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Different dietary fatty acids have dissimilar effects on activity and gene expression of mitochondrial tricarboxylate carrier in rat liver

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Abstract The tricarboxylate carrier (TCC), an integral protein of the mitochondrial inner membrane, transports mitochondrial acetyl-CoA into the cytosol, where lipogenesis occurs. We investigated in rat liver mitochondria the effect of diets enriched with saturated fatty acids (beef tallow, BT), monounsaturated fatty acids (olive oil, OO) or $n-3$ polyunsaturated fatty acids (fish oil, FO), respectively, on the activity and expression of TCC. TCC activity decreased, in parallel with TCC mRNA abundance, only upon FO-feeding. The TCC transcription rate, mRNA turnover and RNA processing indicated that FO administration regulates TCC gene at transcriptional and post-transcriptional steps, whereas BT- and OO-feeding do not seem to affect either TCC activity or gene expression.

Keywords: Dietary fat; gene modulation; mitochondrion; Ribonuclease protection assay; Run-on assay; Tricarboxylate carrier

1. Introduction

Besides its role as an energy source and its effect on membrane lipid composition, dietary fat has remarkable influence on gene expression [1], leading to changes in metabolism, growth and cell differentiation [2,3]. The activities of lipogenic enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), are lower in rats fed a PUFA- than in rats fed a saturated- or monounsaturated-supplemented diet [4,5]. Different molecular mechanisms have been proposed regarding the regulation by dietary PUFA of lipogenic enzymes (for review, see [5]). Most of these enzymes are regulated either at transcriptional or at post-transcriptional steps. FAS [3,6], ACC [3,4] and stearoyl-CoA desaturase [7] are dietary regulated at transcriptional level, whereas PUFA regulation of malic enzyme occurs by changes in mRNA stability [3] and glucose-6-phosphate dehydrogenase is controlled by a post-transcriptional mechanism [8].

In this study, we investigated the effects of different dietary fats on the expression of the hepatic tricarboxylate carrier (TCC), an integral protein of the mitochondrial inner membrane. This carrier protein plays a key role in lipogenesis [9]. It transports, in the form of citrate, acetyl-CoA, mainly derived from sugar sources, from mitochondria to cytosol, providing the carbon units for fatty acid and cholesterol biosyntheses. In addition, TCC supplies NAD^+ and NADPH that support cytosolic glycolysis and lipid biosynthesis, respectively [10]. TCC has been extensively characterized in liver mitochondria from mammals and fish (for review, see [11]). A parallel decrease of TCC activity and lipogenesis in the liver of starved [12] and $n-6$ PUFA-fed rats was reported [13]. The aim of this study was to investigate whether diets with different fatty acid compositions, i.e., rich in either long chain saturated fatty acids (BT) or monounsaturated fatty acids (OO) or $n-3$ PUFA (FO) could influence the hepatic TCC expression and, if so, to characterize the molecular mechanism responsible for TCC gene regulation.

This study showed that while BT and OO administration to rats is practically without effect, FO-supplementation significantly reduced hepatic TCC expression; the TCC gene regulation occurred by both transcriptional and post-transcriptional mechanisms.

2. Materials and methods

2.1. Animal treatments

Male Wistar rats (150–200 g) were randomly assigned to one of the three different groups. The three groups received for 3 weeks a laboratory chow diet enriched with 15% (w/w) diet of OO, BT or FO, respectively. Fatty acid composition of the dietary lipids is reported in Table 1. The experimental design was in accordance with local and national guidelines covering animal experiments.

2.2. Citrate transport in rat liver mitochondria

TCC activity was assayed in freshly isolated rat liver mitochondria as in [14]. The malate–citrate exchange reaction was started by the addition of 0.5 mM [^{14}C]citrate to malate-loaded mitochondria (1–1.5 mg protein) and terminated by adding 12.5 mM of the inhibitor 1,2,3-benzenetricarboxylic acid (BTA) [14].

2.3. Immunoelectrophoretic analysis

Western blots were carried out as reported in [15]. Nitrocellulose membranes were submitted to the reaction with antibody directed against a C-terminal peptide of the rat liver TCC [15] and antibody directed against bovine porin.

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Abbreviations: ACC, acetyl-CoA carboxylase; BT, beef tallow; FAS, fatty acid synthase; FO, fish oil; OO, olive oil; TCC, tricarboxylate carrier

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Table 1
Fatty acid composition (mol%) of experimental diets

Fatty acid	BT	OO	FO
$C_{14:0}$	4.89 ± 0.35[a]	0.16 ± 0.01[b]	6.51 ± 0.46[c]
$C_{16:0}$	29.33 ± 1.93[a]	14.10 ± 1.01[b]	17.62 ± 1.23[c]
$C_{16:1}$ $(n-7)$	2.69 ± 1.91[a]	1.62 ± 0.11[b]	9.12 ± 0.65[c]
$C_{18:0}$	24.21 ± 1.87[a]	1.93 ± 0.11[b]	3.01 ± 0.23[c]
$C_{18:1}$ $(n-9)$	33.77 ± 2.87[a]	72.80 ± 6.88[b]	17.84 ± 1.10[c]
$C_{18:2}$ $(n-6)$	1.91 ± 0.13[a]	4.39 ± 0.34[b]	13.99 ± 1.12[c]
$C_{18:3}$ $(n-3)$	0.30 ± 0.04[a]	0.41 ± 0.04[b]	0.69 ± 0.07[c]
$C_{20:5}$ $(n-3)$	ND	ND	13.55 ± 1.12
$C_{22:5}$ $(n-3)$	ND	ND	2.31 ± 0.12
$C_{22:6}$ $(n-3)$	0.36 ± 0.02[a]	ND	11.76 ± 0.97[b]
∑saturated	58.43 ± 4.89[a]	16.19 ± 1.13[b]	27.14 ± 1.98[c]
∑monounsaturated	36.46 ± 2.88[a]	74.42 ± 6.55[b]	26.96 ± 3.11[c]
∑polyunsaturated	2.57 ± 0.15[a]	4.80 ± 0.23[b]	42.30 ± 4.11[c]

Basal diet was supplemented with 15% of BT, OO or FO, respectively. Fatty acids were extracted from the different diets and analyzed by gas–liquid chromatography. Results are expressed as means ± S.E. of six determinations. Values on the same line with a different superscript differ significantly ($P < 0.05$).
∑saturated = sum of saturated fatty acids.
∑monounsaturated = sum of monounsaturated fatty acids.
∑polyunsaturated = sum of polyunsaturated fatty acids.

2.4. Isolation of RNA and Northern blot analysis

About 15 μg of total RNA, extracted from liver of treated rats according to Chomczynski and Sacchi [16], was electrophoretically separated onto 1% formaldehyde-agarose gel under denaturing conditions and transferred to Hybond N+ nylon membrane. The RNA blots were hybridized with rat liver TCC cDNA probe as reported by Siculella et al. [12]. For normalization of the hybridization signals, the same membranes were hybridized using a probe encoding part of the human β-actin. After autoradiography, the intensity of the bands was evaluated by densitometry with Molecular Analyst Software.

2.5. Isolation of nuclei and nuclear run-on assay

Nuclei were isolated from hepatocytes obtained by liver perfusion and collagenase digestion as reported by Gnoni et al. [17], washed twice with cold PBS (phosphate-buffered saline) and then lysed as in [12]. The crude nuclei were purified as described by Siculella et al. [12] and nuclear run-on assay was carried out as described by Liu et al. [18]. Labelled RNA was extracted as indicated above and hybridized to Hybond N+ nylon membranes as reported by Siculella et al. [12]. Hybridization signals were quantified as described above.

2.6. mRNA turnover assay

Hepatocytes from BT- and FO-fed rats were maintained on plastic Petri dishes (60 mm) until monolayer formation [17]. After 2 h plating, 4 μg/ml actinomycin D in Ham's F12 medium was added. For each time point, total RNA was extracted from 10 plates (approx. 4×10^6 cells) for each group and 10 μg was analyzed by Northern blot hybridization as indicated above, using TCC cDNA as a probe. The same filter was stripped and rehybridized with β-actin cDNA. The autoradiogram was quantified by densitometric scanning.

2.7. Isolation of nuclear RNA

RNA isolation from purified nuclei was carried out as described by Chomczynski and Sacchi [16].

2.8. Probe design for ribonuclease protection assay

Two probes, intron2–exon3 (I2–E3) and exon7–intron7 (E7–I7), designed for use in the ribonuclease protection assay, were obtained by PCR amplification of a genomic clone p5B8 containing the TCC gene (data not shown), using specific primers as previously reported [19]. The amplified products were subcloned into pBlueskript II vector. After linearization, the recombinant plasmids were used in the in vitro transcription reactions.

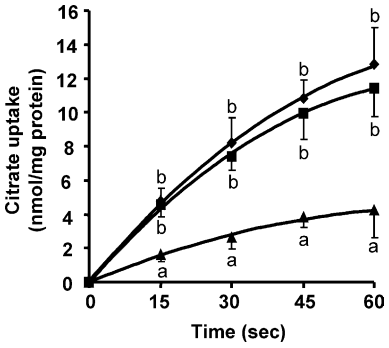


Fig. 1. Time course of citrate uptake in rat liver mitochondria. Malate-loaded mitochondria from BT (♦), OO (■) or FO (▲) treated-rats were incubated with 0.5 mM [^{14}C]citrate for the indicated times. The data are means ± S.E. of six different experiments. Values sharing a different letter were significantly different ($P < 0.001$).

2.9. Ribonuclease protection assay

Antisense RNAs were synthesized by an in vitro transcription reaction as reported in [19]. Nuclear RNA (10 μg) was hybridized with 2×10^5 cpm of ^{32}P-labelled specific antisense probe in 20 μl of hybridization solution at 50 °C for 16 h. For the normalization, a β-actin antisense ^{32}P-labelled RNA probe was added in each hybridization reaction. Probes were also hybridized with 10 μg of yeast RNA used as a control for testing the RNase activity (data not shown). After digestion with RNase A/T1, the protected fragments were separated onto a 6% denaturing polyacrylamide gel. Gels were dried, exposed for autoradiography and the intensity of the bands was evaluated by densitometry with Molecular Analyst Software.

2.10. Statistical analysis

All data are presented as means ± S.E. for the number of experiments indicated in each case. Statistical analysis was performed by Student's t test. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Time course of citrate/malate exchange

Fig. 1 shows the time-course of [^{14}C]citrate uptake by malate-loaded mitochondria from liver of differently treated rats. In the first 15 s, we already observed in FO-treated rats a decrease of the transport activity of about 60% versus both BT- and OO-treatment.

3.2. Effect of BT, OO and FO on the TCC mRNA and on the protein levels in rat hepatocytes

Northern blot analysis showed no significant difference in the TCC mRNA level among BT-, OO- and chow-fed rats (control) (Fig. 2A), while when compared to the other groups, FO administration caused a reduction of about 40% of the hepatic TCC mRNA level. Expression of the house-keeping gene for β-actin was unmodified among the four groups of rats. Analogously, the immunodecoration reported in Fig. 2B revealed that the level of TCC in mitochondria from FO-fed rats was lowered by about 50% with respect to the other groups of rats. By contrast the amount of porin, the mitochondrial outer membrane channel used as a control since its expression is not affected by dietary PUFA [13], was unchanged. These data indicate that inhibition of TCC activity and expression is due specifically to the FO treatment since: (i) the different sources of fat is the sole variable in the diet; (ii) the administration

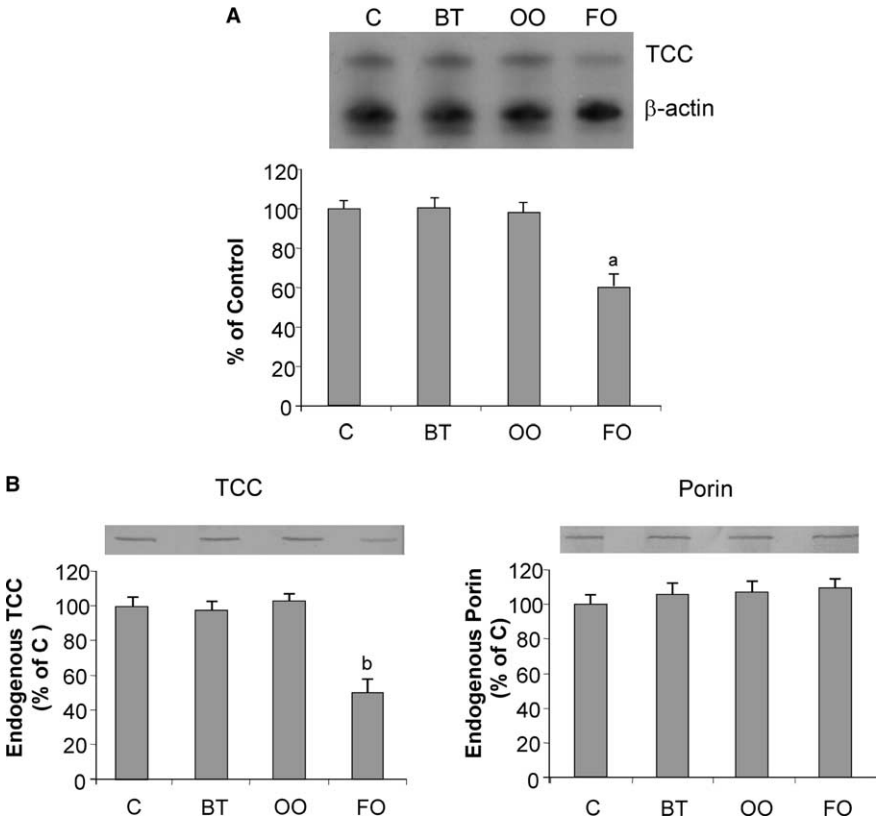


Fig. 2. Effect of different diets on the TCC mRNA and protein levels in rat liver. (A) About 15 μg of RNA from two pools (two animals each) for C (control, chow-fed rats), BT-, OO- or FO-fed rats was analyzed by Northern blot analysis and probed with TCC cDNA or β-actin cDNA fragment. The bars represent an optical scan of the autoradiogram. Data are means ± S.E. of five independent experiments ($^{a}P < 0.05$). (B) Liver mitochondria from C, BT-, OO- or FO-fed rats were immunodecorated with antisera against either a C-terminal peptide of the rat liver TCC or bovine porin by Western blot analysis. The content of mitochondrial TCC and porin was quantified by photodensitometric analysis of blots. Data are means ± S.E. of five independent experiments ($^{b}P < 0.001$).

of BT, rich in long chain saturated fatty acids, or OO, high in oleate (C18:1 $n-9$) failed to inhibit TCC activity. It is worth underlining that rats feeding on coconut oil, rich in short and medium chain saturated fatty acids, showed practically no effect on the TCC activity [15], which on the other hand, was significantly reduced by $n-6$ PUFA-supplemented diet [13]. However, the latter reduction is lower than what we observed in the present study with FO, rich in $n-3$ PUFA. Interestingly, the fact that the mRNA abundance of β-actin used as a control was unmodified by the dietary treatment (Fig. 2A) clearly establishes that FO administration does not have a general effect on all mRNA species.

The Northern and Western experiments showed similar values for BT- and OO- fed rats, which in turn were similar to laboratory chow-fed rats. Therefore, only BT-and FO-supplemented diet effects were compared in all the subsequent experiments.

3.3. Turnover of TCC mRNA

To address the question whether changes of the TCC mRNA turnover occurred upon FO treatment, the decay curve of TCC mRNA (upper panel, Fig. 3) was analyzed. The estimated apparent half-life of TCC mRNA from BT- and FO-fed rat hepatocytes was similar (11.1 ± 0.9 h in BT- vs 10.9 ± 0.8 h in FO-fed rats). In the same RNA preparation, the relative rate of degradation of the β-actin mRNA remained constant (lower panel, Fig. 3). Therefore, in the absence of a

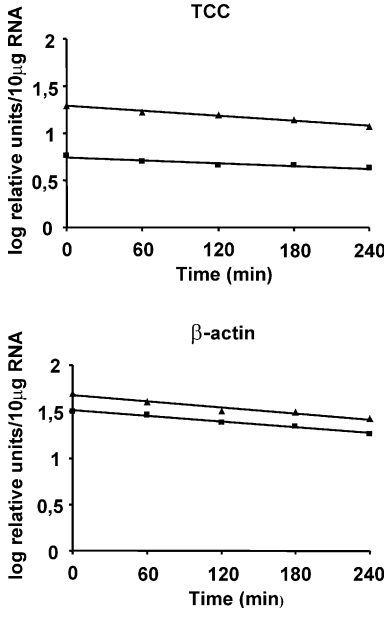


Fig. 3. Turnover of TCC mRNA in cultured hepatocytes from BT- and FO-fed rats. The semi-log plot in the upper and lower panel represents the decay curve of TCC mRNA and β-actin mRNA, respectively. BT-fed rats (▲) and FO-fed rats (■). Each point represents the average of two measurements. The data are from a representative experiment. Similar results were obtained in five independent experiments.

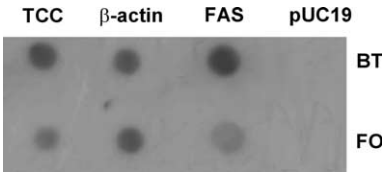


Fig. 4. Effect of BT- and FO-enriched diet on TCC transcriptional activity. Nuclei, isolated from hepatocytes from BT- and FO-fed rats, were allowed to incorporate [α-^{32}P]UTP; TCC transcripts were detected by hybridization to dots (5 μg) of the indicated cDNAs applied to the filters. The data are from a representative experiment. Similar results were obtained in four independent experiments.

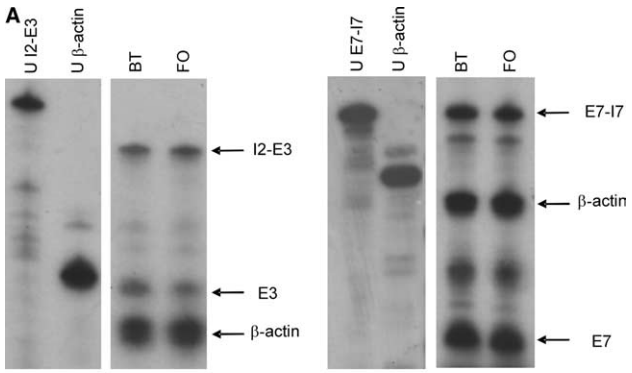
consistent difference in the TCC mRNA half-life, a decrease in TCC mRNA accumulation in FO-fed rats could reflect a transcriptional modulation of TCC gene.

3.4. Run-on assay

To investigate whether FO feeding controlled TCC expression at transcriptional level, nuclear run-on assay was carried out. Nuclei isolated from BT- and FO-fed rat hepatocyte suspensions were allowed to incorporate [α^{32}P]UTP. The labelled RNA was extracted and hybridized to dots (5 μg) of TCC cDNA, β-actin cDNA, FAS cDNA and pUC19 applied to the filters. β-actin and pUC19 were used for normalization and as a negative control, respectively. FAS cDNA was used as a positive control, since it has been reported that $n-3$ PUFA-supplemented diet modulates this gene at transcriptional level [6]. The labelled RNA was transcribed by RNA polymerase II as TCC and β-actin transcripts were specifically suppressed by α-amanitin (4 μg/ml) (data not shown). The dot blot hybridization revealed a remarkable decrease of around 70% in the transcriptional rate of TCC mRNA from FO- vs BT-fed rats (Fig. 4). In good agreement with [6], FAS transcriptional rate decreased by approximately 80% in FO- vs BT-fed rats. The transcriptional rate of β-actin remained constant in the two groups of rats, thus suggesting that FO action was gene-specific.

3.5. Processing of TCC precursor RNA

To study the effects of BT- and FO-supplemented diets on the splicing of TCC RNA, we compared the amount of unspliced and spliced TCC RNA in the nuclei of hepatocytes from FO- vs BT-fed rats. Two probes (I2–E3 and E7–I7) separated by about 1.1 kb were used to investigate the processing of the pre-mRNA (Fig. 5). Each probe hybridized across an exon/intron junction and thus resulted in two types of protected fragments: the unspliced RNA containing both the exon and the intron sequences (intron2–exon 3; exon7–intron7); the spliced RNA containing only the exon sequences (exon3; exon7).



B

Spliced/unspliced		
Probes	I2-E3	E7-I7
BT	2,1±0,3	3,2±0,4
FO	1,1±0,2	3,0±0,2

Fig. 6. Effect of BT- and FO-enriched diet on the processing of TCC precursor RNA. (A) A representative assay is shown. Nuclear RNA (10 μg) was analyzed by two TCC specific probes using RNase protection assay. U, undigested control (hybridization of yeast RNA (10 μg) with the probes without subsequent RNase digestion); BT and FO, hybridization of probes to 10 μg nuclear RNA isolated from liver of, respectively, BT- and FO-fed rats, followed by RNase digestion. (B) Quantitative results of four independent experiments with two rats per treatment per experiment (mean ± S.E.). The hybridization signal was quantified using Molecular Analyst Software. The values were normalized for uridine content of the protected fragment.

The terms unspliced and spliced RNA refer to a RNA mix, containing one or more of all the TCC introns. By using the I2–E3 probe, we found the amount of spliced RNA 2.1-fold greater than that of unspliced RNA in the nuclei from BT-fed rats. To note that, in the nuclei from FO-fed rats, the amount of spliced RNA was only 1.1-fold greater than that of unspliced RNA. Therefore, FO-added diet caused a decrease in the ratio spliced to unspliced RNA (Fig. 6). Furthermore, the reduction in the amount of spliced RNA in the nuclei of FO- versus BT-fed rats was similar to the decrease (around 45%) in the accumulation of the mature TCC RNA, measured in the cytoplasm (Fig. 2). In contrast, by using the second probe (E7–I7), we observed not only the same amount of unspliced RNA but also a similar ratio of spliced/unspliced RNA both in FO- and BT-fed rats (Fig. 6). Experiments are in progress in our laboratory to further elucidate this pathway by using different probes, representing other exon–intron junctions.

The data, consistent with regulation of pre-mRNA processing in rats fed a FO diet, indicate that the splicing reaction itself is inhibited by FO treatment and suggest that: (i) the

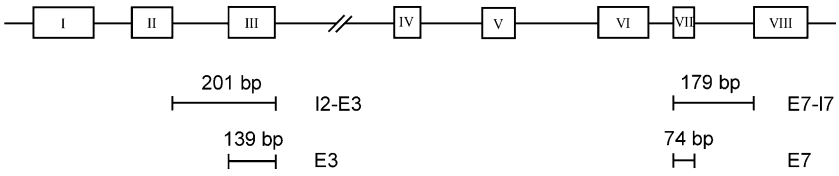


Fig. 5. Probes and predicted fragments for the ribonuclease protection assay. The lines and the boxes depict schematically the structure of the TCC precursor RNA. The lower lines represent TCC specific RNA fragments detected in the RNase protection assay by the two specific TCC probes. The first probe (intron2–exon3) protects a 201 nt fragment of pre-mRNA containing intron2 and in addition a 139 nt processed transcript that has undergone splicing of intron2. The second probe (exon7–intron7) protects a 179 nt fragment containing intron7 and also a spliced transcript of 74 nt without intron.

FO-supplemented diet does not exert its inhibitory effect on the splicing of all the introns from the TCC precursor RNA; (ii) a putative cis acting element, involved in TCC RNA splicing regulation, maps to exon 3; (iii) nuclear-cytoplasmic transport of TCC mRNA seems not to be regulated, and (iv) TCC regulation by FO occurs in the nucleus.

Taken together, the present report indicates that FO-supplemented diet downregulates the expression of TCC gene by both transcriptional and post-transcriptional mechanisms. It is worth underlining that also for the gene expression of the cytosolic lipogenic enzyme ATP-citrate lyase, which works in sequence to TCC, transcriptional and post-transcriptional regulation by nutrients is suggested [20]. On the other hand, gene regulation of ACC [3,4] and FAS [3,6] by dietary PUFA mainly occurs at transcriptional level. A possible speculation to explain these differences is that while ACC and FAS function exclusively in fatty acid synthesis, TCC and ATP-citrate lyase participate in metabolic processes other than lipogenesis such as cholesterol synthesis, and the gluconeogenic pathway.

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