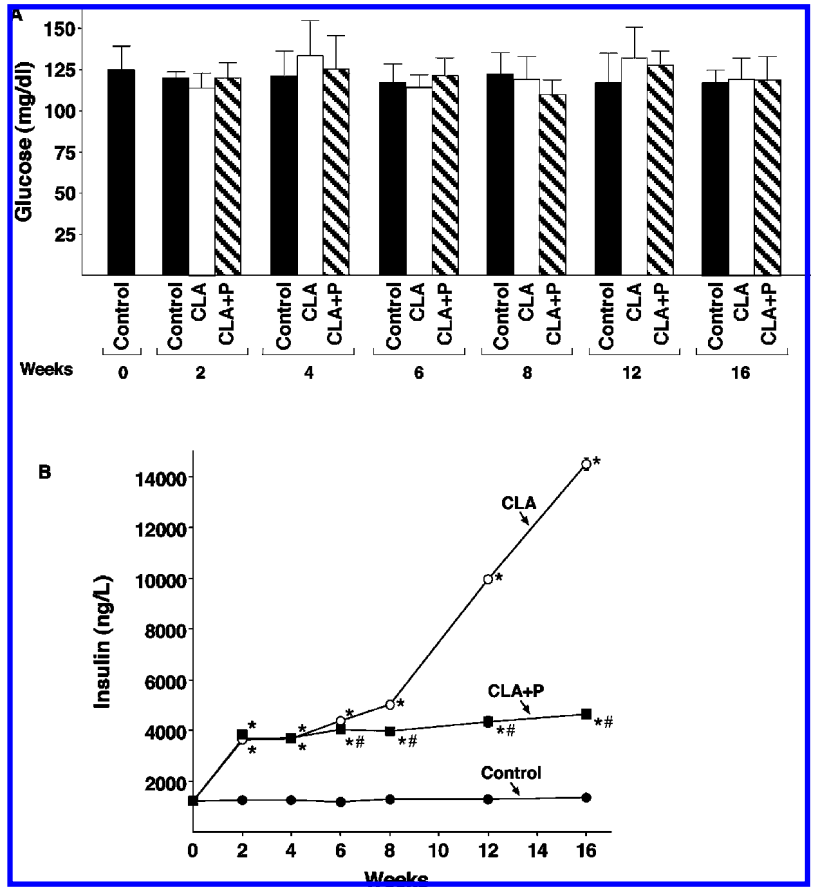

Figure 10. Effect of CLA and CLA + pine nut oil on plasmatic levels of glucose and insulin. (**A**) Blood glucose concentration was determined using reactive strips and a commercial glucometer. (**B**) Plasma insulin concentration was analyzed with commercial enzyme-linked immunosorbent assay kits. The values reported in the figure represent the means ± SE ($n = 5$). ∗, $P < 0.05$ versus mice fed control diet; #, $P < 0.05$ versus mice fed CLA diet.

although significantly, lowered the liver triglycerides even in comparison to control animals. An added value of the CLA + pine nut oil dietary combination is further present in liver, where lower levels of both cholesterol and phospholipids were detected.

The effects exerted by the CLA + pine nut oil coadministration on the plasma lipid levels are also remarkable. In fact, this dietary association reinforced the ability of CLA, which it is known to have, to decrease the plasmatic concentration of triglycerides (**Figure 9A**). Whereas CLA did not influence the plasmatic levels of cholesterol and phospholipids at all, the mixture of CLA + pine nut oil was instead able to slightly, yet significantly, decrease these parameters with respect to control animals. To sum up, these results clearly indicate that the association of CLA with pine nut oil modifies the plasma lipid parameters in such a way that healthy beneficial effects for the organism are to be expected.

The changes occurring in plasma insulin concentration merit particular attention. It has been reported that this hormone strongly increases in mice under CLA treatment, and this event has been interpreted as the progressive appearance of insulin resistance. In this study, the CLA-fed animals showed a 3-fold increase in the plasma insulin level in the first 6 weeks of dietary treatment, followed by a further dramatic increase in this parameter starting from the 8th week. On the contrary, the CLA and pine nut oil association stabilized the plasmatic insulin level over time, although the amount of this hormone was 3 times higher than that of the control animals.

Some attempts have also been made in the past to overcome the adverse effects of CLA, such as fatty liver, by using appropriate mixtures of CLA and other dietary fatty acids (*27*−*29*). In one study (*27*) a mixture of CLA and γ-linolenic acid prevented CLA-induced fatty liver in mice, even if the antiobesity properties of CLA were only partially retained. In another investigation (*28*), on the other hand, the fatty liver and the high hepatic triglyceride content induced by CLA in mice were only slightly alleviated by the coadministration of docosahexaenoic acid (DHA), even if with this dietary mixture the antiobesity effects of CLA were fully retained. None of these studies (*27*−*29*), however, was carried out over periods longer than 4 weeks, supplying therefore only limited information on the effects of the dietary combinations tested. Indeed, the present investigation as well as previous papers (*10*) revealed that the effects of dietary fatty acids on lipid metabolism are spread-out over periods which exceed the first 4 weeks of feeding.

It has also been hypothesized that adipose tissue reduction and liver enlargement, both induced by dietary CLA, are strictly interconnected in such a way that the liver is forced to become a sort of metabolic buffer system for body fat in the absence of active adipocytes (*30*). Moreover, the increase in hepatic fatty acid synthesis was retained to be the main mechanism responsible for fatty liver in CLA-fed mice (*30*). In line with these studies, Ide et al. (*29*) found that the addition of 6% fish oil to a diet containing CLA was able to efficiently decrease hepatic fatty acid synthesis and consequently the accumulation of liver triglycerides. However, this dietary combination reversed the antiobesity effects of CLA, leading to a reaccumulation of fat in the adipose tissue. These

findings, therefore, were interpreted as proof of the strict relationship existing between liver and adipose tissue in body fat management (*29, 30*). The results obtained in this study, however, demonstrate for the first time a clear and complete dissociation between these two phenomena, the liver maintaining its normal size and a lipid content even better than that of control animals in the presence of a drastic adipose tissue reduction.

It is important to emphasize that the CLA + pine nut oil diet used in this investigation differed mainly in the fatty acid composition when compared to the CLA diet. In fact, in the CLA + pine nut oil diet the content of saturated and monounsaturated (oleic acid) fatty acids was lower (about −33 and −57%, respectively), whereas that of polyunsaturated fatty acids (linoleic acid) was higher (about +102%) with respect to the CLA diet. Furthermore, the CLA + pine nut oil diet also differed for the specific presence of 1.12% pinolenic acid. Such a peculiar composition in the fatty acids may have a fundamental impact on several parameters in treated animals, such as specific enzymatic activities, or even on cell survival parameters. In addition, it must be considered that other compounds present in the CLA + pine nut oil diet, such as polyphenolic constituents, may have a role in triggering the effects observed in this study. The oxidative status of liver can indeed be altered by the presence of these compounds and the decreased level of oleic acid. Finally, we cannot exclude that some of the effects observed in the CLA + pine nut oil-fed animals may derive from unpredictable interactions between the different fatty acids. Further experiments are therefore necessary to gain insights into these phenomena. In this respect, a study carried out with a group of mice fed only pine nut oil would be particularly helpful.

Molecular Mechanisms of CLA Action. In the first 6 weeks of CLA administration, regardless of whether it was alone or in combination with pine nut oil, a 3-fold increase in the level of plasma insulin in comparison to control animals was clearly found. This increase most probably stimulated, to the same extent, the hepatic lipogenesis in treated animals. In previous work (*28*), the contemporaneous administration of DHA reduced the CLA-induced hepatic fatty acid synthesis, thereby inducing these authors to conclude that the attenuation of fatty liver was due to the suppressive effect of DHA on hepatic lipogenesis. This conclusion is not consistent with the present findings. In fact, in the first period of CLA + pine nut oil coadministration we found an increase in hepatic lipogenesis, identical to that induced by the sole CLA, but no liver enlargement and no hepatic triglyceride accumulation. This suggests that in the first period of CLA feeding the liver enlargement and the hepatic triglyceride accumulation occur independently of the fatty acid synthesis stimulation.

The time-dependent changes observed in the case of fatty acid oxidation were also interesting. The activity of liver and muscle CPT I increased over time in the first 2 months of dietary treatment both in CLA-fed and in CLA + pine nut oil-fed animals. From this time onward a reversal in the activity of this enzyme was clearly observed in both groups of animals. Furthermore, it is remarkable that the CLA + pine nut oil-induced reduction in the CPT I activity was stronger than that provoked by CLA and also that muscle was less responsive in this respect than liver.

A sharp rise in the plasma insulin levels occurred in the CLA-fed mice at the eighth week of treatment, whereas the level of this hormone remained stable in the CLA + pine nut oil-fed animals. Notably, just at the eighth week a differential change in the hepatic lipogenic activities also occurred in these groups of mice. Whereas the activities of the mitochondrial CIC and of the cytosolic lipogenic enzymes started to decrease in the CLA + pine nut oil-fed mice, those of the CLA-fed group of animals started to increase. Moreover, the level of triglycerides in the liver of CLA-fed mice increased dramatically, passing from values quite constant and approximately 2 times higher than those of control animals during the first 8 weeks of CLA administration to values 3.2 times higher at the 12th week and 4.7 times higher at the 16th week. These results suggest that the insulin-induced stimulation of hepatic lipogenesis, possibly in addition to other factors, plays an important role in the accumulation of triglycerides in the liver of CLA-fed animals at longer times of dietary treatment.

These findings also suggest that another regulatory element, appearing at the eighth week of the CLA + pine nut oil dietary treatment, provokes the decrease in the hepatic lipogenesis and prevents the increase in the insulin level found, instead, in the CLA-fed mice at the 12th and 16th weeks of dietary treatment.

ABBREVIATIONS USED

ACC, acetyl-CoA carboxylase; 1,2,3-BTA, 1,2,3-benzenetricarboxylate; CIC, mitochondrial tricarboxylate or citrate carrier; CLA, conjugated linoleic acid; FAS, fatty acid synthetase; CPT, carnitine palmitoyl-CoA transferase; FFA, free fatty acids; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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