

Differential effects of coconut oil- and fish oil-enriched diets on tricarboxylate carrier in rat liver mitochondria

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Abstract The mitochondrial tricarboxylate carrier (TCC) plays an important role in lipogenesis being TCC-responsible for the efflux from the mitochondria to the cytosol of acetyl-CoA, the primer for fatty acid synthesis. In this study, we investigated the effects of two high-fat diets with different fatty acid composition on the hepatic TCC activity. Rats were fed for 3 weeks on a basal diet supplemented with 15% of either coconut oil (CO), abundant in medium-chain saturated fatty acids, or fish oil (FO), rich in n-3 polyunsaturated fatty acids. Mitochondrial fatty acid composition was differently influenced by the dietary treatments, while no appreciable change in phospholipid composition and cholesterol level was observed. Compared with CO, the TCC activity was markedly decreased in liver mitochondria from FO-fed rats; kinetic analysis of the carrier revealed a decrease of the V_{max} with no change of the K_m . No difference in the Arrhenius plot between the two groups was observed. Interestingly, the carrier protein level and the corresponding mRNA abundance decreased following FO treatment. These data indicate that FO administration markedly decreased the TCC activity as compared with CO. This effect is most likely due to a reduced gene expression of the carrier protein.—Giudetti, A. M., S. Sabetta, R. di Summa, M. Leo, F. Damiano, L. Siculella, and G. V. Gnoni. Differential effects of coconut oil- and fish oil-enriched diets on tricarboxylate carrier in rat liver mitochondria. *J. Lipid Res.* 2003. 44: 2135–2141.

Supplementary key words cholesterol • fatty acids • lipogenesis • lipogenic gene regulation • phospholipids • n-3 polyunsaturated fatty acid • saturated fat

A large body of evidence shows that hepatic lipogenesis is regulated by both nutritional and hormonal factors [for review see Ref. (1)]. In particular, it is well documented that dietary polyunsaturated fatty acids (PUFAs) are noticeably effective in inhibiting hepatic lipogenesis and in lowering hypertriglyceridemia, n-3 fatty acids being more potent than n-6 lipids in this respect (2, 3). The activities of the lipogenic enzymes, such as acetyl-CoA carboxylase,

fatty acid synthase (FAS), ATP-citrate lyase, stearoyl-CoA desaturase, malic enzyme, glucose 6-phosphate dehydrogenase, and the S14 protein, are greatly reduced by PUFA administration (4–6). Furthermore, dietary PUFAs coordinately decrease the expression of hepatic genes encoding glycolytic and lipogenic regulatory enzymes involved in the flux of glucose to fatty acids (7–10). The n-3 PUFAs also play a crucial role as “fuel partitioners,” in that they direct fatty acids away from triacylglycerol storage and toward oxidation (11). They act by upregulating the expression of genes encoding proteins involved in fatty acid oxidation while downregulating genes encoding proteins of lipid synthesis (11). On the other hand, monounsaturated fatty acids like oleate (C18:1, n-9) or saturated fatty acids like palmitate (C16:0) and medium-chain fatty acids [as present in coconut oil (CO)] do not inhibit either the activities or the expression of the lipogenic enzymes (6, 8, 12).

Lipogenesis requires cooperation between mitochondrial and cytoplasmic enzymes and involves fluxes of metabolites across the mitochondrial membranes (13). The mitochondrial tricarboxylate carrier (TCC) (also known as citrate carrier), an integral protein of the mitochondrial inner membrane, catalyzes the efflux of citrate from the mitochondrial matrix in exchange for tricarboxylates, dicarboxylate (malate), or phosphoenolpyruvate across the mitochondrial inner membrane (14). This carrier protein plays a pivotal role in intermediary metabolism by connecting carbohydrate with the lipid metabolism. In fact, it transports in the form of citrate acetyl-CoA, mainly derived from the sugar source, from mitochondria to the cytosol. Here, by the action of ATP-citrate lyase, citrate provides the carbon units for fatty acid and cholesterol biosynthesis. In addition, it supplies NAD^+ and $NADPH$, which support cytosolic glycolysis and lipid biosynthesis, respectively (15). TCC has been extensively characterized in mammalian (16–18) and fish (19, 20) liver mitochondria.

Abbreviations: 1,2,3-BTA, 1,2,3-benzenetricarboxylic acid; BF₃, boron trifluoride; FAME, fatty acid methyl ester; TCC, tricarboxylate carrier.

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dria. The rat liver cDNA was cloned (21) and overexpressed in bacteria (22). The cDNA sequence of yeast (23), cow (24), and human (25) are also known. The nucleotide sequence of the human TCC gene has been determined (26). However, despite its important metabolic role and unlike the lipogenic enzymes, little is known about the regulation of TCC activity. A coordinate regulation of lipogenic enzyme activities in the cytosol and citrate transport activity across the mitochondrial inner membrane by nutritional factors was found (27, 28). In particular, a decrease of TCC activity and lipogenesis in the liver cytosol of PUFA (n-6)-fed rats was observed (28). Moreover, a recent study from our laboratory showed that the starvation-induced decrease of TCC activity in rat liver is parallel with a reduction of TCC mRNA accumulation. This latter effect was due to a posttranscriptional control of the carrier gene expression (29).

In the present study, we showed that the fatty acid composition of a high-fat diet specifically affects TCC activity in rat liver mitochondria. Compared with CO-fed rats, a fish oil (FO)-supplemented diet markedly reduced the TCC activity in these organelles. This reduction can most likely be attributed to a lower content of both an immunoreactive carrier protein and an mRNA abundance in rat liver cells.

EXPERIMENTAL PROCEDURES

Materials

Bio-Rad protein assay was purchased from Bio-Rad Laboratories (Milano, Italy). Nylon filters, Hybond N+, and nitrocellulose paper (0.45 μm) were purchased from Amersham Biosciences (Milano, Italy). [1,5- ^{14}C]citrate (specific activity, 100 mCi/mmol) was obtained from Amersham Pharmacia Biotech (Milano, Italy). [α - ^{32}P]dATP (specific activity 3,000 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Milano, Italy). The 1,2,3-benzenetricarboxylic acid (1,2,3-BTA) and horseradish peroxidase-conjugated anti-rabbit immunoglobulin secondary antibodies were obtained from Sigma-Aldrich Co. (Milano, Italy). CO and FO were from Mucedola (Milano, Italy). All other reagents were of analytical grade.

Animal treatments

Male Wistar rats (150–200 g) were used throughout this study. They were housed in cages in a temperature ($22 \pm 1^\circ\text{C}$)- and light (light on 8:00–20:00)-controlled room and randomly assigned to one of two different groups. The first group received a basal pellet diet (Morini, S. Polo D'Enza, Reggio Emilia, Italy) enriched with 15% (wt/wt) CO for three weeks, while the second group was fed on a 15% (wt/wt) FO-enriched basal diet for the same treatment period. The basal diet consisted of: 18.8% crude protein, 3.5% crude fat with adequate amounts of essential fatty acids, 4% crude fiber, 6% ash, and a salt and vitamin mixture.

The diets were prepared weekly and stored at -20°C . Fatty acid composition of the dietary lipids, determined by gas-liquid chromatographic analysis of the fatty acid methyl ester (FAME) derivatives, is reported in **Table 1**. In the CO-enriched diet, lauric acid (C12:0) was the most representative fatty acid, which was absent in the FO diet. Together with myristic acid (C14:0), it represented almost 60% of total CO fatty acids; the Σ saturated/ Σ unsaturated fatty acid ratio was much higher in the CO diet than in the FO diet. In the latter, the n-3 series fatty acids were particu-

TABLE 1. Fatty acid composition (mol%) of experimental diets

Fatty Acid	CO	FO
C _{10:0}	4.95 \pm 0.33	ND
C _{12:0}	40.78 \pm 0.39	ND
C _{14:0}	15.65 \pm 0.30	6.51 \pm 0.34 ^a
C _{16:0}	11.43 \pm 0.10	17.62 \pm 0.82 ^a
C _{16:1} (n-7)	0.28 \pm 0.05	9.12 \pm 0.38 ^a
C _{18:0}	9.08 \pm 0.09	3.39 \pm 0.18 ^a
C _{18:1} (n-9)	5.18 \pm 0.48	17.84 \pm 1.2 ^a
C _{18:2} (n-6)	12.90 \pm 0.72	13.99 \pm 1.1
C _{18:3} (n-3)	ND	2.44 \pm 0.15
C _{20:5} (n-3)	0.42 \pm 0.13	13.55 \pm 0.90 ^a
C _{22:5} (n-3)	ND	2.31 \pm 0.15
C _{22:6} (n-3)	0.43 \pm 0.10	11.76 \pm 0.92 ^a
Σ Saturated	81.89 \pm 0.45	27.52 \pm 0.40 ^a
Σ Unsaturated	18.31 \pm 0.56	71.01 \pm 0.77 ^a
Σ Saturated/ Σ unsaturated	4.48 \pm 0.17	0.39 \pm 0.02 ^a

CO, coconut oil; FO, fish oil; ND, not detected; Σ saturated, sum of saturated fatty acids; Σ unsaturated, sum of unsaturated fatty acids. Results are expressed as means \pm SD of six determinations. Basal diet was supplemented with 15% CO or 15% FO, respectively. Fatty acids were extracted from the two different diets and analyzed by gas-liquid chromatography.

^a $P < 0.001$, statistically significant diet effect.

larly abundant and represented about 30% of the total fatty acids. The animals had free access to food and water. Food consumption was monitored daily, and no significant difference was recorded between the two groups. The experimental design was in accordance with local and national guidelines covering animal experiments.

Citrate transport in rat liver mitochondria

Rat liver mitochondria were prepared following standard procedures. TCC activity was assayed in freshly isolated rat liver mitochondria as reported in Palmieri et al. (30). Briefly, the mitochondria (40–50 mg protein) were incubated for 2 min at 20°C in 10 ml 100 mM KCl, 20 mM HEPES, 1 mM EGTA (pH 7.0), and 2 $\mu\text{g}/\text{ml}$ rotenone and loaded with L-malate (0.75 mM) essentially as described (27). The exchange reaction was started by the addition to malate-loaded mitochondria (1–1.5 mg protein) of 0.5 mM [^{14}C]citrate, unless otherwise specified, and terminated by adding 12.5 mM of the inhibitor 1,2,3-BTA (30). Mitochondria were then isolated by centrifugation at 18,000 g for 5 min at 2°C , washed, and extracted with 20% HClO_4 . The mixture was centrifuged, and the radioactivity in the supernatant was counted.

Phospholipid and fatty acid analysis

Total lipids were extracted from mitochondria (10 mg protein) by the Bligh and Dyer procedure (31). The extracts were dried under N_2 flow and resuspended in a proper volume of CHCl_3 . Phospholipids were separated by HPLC, as previously described (32), by using a Beckman System Gold chromatograph equipped with an ultrasil-Si column (4.6 \times 250 mm) (Chemtek Analytica, Bologna, Italy). The chromatographic system was programmed for gradient elution by using two mobile phases: solvent A, hexane-2-propanol (6:8; v/v), and solvent B, hexane-2-propanol-water (6:8:1.4; v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0% to 100%. Flow rate was 2 ml/min, and detection was at 206 nm. Single phospholipids were identified by using known standards and quantitatively assayed by determining inorganic phosphate by the procedure reported in Nakamura (33). To analyze fatty acids, liver mitochondria were saponified with ethanolic KOH for 2 h at 90°C . Fatty acids were extracted as in Muci et al. (34), and their corresponding methyl esters were prepared by *trans*-esterification with

methanolic boron trifluoride (17% BF_3) at 65°C for 30 min. FAMES were then analyzed by gas-liquid chromatography. The helium carrier gas was used at a flow rate of $1 \text{ ml} \cdot \text{min}^{-1}$. FAMES were separated on a $30 \text{ m} \times 0.32 \text{ m}$ HP5 (Hewlett Packard) capillary column. The injector and detector temperatures were maintained at 250°C . The column was operated isothermally at 150°C for 4 min and then programmed to 250°C at $4^\circ\text{C}/\text{min}$. Peak identification was performed by using known standards, and relative quantitation was automatically carried out by peak integration. Cholesterol was assayed by HPLC as described (34).

Isolation of RNA and Northern blot analysis

Approximately 15 and 30 μg of total RNA, extracted from livers of CO- and FO-treated rats according to Chomczynski and Sacchi (35), were electrophoretically separated onto 1% formaldehyde-agarose gel under denaturing conditions and transferred to Hybond N+ nylon membrane. The RNA blots were hybridized with an $\alpha\text{-}^{32}\text{P}$ -labeled probe corresponding to nucleotides 459–1421 of the rat liver TCC cDNA (21). After hybridization, the membranes were washed twice in $2\times \text{SSC}$, 1% SDS at room temperature for 15 min and in $0.1\times \text{SSC}$, 0.1% SDS at 65°C for 30 min. For the normalization of the hybridization signals, the same membranes were stripped by washing them twice in a boiling solution of 0.1% SDS and rehybridized using a probe encoding part of the human β -actin. The membranes were stripped again and rehybridized with a randomly primed, $\alpha\text{-}^{32}\text{P}$ -labeled, 800 bp FAS cDNA as a positive control. After autoradiography, the intensity of the bands was evaluated by densitometry with Molecular Analyst Software.

Immunoelectrophoretic analysis

Equal amounts of mitochondrial proteins from CO- and FO-fed animals were loaded into the wells of 15% polyacrylamide gel (0.75 mm thick). After the electrophoretic run (25 mA/gel), proteins were electroblotted on nitrocellulose membrane and stained with Ponceau. After destaining with water, membranes were subjected to the reaction with antibody directed against a C-terminal peptide of the rat-liver TCC (36) and antibody directed against bovine porin. Porin, the mitochondrial outer membrane channel, was used as a control, because it has been reported that its expression is not affected by dietary PUFA (28). The bound antibody was revealed by peroxidase-conjugated anti-rabbit IgG antibody by using 3,3'-diaminobenzidine and hydrogen peroxide as substrates. Blots were evaluated by densitometric analysis with Molecular Analyst Software.

Other methods

Protein was determined by using the method of Lowry et al. (37) with BSA as a standard.

Statistical analyses

Data are the means \pm SD of the indicated number of experiments. The Student's *t*-test was performed to detect significant differences between the two treated groups of animals.

RESULTS

Citrate uptake in rat liver mitochondria

Figure 1 shows the time course of citrate uptake by malate-loaded mitochondria from liver of the two groups of animals. In all the experiments, the incubation mixture contained equal amounts of mitochondrial proteins and an external citrate concentration of 0.5 mM. When the TCC inhibitor 1,2,3-BTA was added to the incubation mix-

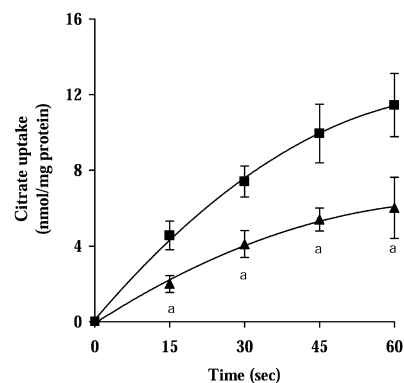


Fig. 1. Time course of citrate uptake in rat liver mitochondria. Malate-loaded mitochondria from coconut oil (CO)- (closed square) or fish oil (FO)- (closed triangle) treated rats were incubated with 0.5 mM ^{14}C citrate for the indicated times. The data are the means \pm SD of six different experiments. Statistically significant diet effect: $a P < 0.001$.

ture before starting the assay with ^{14}C citrate, the amount of citrate bound to the mitochondria was always the same. This indicates that dietary treatment was without effect on nonspecific citrate binding. As shown in Fig. 1, citrate uptake was strongly reduced in the liver mitochondria of animals fed the FO diet versus the CO diet.

In particular, in the first 15 s from starting the reaction (i.e., during the linear range of the citrate uptake curve), the decrease of the transport activity in FO-treated rats was reproducibly found to be $\sim 60\%$ of that found in the mitochondria from CO-treated rats ($2.0 \pm 0.45 \text{ nmol } ^{14}\text{C}$ citrate-transported/mg protein vs. $4.6 \pm 0.75 \text{ nmol } ^{14}\text{C}$ citrate-transported/mg protein; $P < 0.001$). To identify possible changes in the kinetic parameters of the carrier, the rate of ^{14}C citrate/malate exchange was studied at different external citrate concentrations. On the basis of experimental data (see Fig. 2 for a typical experiment),

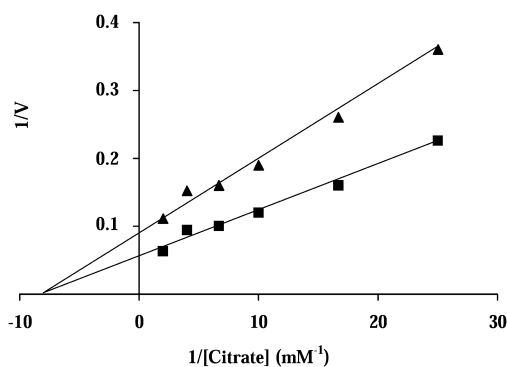


Fig. 2. Dependence of the rate of citrate uptake on substrate concentration in liver mitochondria from differently treated rats. Malate-loaded mitochondria (1–1.5 mg protein) from CO- (closed square) or FO- (closed triangle) fed rats were incubated at the external ^{14}C citrate concentration indicated in the figure. The citrate-malate exchange was stopped 15 s after the ^{14}C citrate addition by 12.5 mM 1,2,3-benzenetricarboxylic acid. Velocity of reaction (V) is expressed as $\text{nmol } ^{14}\text{C}$ citrate transported $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

the concentration dependence of citrate uptake by all the mitochondrial preparations is represented by straight lines, thus revealing hyperbolic saturation characteristics. The FO diet induced a remarkable decrease in the V_{max} value as compared with the CO diet (8.88 ± 1.49 nmol/min \times mg protein vs. 19.52 ± 2.98 nmol/min \times mg protein; $P < 0.001$), while no difference in the affinity of the carrier for citrate, as evidenced by the same K_m value (0.11 mM), was observed in liver mitochondria from the two groups of rats.

The TCC activity in CO- and FO-treated rats was also studied at different temperatures, and the corresponding Arrhenius plots of a representative experiment are reported in Fig. 3. The results demonstrated that citrate transport was reduced in mitochondria from FO-treated rats at all the tested temperatures compared with CO-fed animals. However, the biphasic profile of the two Arrhenius plots was identical: two linear portions with different slopes intersecting at the same temperature ($\sim 17^\circ\text{C}$).

Phospholipid analysis and fatty acid composition of the mitochondria

It has been shown that the transport activities of several mitochondrial carriers are influenced by the lipid composition of the mitochondrial membranes (38, 39). The phospholipid and fatty acid composition, as well as the cholesterol content of mitochondrial membranes from treated animals, were, therefore, investigated. No significant variation in the percentage of the main phospholipids of rat liver mitochondria as well as in the levels of total phospholipids and cholesterol (both calculated relative to mitochondrial protein) between the two groups of rats was observed (Table 2). However, the mitochondrial fatty acid composition was noticeably different in the two groups of animals (Table 3). A lack of incorporation of

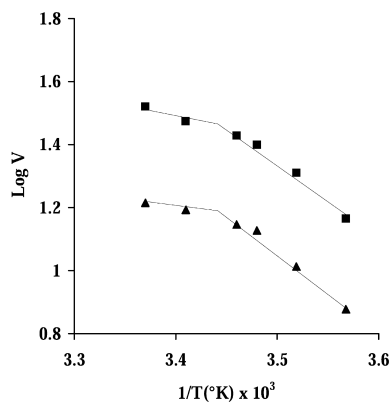


Fig. 3. Arrhenius plot of the temperature dependence of the rate of citrate uptake in liver mitochondria. Malate-loaded mitochondria from CO- (closed square) or FO- (closed triangle) treated rats were incubated with 0.5 mM [^{14}C]citrate at the indicated temperatures. The rates of citrate uptake were calculated from the amount of citrate taken up within the initial time period (15 s), during which citrate uptake was linear. Velocity of reaction (V) is expressed as nmol [^{14}C]citrate transported \cdot min $^{-1}$ \cdot mg protein $^{-1}$. The data are from a representative experiment. Similar results were obtained in six independent experiments.

TABLE 2. Phospholipid and cholesterol content of mitochondrial membranes

	CO	FO
Cardiolipin	8.40 ± 2.23	8.20 ± 1.60
Phosphatidylethanolamine	25.40 ± 4.50	26.50 ± 3.41
Phosphatidylinositol	9.90 ± 3.40	9.20 ± 3.00
Phosphatidylserine	2.40 ± 0.74	2.30 ± 0.80
Phosphatidylcholine	53.90 ± 12.41	53.80 ± 8.10
Phospholipids:protein	189.94 ± 40.53	184.40 ± 23.58
Cholesterol:protein	9.53 ± 1.30	10.63 ± 1.05
Cholesterol:phospholipids	0.096 ± 0.02	0.110 ± 0.02

Phospholipids:protein ratio is expressed as $\mu\text{g}/\text{mg}$ protein; cholesterol:protein ratio is expressed as $\mu\text{g}/\text{mg}$ protein; cholesterol:phospholipids are expressed as a molar ratio. The data are the means \pm SD of 10 independent analyses. Phospholipids were extracted from mitochondrial membranes of CO- and FO-fed rats. Single phospholipids were separated by HPLC, quantified by determining inorganic phosphate, and expressed as a percentage of the total phospholipids. Cholesterol was quantified by HPLC, whereas total phospholipids were quantified by determining inorganic phosphate.

C12:0, the most abundant fatty acid in the CO diet, into mitochondrial phospholipids of CO-fed rats was observed. This confirms earlier observations, which suggest that fatty acids shorter than C14:0 are not incorporated into hepatic phospholipids (40, 41). Moreover, in the CO-enriched mitochondria, palmitic acid (C16:0) and stearic acid (C18:0) were the most prominent saturated fatty acids. In the latter mitochondria, linoleic acid (C18:2, n-6) and arachidonic acid (C20:4, n-6) were more present than in mitochondria from FO-treated rats. Mitochondria from the latter animals showed a content of n-3 fatty acids more than twice as high as those from CO-fed animals. In fact, the FO diet significantly increased the mitochondrial level of eicosapentaenoic acid (C20:5, n-3), docosapentaenoic acid (C22:5,

TABLE 3. Fatty acid composition (mol%) of mitochondrial membrane phospholipids

Fatty Acid	CO	FO
C _{14:0}	2.21 ± 0.48	0.75 ± 0.34^a
C _{16:0}	18.82 ± 0.87	20.20 ± 0.96^b
C _{16:1} (n-7)	0.88 ± 0.26	2.24 ± 0.34^a
C _{18:0}	22.82 ± 1.67	18.79 ± 0.84^a
C _{18:1} (n-9)	6.86 ± 0.47	9.23 ± 0.80^a
C _{18:2} (n-6)	21.36 ± 0.61	14.39 ± 0.94^a
C _{18:3} (n-3)	0.34 ± 0.05	0.31 ± 0.11
C _{20:4} (n-6)	17.18 ± 1.19	12.40 ± 1.28^a
C _{20:5} (n-3)	1.18 ± 0.19	7.11 ± 0.46^a
C _{22:5} (n-3)	1.36 ± 0.21	2.37 ± 0.27^a
C _{22:6} (n-3)	7.48 ± 0.44	12.84 ± 1.31^a
Σ Saturated	43.85 ± 1.03	39.74 ± 0.97^a
Σ Unsaturated	56.84 ± 0.52	60.89 ± 0.83^a
Σ Saturated/ Σ unsaturated	0.77 ± 0.02	0.65 ± 0.02^a
Unsaturation index	178.78 ± 1.39	215.22 ± 7.83^a

Unsaturation index, Σ mol% of each fatty acid \times number of double bonds of the same fatty acid. The data are the means \pm SD of six independent experiments. Fatty acids were extracted from mitochondrial membrane phospholipids after saponification. After derivatization with methanolic boron trifluoride, fatty acid methyl esters were separated by gas-liquid chromatography and identified by using known standards.

^a $P < 0.001$, statistically significant differences between CO- and FO-treated rats.

^b $P < 0.05$, statistically significant differences between CO- and FO-treated rats.

n-3), and docosahexaenoic acid (C22:6, n-3) and, consequently, decreased the proportion of n-6 PUFA.

Effect of dietary treatment on mRNA abundance and protein level of TCC in rat liver cells

To investigate the molecular mechanism responsible for the regulation of the TCC activity by dietary fats, Northern blot and Western blot analyses were performed. Total RNA from liver of CO- and FO-treated rats was hybridized with TCC [α - 32 P]cDNA and then with [α - 32 P] β -actin cDNA. The latter was used for normalization, as it has been reported that the hepatic abundance of its mRNA is hardly affected by PUFA feeding (6, 28, 42). Densitometric analysis of the autoradiogram revealed that FO administration caused a reduction of \sim 40% of the liver TCC mRNA abundance as compared with CO-fed animals (Fig. 4A). By using the FAS cDNA as a positive control in the Northern blot analysis in agreement with previous reports (5, 6, 12), we found an impressive decrease in the level of FAS mRNA in the FO-treated rats compared with CO-fed animals (data not shown). Similarly, the immunodecoration reported in Fig. 4B revealed that the level of TCC in mitochondria from FO-treated rats was reduced by \sim 50% as compared with CO-fed rats. The amount of porin, the mitochondrial outer membrane channel used as a control, was unmodified.

DISCUSSION

The results reported in this study show for the first time that the activity of TCC, a protein of the mitochondrial inner membrane strictly correlated with lipogenesis, is specifically affected by dietary fatty acid composition. It was strongly reduced by the FO diet, rich in n-3 PUFA, as compared with a diet enriched with medium-chain, saturated fatty acids, as in the CO diet. Kinetic analysis of TCC activity revealed that in mitochondria from FO-treated rats the V_{max} of the citrate uptake was reduced by \sim 55%. This decrease in the V_{max} was larger than that previously found in rats for a high-fat, n-6 fatty acid-enriched diet, which triggered a 30% reduction of TCC activity (28). However, it is important to underline that there was practically no change in the affinity of the carrier protein for its substrate, as evidenced by the same K_m (0.11 mM) found in rats treated with either CO- or FO-enriched diets. A very similar K_m value not affected by dietary treatment has been reported for TCC in mitochondria from the n-6 fatty acid-fed rats (28). The present results suggest that the decreased transport activity observed in mitochondria from FO-fed rats did not reflect a general change in the intrinsic properties of the transport protein. On the basis of these results and of previous data regarding an n-6 fatty acid (present in safflower oil)-enriched diet (28), we may hypothesize that TCC activity is influenced by the fatty acid composition of a high-fat diet rather than by the fact that this diet is hypercaloric. This notion is supported by the observations that *i*) TCC activity is hardly affected by a CO-enriched diet, as its activity is very similar to that ob-

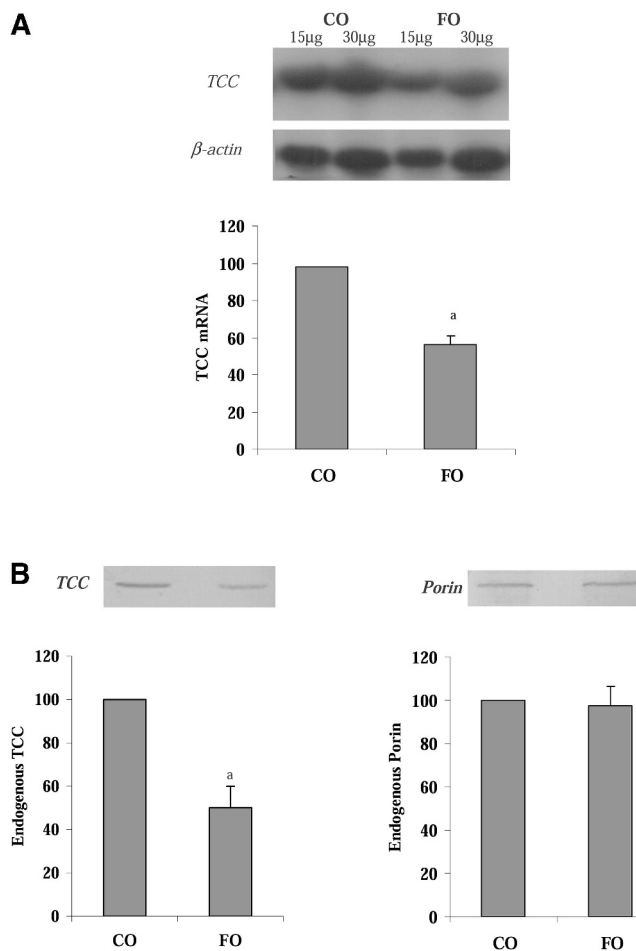


Fig. 4. Effect of CO or FO feeding on mRNA and protein levels of the mitochondrial tricarboxylate carrier (TCC). A: Accumulation of mature cytoplasmic mRNA for the TCC in liver from CO- or FO-fed rats. Approximately 15 μ g and 30 μ g of total RNA from liver was analyzed by Northern blot analysis and probed with [α - 32 P]TCC cDNA or with a [α - 32 P] β -actin cDNA fragment. The bars represent the optical scan of the autoradiograms. Data are expressed as a percentage of TCC mRNA abundance of CO (100%)-fed rats and are the means \pm SD of five independent experiments. Statistically significant diet effect: ^a $P < 0.001$. B: Liver mitochondrial proteins from CO or FO-fed rats were immunodecorated with antisera against either a C-terminal peptide of the rat TCC or bovine porin. The content of TCC and porin was quantified by photodensitometric analysis of blots. Data are expressed as percentage of TCC and porin abundance of CO (100%)-fed rats and are the means \pm SD of five independent experiments. Statistically significant diet effect: ^a $P < 0.001$.


served in mitochondria from laboratory chow-fed rats (data not shown), and *ii*) the inhibitory effect of an n-3 fatty acid-enriched diet on TCC activity observed in this study is higher than that reported for an n-6 fatty acid-supplemented diet (28).

Alterations in activities of membrane proteins could be generally ascribed either to a change in membrane lipid composition or to a modification of the functional protein level in the membrane (43). Phospholipid:protein ratio, cholesterol:phospholipid molar ratio, phospholipid composition, and degree of fatty acid unsaturation are

among the main parameters known to affect membrane fluidity (32, 44, 45). Under our experimental conditions, no significant changes by dietary treatments in almost all of these parameters were observed. However, mitochondrial membrane fatty acid composition was significantly influenced by the administration to rats of an FO- or CO-supplemented diet (Table 3). The observed diet-induced changes in phospholipid fatty acid composition could suggest an overall variation in mitochondrial membrane fluidity. The Arrhenius plots of Fig. 3 indicated that this is not the case. It should be noted that several mitochondrial carriers require a lipid microenvironment that may remain strictly bound to the protein even after its solubilization and purification (46). A lipid microenvironment unmodified by dietary manipulation may explain why the pattern of the Arrhenius plot of TCC (Fig. 3) was almost identical in the two groups of animals. Moreover, this finding is in agreement with a previous study (28) in which a reduced TCC activity, without appreciable changes in mitochondrial membrane fluidity following 3 weeks n-6 PUFA administration to rats, was observed. At any rate, it must be considered that the temperature-dependence study of TCC activity, via Arrhenius plot analysis, represents only an indirect method for analyzing the membrane fluidity. Further studies, using a more direct experimental tool, such as electron spin resonance, are required to better define this aspect.

Therefore, without also ruling out that different mechanisms may be involved in the regulation of TCC activity by n-3 dietary PUFA, the above-mentioned observations convinced us to hypothesize that a specific, direct effect on the carrier activity could be exerted by n-3 PUFA. Indeed, the total number of carrier molecules in the mitochondrial membrane may be decreased by the addition of FO to the diet. To verify this hypothesis, we quantified TCC levels in liver mitochondria of both FO- and CO-treated rats by using specific polyclonal antibodies. We showed that the diet-induced variations in TCC-specific activity were due to changes in mitochondrial carrier content in FO-treated rats. Interestingly, Northern blot analysis with total RNA from rat liver showed that the amount of hepatic carrier mRNA noticeably decreased following FO treatment. Analysis of the abundance of β -actin mRNA used as a control showed no difference, thereby establishing that dietary n-3 PUFA did not have a general effect on all mRNA species. Therefore, our results clearly show that expression of TCC is reduced by dietary n-3 PUFA. In agreement with previous findings (6), the observed n-3 PUFA effects on gene transcription are thus targeted to specific metabolic pathways and are not due to generalized changes in hepatic function. It is worth underlining that the observed decrease in both TCC mRNA abundance and protein level, due to feeding an n-3 fatty acid-enriched diet, is higher than that reported for an n-6 fatty acid-supplemented diet (28). Taken together, these results suggest that n-3 fatty acids are more potent inhibitors than n-6 lipids of both TCC activity and expression. Carbohydrates, through glycolysis and the pyruvate dehydrogenase complex that generates acetyl-CoA, are generally

considered a source of carbon for hepatic lipogenesis. It has been shown that dietary PUFA caused a marked inhibitory effect on total and active pyruvate dehydrogenase complex (47). TCC represents a link between carbohydrate catabolism and fatty acid synthesis (48). In this respect, it is relevant to note that cytosolic citrate, exported by TCC from the mitochondrial matrix to the cytosol, is also an activator of acetyl-CoA carboxylase, the first committed step in the de novo fatty acid synthesis (48). Therefore, the reduction of lipogenesis observed by several others (1, 5, 10) and confirmed by us (data not shown) following n-3 PUFA administration to rats could be ascribed, at least in part, to the reduced activity of TCC. Unlike FO feeding, CO feeding seems not to have an appreciable effect on both TCC activity and expression. It could be of interest to investigate the effect on TCC of a saturated fat of a different origin, i.e., beef tallow rich in long-chain, saturated fatty acids. To this aim, experiments are in progress in our laboratory.

In conclusion, the modulation of TCC activity by dietary fatty acids reported in this paper can be important for defining the overall mechanism of regulation of hepatic lipogenesis. Therefore, the molecular basis involved in the regulation of TCC activity by nutrients awaits further investigation. 

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