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ORIGINAL ARTICLE

L-Carnitine induces recovery of liver lipid metabolism in cancer cachexia

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Abstract Cancer cachexia causes metabolic alterations with a marked effect on hepatic lipid metabolism. L-Carnitine modulates lipid metabolism and its supplementation has been proposed as a therapeutic strategy in many diseases. In the present study, the effects of L-carnitine supplementation on gene expression and on liver lipid metabolism-related proteins was investigated in cachectic tumour-bearing rats. Wistar rats were assigned to receive 1 g/kg of L-carnitine or saline. After 14 days, supplemented and control animals were assigned to a control (N), control supplemented with L-carnitine (CN), tumour-bearing Walker 256 carcinosarcoma (TB) and tumour-bearing supplemented with L-carnitine (CTB) group. The mRNA expression of carnitine palmitoyltransferase I and II (CPT I and II), microsomal triglyceride transfer protein (MTP), liver fatty acid-binding protein (L-FABP), fatty acid translocase (FAT/CD36), peroxisome proliferator-activated receptor-alpha (PPAR-alpha) and organic cation transporter 2 (OCTN2) was assessed, and the maximal activity of CPT I and II in the liver measured, along with plasma and liver triacylglycerol content. The gene expression of MTP, and CPT I catalytic activity were reduced in TB, who also showed increased liver (150%) and plasma (3.3-fold) triacylglycerol content. L-Carnitine supplementation was able to restore these parameters back to control values ($p < 0.05$). These data show that L-carnitine preserves hepatic lipid metabolism in tumour-bearing animals, suggesting its supplementation to be of potential interest in cachexia.

Keywords L-Carnitine · Cachexia · Liver · Lipid metabolism · Steatosis

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Introduction

Cancer cachexia is a paraneoplasic syndrome affecting the large majority of terminally ill cancer patients (Argilés et al. 1997; Barber et al. 1999) This syndrome is clinically characterised by a number of symptoms and signs which are not overcome by standard nutritional supplementation nor by any single therapy known to date, including anorexia, weight loss, wasting, fatigue, poor mental and physical performance, and compromised quality of life (Inui 2002). Cancer cachexia is markedly associated with adverse prognosis and shortened survival time (Barber et al. 1999), and comprises a plethora of metabolic alterations, which profoundly affect carbohydrate, protein, and lipid metabolism (Argilés et al. 1997).

In experimental cancer cachexia, the capacity for oxidation of fatty acids in the liver is decreased (Siddiqui and Williams 1989), resulting in steatosis and alteration in the production of ketone bodies (Siddiqui and Williams 1989; Kazantzis and Seelaender 2005). The carnitine palmitoyltransferase system is the main step in the control of these processes, playing an essential role in the transport of long-chain fatty acids into mitochondria (McGarry 1995). One such transport involves two enzymes, associated with the mitochondrial membranes: carnitine palmitoyltransferase I (CPT I) catalyses the transfer of acyl groups from

acyl-CoA to carnitine to synthesise acylcarnitine, yielding free CoA. The protein carnitine/acylcarnitine translocase (CACT) exchanges cytoplasmic acylcarnitine for mitochondrial-free carnitine; and finally, the enzyme carnitine palmitoyltransferase II (CPT II) reconverts acylcarnitine to acyl-CoA, which is conveyed to β-oxidation (McGarry 1995). In cachectic Walker 256 tumour-bearing rats, the activities of CPT I and CPT II have been found to be reduced (Seelaender et al. 1998; Kazantzis and Seelaender 2005), with concomitant steatosis.

Because the hepatocyte relies upon long-chain fatty acid oxidation to support its own energy demands during cachexia, there is disruption of liver function. These demands are still further enhanced by increased flux through the Cori cycle (Kazantzis and Seelaender 2005), through which the hepatocyte produces glucose de novo from tumour metabolism-derived lactate. Therefore, the compromised oxidative capacity related with decreased CPT system activity greatly contributes to aggravation of the wasting induced by cachexia.

L-Carnitine (β-hydroxy-(γ-*N*-trimethylamino) butyrate) is fundamental for lipid β-oxidation as its takes part in the above-mentioned process of long-chain fatty acid transport into mitochondria (Bremer 1981). Patients with senile dementia, metabolic neuropathies, HIV infection, renal failure, as well as other clinical conditions have been shown to benefit from L-carnitine supplementation (Evangeliou and Vlassopoulos 2003).

In cancer patients, low serum levels of carnitine have been reported (Malaguarnera et al. 2006; Vinci et al. 2005; Hockenberry et al. 2009). In experimental cachexia, carnitine supplementation significantly lowered the tumour-induced increase in plasma triacylglycerol concentration, as well as cytokine production (Winter et al. 1995). Because cachexia is currently accepted to be a chronic inflammatory condition, eventual effects of carnitine on parameters related with inflammation would also recommend its use to reduce the symptoms of this syndrome.

Based on these promising data, carnitine supplementation has been tested in preliminary studies in human cachexia, resulting in improved fatigue and quality of life (Cruciani et al. 2004, 2006; Gramignano et al. 2006; Graziano et al. 2002; Mantovani et al. 2008). However, none of these studies investigated the possible effects of carnitine supplementation on liver lipid metabolism during cancer-associated cachexia.

Bearing in mind the reported positive effects of L-carnitine supplementation on liver steatosis described by Bykov et al. (2003) and Romano et al. (2008), we examined whether L-carnitine supplementation would affect the deleterious cancer cachexia-related alterations of the CPT system and other parameters of lipid metabolism. For that purpose, we adopted the Walker 256 carcinosarcoma, the most widely studied animal model of cancer cachexia, know to induce most of the clinical and metabolic symptoms also present in humans.

The results show that carnitine supplementation was effective in reversing the cachexia-associated disruption in the examined parameters.

Materials and methods

Animals and experimental design

Forty-eight animals were divided into four groups: control (N), control supplemented with L-carnitine (CN), tumour-bearing (TB) and tumour-bearing supplemented with L-carnitine (CTB).

Male Wistar rats, weighing between 200 and 250 g, consuming water and commercial chow (NUVILAB 1, Nuvital, Brazil) ad libitum, were kept under a light/dark cycle of 12 h, temperature of 22 ± 2°C and relative humidity at 60%, in the Animal House of the Cell and Developmental Biology Department of the Institute of Biomedical Sciences, University of São Paulo. All experimental procedures were approved by the Committee for Ethics in Animal Experimentation of the Institute of Biomedical Sciences (Protocol no. 041/2005), in accordance with the Brazilian College for Animal Experimentation (COBEA).

Rats were supplemented by intragastric administration of 1 g kg body weight^{-1} day^{-1} of L-carnitine (ICN-USA) diluted in 1.0 mL of saline, always at the same time of the day, for 28 days. For the tumour-bearing animals, supplementation started 14 days before tumour cell inoculation. N and TB received saline in substitution to L-carnitine. For the induction of cachexia, Walker 256 tumour cells (2×10^7 cells in 1.0 mL of 0.9% NaCl) were injected subcutaneously into the right flank of the rats (Seelaender et al. 1996). Experimental procedures were carried out on the 14th day following tumour inoculation (Seelaender et al. 1996). After killing (decapitation without anaesthesia), the tumour was excised and weighted, and the plasma and the whole liver, collected.

Assessment of body and tumour weight, and of food intake

For all animals, body weight was assessed three times a week and weight gain calculated. Food intake was assessed daily. The weight of the tumour, liver, white adipose tissue (retroperitoneal and epididymal fat pads) and brown adipose tissue was measured at sacrifice, i.e., 14 days after inoculation. Weight gain and relative tissue weight were calculated after subtracting the weight of the tumour from the value obtained for total body weight.

Enteral absorption and liver incorporation of ^{3}[H]-L-carnitine

N ($n = 6$) and TB ($n = 6$) rats received intragastrically 1.0 mL of saline with 20 μCi of ^{3}[H]-L-carnitine. After 6 h, the animals were killed and the intestinal tract and liver removed. The plasma was obtained after centrifugation. Assessment of enteral absorption of ^{3}[H]-L-carnitine: the intestinal tract and faeces were homogenised with 0.5 mL of KOH 30% (*w*/*v*) and incubated at 70°C for 30 min. Hydrogen peroxide (130 V), HCl and scintillation fluid were added to the samples. ^{3}[H]-L-carnitine absorption was determined by subtracting the radioactivity remaining in the intestinal tract from the total amount given. Measurement of total radioactivity in the liver: to 300 mg of tissue 0.5 ml of NaOH (1 N) was added and the samples were incubated for 30 min at 70°C. 100 μL of the sample was then pipetted into a scintillation vial, after the addition of two drops of hydrogen peroxide (130 V), 100 μL of HCI, and scintillation fluid. The radioactivity found in the liver was subtracted from the total absorbed by the animal, yielding a measurement of the amount of the incorporated labelled L-carnitine.

Reverse transcription and real-time polymerase chain reaction

Total RNA was obtained from aliquots of 100 mg of liver by Trizol reagent extraction according to the manufacture's instructions. RNA concentration was determined spectrophotometrically (UVmini 1240, Shimadzu). The reverse transcriptase and real-time polymerase chain reaction methods were used for the estimation of the concentration of CPT I and CPT II, microsomal triglyceride transfer protein (MTP), liver fatty acid-biding protein (L-FABP), fatty acid translocase (FAT/CD36), peroxisome proliferator-activated receptor-alpha (PPAR-alpha) and organic cation transporter 2 (OCTN2) mRNA. Complementary DNA synthesis was carried out using a 33 μL assay mix containing 3 μg total RNA, 10 U RNAse inhibitor, 2 μL random primers, 2 μL dNTP (10 nmol), 2 μL dithiothreitol, 10 U M-MLV reverse transcriptase and 4 μL 10× reaction buffer (100 mM Tris-HCl, 500 mM KCl; 150 mM $MgCl_2$ in nuclease-free water) (Invitrogen), in a final volume of 25 μL, 5 μL cDNA was mixed with 2× SYBR Green PCR master mix (Applied Biosystems) and primers (Invitrogen). The sequences for the primers were obtained from Genebank (Table 1). Quantitative real-time PCR was performed with an ABI 7300 Real-Time PCR Systems (Applied Biosystems). The mRNA levels were determined by a comparative C_t method. For each sample, a ΔC_t value was obtained by subtracting GAPDH values from those of the gene of interest. The average ΔC_t value of the control group was then subtracted from the sample to derive a $\Delta - \Delta C_t$ value. The expression of each gene was then evaluated by $2^{-(\Delta - \Delta Ct)}$.

Measurement of CPT I and II maximal activity

To isolate the mitochondria, the livers were minced with scissors and homogenised manually in isolation buffer (220 mM Mannitol, 70 mM sucrose, 2 mM Hepes, 0.1 mM EDTA, pH 7.4). The homogenate was filtered and centrifuged twice at 1,000 g (12 min). The supernatant was then centrifuged twice at 10,000 *g* for 15 min. The isolated mitochondria were suspended in a buffer consisting of 0.15 mM KCl and 5 mM Tris HCl (pH 7.5), centrifuged (10,000 *g*, 15 min), resuspended in 10 mM phosphate buffer (pH 7.5), frozen in liquid nitrogen and thawed. Samples were then ultracentrifuged (100,000 *g*, 1 h—Hitachi). The resulting pellet was suspended in phosphate buffer to which Tween 20 (1% *w*/*v*) had been added, and stirred on ice for 30 min, to separate CPT I (membrane bound) from CPT II. Another ultracentrifugation followed, after which the fractions containing CPT I (pellet) and CPT II (supernatant) were obtained.

CPT activity was measured with the method of Bremer (1981). The assay medium consisted of 60 mM KCl, 40 mM Mannitol, 20 mM Hepes, 0.15 mM EGTA, 1.5 mM KCN, fat-free bovine serum albumin (0.5%), 42 μM palmitoyl CoA, 0.35 mM carnitine (0.6 Ci ^{3}H-methylcarnitine) and approximately 0.03 mg of the

Table 1 Sequences of primers used for real-time PCR analysis

Gene (NCBI Genbank)	Forward primer	Reverse primer
CPT I (NM_031559)	CCGAGCTCAGTGAGGACCTA	ATCTGTTTGAGGGCTTCGTG
CPT II (NM_012930)	GAGCCCCTAGTAGGCCCTTA	AGGCTTCTGTGCATTGAGGT
MTP (NM_001107727)	GCCGTTATCGTGACTTGGAT	GTCACACAACTGGCCTCTCA
L-FABP (NM_012556)	ACCTCATTGCCACCATGAAC	CTTCCCTTCATGCACGATTT
CD36 (NM_031561)	CTGTGAATTGGCAAGAAGCA	TCTCAATGAGCAGGTCTCCA
PPAR-alpha (NM_013196)	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA
OCTN2 (NM_019269)	TCTGAGAGCTGTCCCGAAAT	AGCAGCTGGAGCAAGAGAAC
GAPDH (NM_017008)	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

isolated enzyme fraction or distilled water (blanks). The final volume of the assay mixture was 0.5 mL, and the pH 7.3. The assay was stopped by the addition of 1.5 mL of 7% perchloric acid, and the acylcarnitine formed was extracted with *n*-butanol. CPT activity is expressed as nmol/min per mg of protein in the isolated enzyme fraction.

Measurement of plasma and liver neutral lipid content

Blood was collected into tubes containing EDTA and plasma samples were obtained after centrifugation. Plasma cholesterol and triacylglycerol were quantified using commercial kits (Labtest®, Brazil). Liver triacylglycerol content was assessed with the method described by Folch et al. (1957).

Electron microscopy

The ultrastructure of the hepatocytes was studied after fixation of the samples with 3% glutaraldehyde, followed by post-fixation with 1% osmium tetroxide. Successive dehydration steps with ethanol (70, 90, 100%) and acetone were followed by embedding in Spurr. Ultra-thin slices were contrasted with uranyl acetate and lead citrate for 5 min. The samples were observed in a transmission electron microscope (Jeol 10101).

Statistical analysis

The data obtained were compared using two-way ANOVA, and partitioned into main effects (control vs. supplemented group effects and tumour bearing vs. control group effects). When a significant F value was found by two-way ANOVA, a Tukey post hoc test was performed to demonstrate all pairwise multiple comparisons between the means. The 0.05 probability level was considered to indicate statistical significance. The data are presented as mean ± SE.

Results

Assessment of body and tumour weight and food intake

Body weight gain during the experimental period was decreased (after tumour tissue weight was subtracted) in TB, when compared with N (63.67 ± 3.05 vs. 92.34 ± 7.45 g, respectively; $p < 0.05$). L-Carnitine supplementation allowed a pattern of body weight gain similar to that of N (90.57 ± 3.49 g). Furthermore, L-carnitine supplementation reduced tumour weight (17.09 ± 3.46 g TB vs. 6.50 ± 1.79 CTB, $p < 0.01$, $n = 8$ and $n = 12$, respectively).

Cancer cachexia had no effect on total or relative weight (percentage of total body weight) of the white adipose tissue depots (epididymal and retroperitoneal), nor upon brown adipose tissue weight. Relative liver weight, however, increased in TB when compared with N (3.02 ± 0.12 vs. 2.75 ± 0.06%, respectively; $p < 0.05$). No differences were found regarding total mean food intake during the experimental period.

Enteral absorption and liver incorporation of ^{3}[H]-L-carnitine

The enteral absorption of the supplemented ^{3}[H]-L-carnitine was lower in TB ($n = 6$; $p < 0.01$), whereas incorporation in the liver was increased in the same animals compared with N ($n = 6$; $p < 0.05$) (Table 2). Labelled carnitine concentration in the plasma did not differ between groups, corresponding to 0.206 ± 0.172 and 0.546 ± 0.264% ($p > 0.05$) from the total amount absorbed after 6 h of the administration, for N and TB, respectively.

Analysis of gene expression

Because carnitine shows a direct effect on the regulation of gene expression, we analysed L-FABP, MTP, FAT/CD36 and PPAR-alpha mRNA expression, all of which proteins related with the main pathways in lipid metabolism (Fig. 1). L-FABP, FAT/CD36 and PPAR-alpha gene expression showed no difference among the groups. However, cancer cachexia decreased MTP gene expression (67%, $p < 0.001$), when compared with N. This parameter was restored to control values when animals were supplemented with L-carnitine ($p < 0.05$, TB vs. CTB). In a control group, in which supplementation was initiated at the same time of tumour cell injection, we found that MTP expression was equally enhanced by L-carnitine supplementation ($p < 0.01$).

The gene expression of OCTN2, an important carnitine transporter, was also examined. However, there were no differences between groups ($n = 1.0 \pm 0.13$; TB = 0.64 ± 0.21; CTB = 1.23 ± 0.13; CN = 0.99 ± 0.28; $p > 0.05$).

Table 2 Enteral absorption of ^{3}[H]-L-carnitine (as percentage of the total ingested bolus) and liver incorporation (as a percentage of the total amount absorbed by the intestine)

	Enteral absorption (%)	Liver incorporation (%)
N	60.81 ± 3.08	2.46 ± 1.51
TB	33.61 ± 5.98[a]	6.78 ± 1.00[b]

Results are means ± SE ($n = 6$ per group)

N control, *TB* tumour bearing

[a] $p < 0.01$

[b] $p < 0.05$

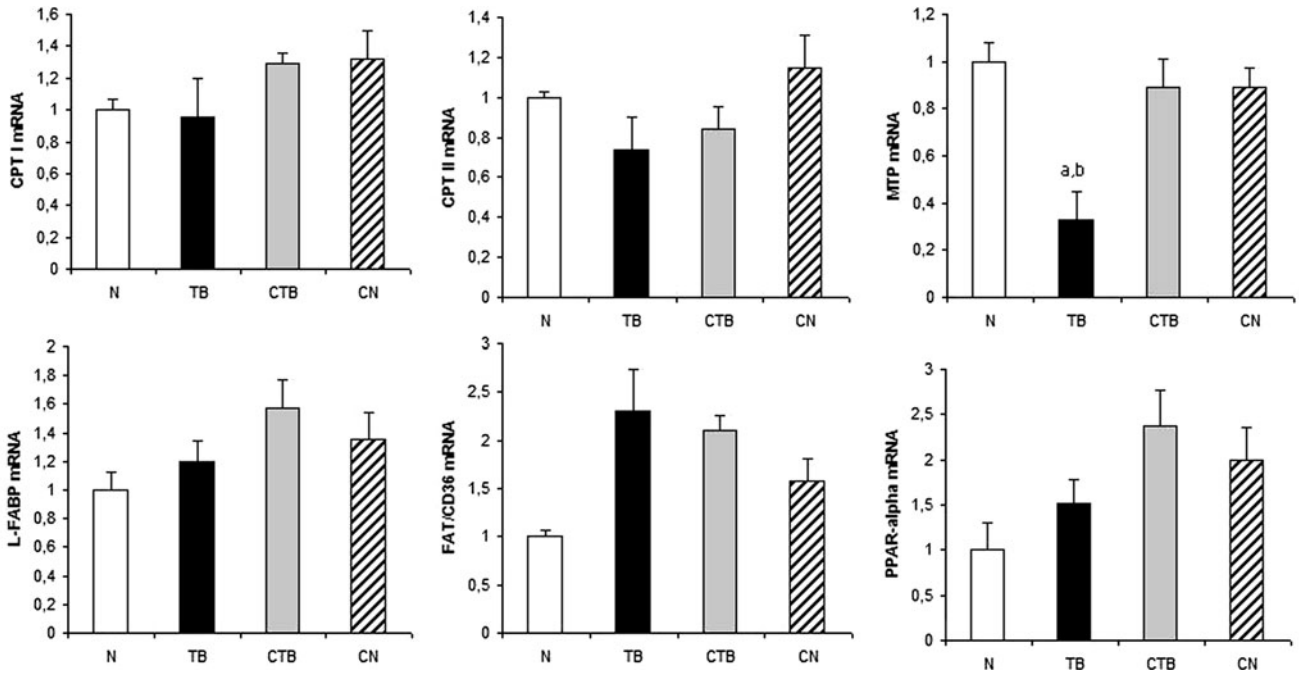

Fig. 1 mRNA expression of lipid metabolism-related proteins in the liver of control (*N*), control supplemented with L-carnitine (*CN*), tumour bearing (*TB*) and tumour-bearing supplemented with L-carnitine (*CTB*) groups. mRNA levels of target genes were normalised to GAPDH. Values are expressed as mean ± SE presented as fold changes relative to controls (TB, $n = 7$; other groups, $n = 5$). Significance at $^{a}p < 0.001$ compared with N. Significance at $^{b}p < 0.05$ compared with CTB

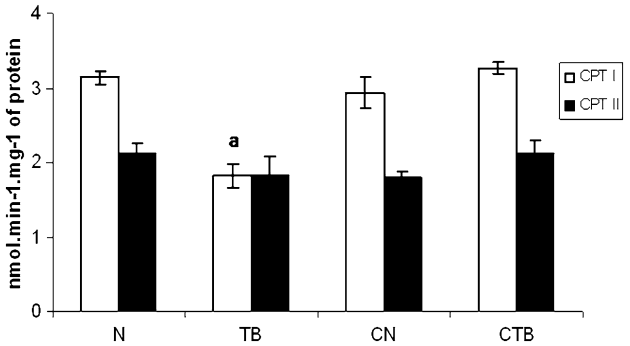

Fig. 2 Maximal activity of liver carnitine palmitoyltransferases I and II. Control (*N*), control supplemented with L-carnitine (CN), tumour bearing (*TB*) and tumour-bearing supplemented with L-carnitine (*CTB*). Data are mean ± SE ($n = 4$). $^{a}p < 0.01$ for comparison with all groups

Measurement of CPT I and II activity and mRNA expression

We examined the maximal activity and the gene expression of CPT I and II (Figs. 1, 2, respectively). TB showed reduced maximal activity of CPT I (42%, $p < 0.01$) in relation to N. L-carnitine supplementation prevented the decrease in CPT I activity, which was enhanced by 1.4 fold ($p < 0.01$), when compared with TB. The maximal activity of CPT II and the gene expression of CPT I and II did not differ among the groups.

Table 3 Liver and plasma triacylglycerol content

	Liver (mg/g)	Plasma (mg/dL)
N	5.74 ± 0.68 ($n = 9$)	44.10 ± 3.07 ($n = 11$)
CN	4.04 ± 0.42 ($n = 8$)	49.10 ± 4.20 ($n = 10$)
TB	14.37 ± 1.27^{abc} ($n = 9$)	145.69 ± 13.20^{d} ($n = 11$)
CTB	6.66 ± 0.63 ($n = 9$)	58.66 ± 5.38 ($n = 11$)

Data are mean ± SE

N control, *CN* control supplemented with L-carnitine, *TB* tumour bearing, *CTB* tumour-bearing supplemented with L-carnitine

[a] $p < 0.01$ compared with N

[b] $p < 0.001$ compared with CN

[c] $p < 0.05$ compared with CTB

[d] $p < 0.05$ compared with all groups

Assessment of plasma triacylglycerol and cholesterol and liver triacylglycerol content

Plasma triacylglycerol levels were increased in TB ($p < 0.01$), while after L-carnitine supplementation the levels were comparable to N (Table 3). Plasma cholesterol levels were not affected by cachexia, neither by L-carnitine supplementation.

Liver triacylglycerol content increased by approximately 150% in TB compared with N ($p < 0.01$), and L-carnitine supplementation significantly reduced this parameter in CTB (Table 3).

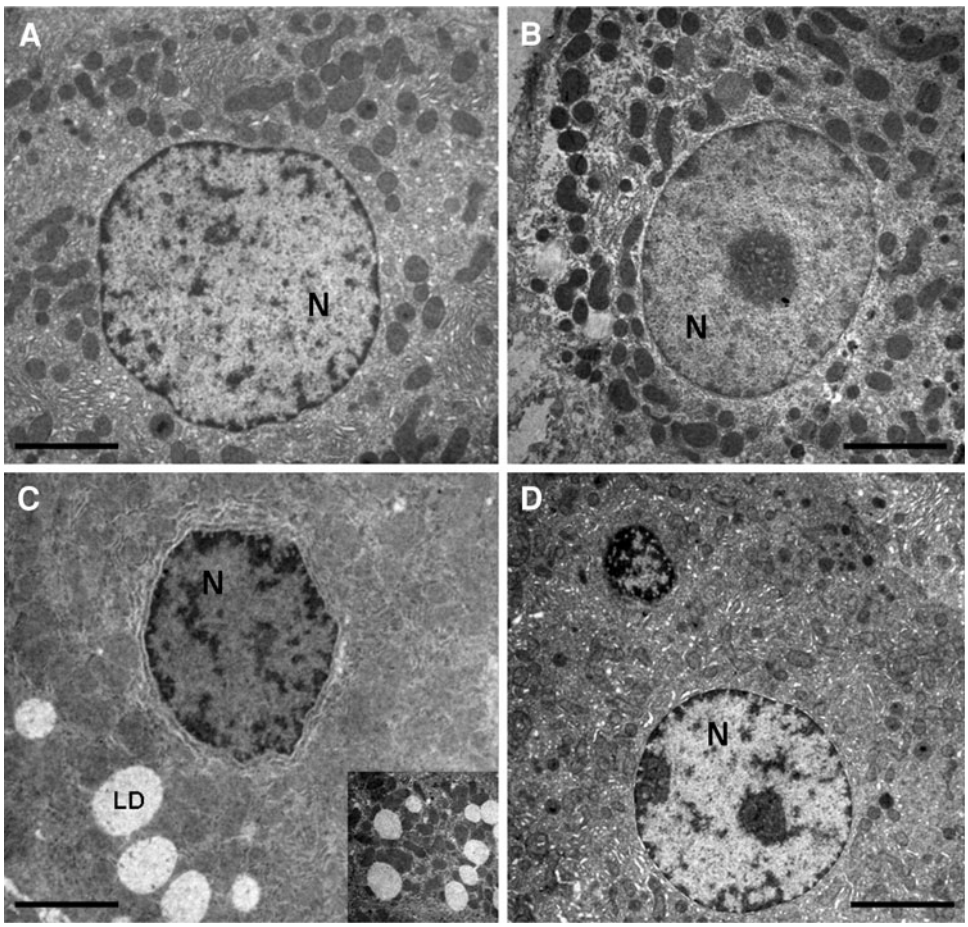

Fig. 3 Ultrastructural aspect of hepatocytes from control (**a**), control supplemented with L-carnitine (**b**), tumour-bearing (**c**) and tumour-bearing supplemented with L-carnitine (**d**) rats. Note the presence of lipid droplets in the cytoplasm of tumour-bearing rats. *LD* lipid droplets, *N* nucleus. *Bars* 1 μm

Electron microscopy

Figure 3 illustrates the main morphologic characteristics of the hepatocytes from N (A), CN (B), TB (C) and CTB (D). The ultrastructural analysis of cells showed that, in all groups, the nucleus was rounded with predominance of euchromatin. Abundant rough and smooth-surfaced endoplasmic reticulum was found, as well as many rounded mitochondria. In N, CN and CTB hepatocytes, the mitochondria were highly electrondense, in contrast to what was found in TB. An abundant number of lipid droplets were found in the cytoplasm of TB hepatocytes, always in intimate contact with mitochondria, as also previously reported by our group (Seelaender et al. 1996).

Discussion

Cancer cachexia is a multifaceted syndrome characterised, among many symptoms, by marked abnormalities in lipid metabolism (Argilés et al. 1997; Barber et al. 1999). Several lines of evidence suggest that the liver plays a crucial role in the establishment and maintenance of the cachectic state (Kazantzis and Seelaender 2005; Martignoni et al. 2009). We previously demonstrated disrupted liver lipid metabolism in experimental cachexia, leading to reduced oxidation of fatty acids and decreased incorporation into very low density lipoproteins (VLDL) (Seelaender et al. 1996, 1998; Kazantzis and Seelaender 2005; Lira et al. 2008). We now demonstrate that L-carnitine supplementation is able to counteract cachexia-related hypertriglyceridemia and steatosis, by modulating CPT I activity and MTP gene expression.

In cachectic tumour-bearing rats, the liver and plasma triacylglycerol levels are consistently increased and in the Walker 256 model, the same features were found. In cachectic rats, fat accumulation in the liver is associated with the decrease in mitochondrial CPT activity, as we have previously reported (Kazantzis and Seelaender 2005). Carnitine palmitoyltransferase I is generally accepted as the main site of control of long-chain fatty acid oxidation, and the reduction of activity leads to lipid accumulation in the hepatocyte cytoplasm (Kang et al. 1990; Stefanovic-Racic et al. 2008). In addition, we have also previously shown that the liver of cachectic rats uptakes exogenous long-chain fatty acid more avidly (104%) than control rat liver (Seelaender et al. 1996). Therefore, the reduction in the oxidation of this substrate, along with increased

incorporation, appears as direct causes of steatosis (Letteron et al. 2003). However, long-chain fatty acid partition in the hepatocyte comprises as well, the secretion in VLDL. We demonstrated that this process is also compromised in the cachectic rats (Lira et al. 2008).

In cancer patients with cachexia, low-serum carnitine levels have been often reported (Vinci et al. 2005; Malaguarnera et al. 2006), and this change has been suggested to play an important contributory role in the development of cachexia (Vinci et al. 2005). In addition, many studies demonstrate that L-carnitine supplementation yields clinical benefits to cancer patients (Cruciani et al. 2004; Gramignano et al. 2006; Laviano et al. 2006; Hoang et al. 2007; Mantovani et al. 2008). It was our aim to examine the effect of L-carnitine supplementation upon the disrupted lipid pathways in the liver of tumour-bearing rats.

It is generally thought that reduced serum carnitine levels are the likely result of decreased availability of carnitine in the diet or altered endogenous biosynthesis. Quite surprisingly, no data are available on intestinal L-carnitine transport in either experimental or human cachexia. Disrupted intestinal absorption of carnitine could, per se, be of marked relevance to the decreased plasma concentration found in cachectic patients. Our study demonstrates for the first time, that enteral ^{3}[H]-L-carnitine uptake is reduced in cachectic rats when compared with control animals.

Reduced absorption is not necessarily related with diminished tissue concentration of carnitine, and tumour-bearing rats showed greater ^{3}[H]-L-carnitine liver incorporation than control animals. A similar pattern was previously demonstrated by our group in regard to incorporation of an exogenous bolus of ^{14}[C]-oleate, when a greater fraction (104%) of ^{14}[C]-oleate was found in cachectic rat liver in comparison with the control, despite reduced enteral absorption in the former (Seelaender et al. 1996). It is tempting to speculate that the higher liver incorporation of L-carnitine could represent a compensatory mechanism to offset the decreased absorptive capacity of the small intestine and the possible reduction of endogenous biosynthesis. Indeed, tumour cells are known to avidly uptake methionine (Nerini-Molteni et al. 2000), which is the precursor of L-carnitine, thereby possibly decreasing the host's L-carnitine synthesis capacity. Therefore, tumour growth may mimic the fasting state when the supply of L-carnitine and its precursors is diminished and L-carnitine incorporation by the perfused rat liver is enhanced (Luci et al. 2008).

Distribution of carnitine within the body and intracellular homeostasis of carnitine are controlled by the novel organic cation transporters (OCTN). Three OCTN have been identified so far: OCTN1, OCTN2 and OCTN3; however, OCTN2 is the most physiologically important carnitine transporter due to its high binding affinity for carnitine (Kispal et al. 1987; Wen et al. 2010). Some conditions, as well as starvation, increase the concentration of carnitine in the liver, due to increased uptake of carnitine from blood into the liver. In the present study, we showed higher carnitine incorporation by the liver of TB, which was not associated with increased OCTN2 gene expression. Nevertheless, even if the mRNA expression is unchanged it is not possible to discard increased transport as determined by increased flux, a measurement we did not carry out. The lack of modification regarding OCTN2 expression is possibly linked to the absence of alterations on PPAR-alpha mRNA expression presently observed, since it has been shown that OCTN2 is a direct target gene of this transcription factor, at least in the mouse (Kispal et al. 1987). Another possibility is that different transporters are involved in carnitine transport in the liver, such as the one described by Scaglia et al. (1999) in a hepatocyte lineage. It is tempting to speculate that carnitine incorporation was augmented in the liver as an attempt to prevent steatosis as induced by disrupted VLDL assembly and reduced CPT I activity.

Liver triacylglycerol content was presently found to be decreased after L-carnitine supplementation as assessed by biochemical and morphological analysis. Similar results were reported for normal rats (Clouet et al. 1996), and in clinical and animal models of hepatic steatosis after L-carnitine supplementation (Mantovani et al. 2008; Bykov et al. 2003).

Because L-carnitine is essential for lipid oxidation, several groups have studied the potential effects of its supplementation on the enzymatic activity of the CPT system. Although it is apparently a promising idea, conflicting results have been found. Increases in tissue carnitine concentration occur only after chronic L-carnitine supplementation (Bernard et al. 2008; Fischer et al. 2009). In addition, increased liver carnitine content has been shown to influence mitochondrial fatty acid oxidation (Cook et al. 2000). However, most of studies demonstrate that L-carnitine supplementation does not affect liver and muscle CPT system in the absence of disease (Negrao et al. 1987). This observation is consistent with the present finding that L-carnitine supplementation did not affect the maximal activity of CPT I and II in the liver of non-tumour bearing rats (CN). However, when the supplementation was given to tumour-bearing animals, CPT I activity was restored to normal values. This result may partially explain the decrease in triacylglycerol liver content presently reported.

Measuring CPT activity in an isolated system presents the advantage of discarding the influence of alterations in malonyl CoA concentration and in CPT I sensitivity to malonyl CoA-induced inhibition (Ramsay et al. 2001). In contrast with the analysis of catalytic activity, no difference was observed in CPT I and II mRNA expression among groups. Therefore, in our model, short-term

regulation of enzyme activity was not due to its interaction with malonyl CoA, neither to alterations concerning CPT I concentration. We have previously reported lower activity of CPT due to the appearance of isoforms (Seelaender et al. 1998), and therefore, we may hypothesise that L-carnitine supplementation prevented the induction of isoforms with decreased substrate affinity. In addition, L-carnitine supplementation may protect hepatocytes of tumour-bearing rats from oxidative damage, since free fatty acids are able to produce damage on cell and mitochondrial surfaces (Bremer 1981). In addition, we have recently published a study in which we show that increased CPT activity is found in association with reduction in the enhancement in liver prostaglandin E2 content which is related with cachexia (Lira et al. 2010). Roy et al. (2009) found decreased PGE2 production by Caco-2 cells following treatment with carnitine. It is therefore, possible that the effect of supplementation on CPT was exerted through this mechanism.

L-Carnitine is directly involved in the regulation of gene expression, but may also exert its effects via the modulation of intracellular fatty acid concentration (Mitsuyoshi et al. 2009). Cook et al. (2000) previously demonstrated that increased hepatic carnitine content affects mitochondrial fatty acid oxidation through PPAR-alpha mediation. Because no differences were found for PPAR-alpha expression among the groups, we suggest that is more likely that supplementation acted directly. However, as we did not examine PPAR-alpha/RXR biding to DNA, we may not discard the hypothesis that this parameter was affected by the protocol.

Hypertriglyceridemia, a hallmark of cachexia (Argilés et al. 1997), could be the consequence of the impairment of many different metabolic pathways. Another important pathway contributing to steatosis, in addition to diminished oxidation, is the disruption of VLDL assembly and secretion. These processes are dependent on MTP and apolipoprotein B (apoB), whose expression is decreased in the herein adopted model of cachexia (Lira et al. 2008). L-Carnitine supplementation was able to counteract the decrease in MTP mRNA expression in tumour-bearing rats. Both TNF-alpha and IL-1 have been shown to reduce in vivo liver MTP mRNA levels, whereas incubation with IL-6 and IL-1 reduces MTP levels in HepG2 cells (Navasa et al. 1998). Bearing this in mind, it is conceivable that L-carnitine modulated MTP gene expression in tumour-bearing rats due to its capacity to reduce the levels of proinflammatory cytokines. The anti-inflammatory effect of L-carnitine supplementation is well documented (Savica et al. 2005), and has been exploited with clinical benefits in senile dementia, metabolic neuropathies, HIV infection, tuberculosis, myopathies, cardiomyopathies, renal failure and anaemia (Evangeliou and Vlassopoulos 2003). Consistent experimental and clinical evidence suggests that L-carnitine may directly modify cytokine response. In surgical and in HIV-positive patients, L-carnitine administration lowered the circulating levels of cytokines (De Simone et al. 1993; Delogu et al. 1993). Similarly, in rats exposed to LPS or methylcholanthrene, L-carnitine treatment blunted the increase in serum TNF-alpha (Winter et al. 1995), whereas dietary supplementation with L-carnitine of ethanol-treated rats inhibited TNF-alpha production by Kupffer cells (Bykov et al. 2003).

Four weeks of L-carnitine supplementation decreased final tumour weight (by 62%). The exact mechanism by which L-carnitine supplementation inhibits tumour growth is unknown. It has been known for many years that cancer cells activate glycolysis in the presence of adequate oxygen levels (aerobic glycolysis or Warburg effect) (López-Lázaro 2008). Diminished mitochondrial oxidation of acetyl-CoA from glucose and fatty acid breakdown is a hallmark of cancer cell metabolism. Because low mitochondrial respiration also reduces the reactive oxygen species (ROS) burden, tumour cells appear to be particularly well protected from oxidative stress (Wenzel et al. 2005). Redirecting cancer cell metabolism towards a normal phenotype could therefore result in specific apoptosis induction by an increased ROS production (Wenzel et al. 2005). It is, nevertheless, unlikely that L-carnitine supplementation, by reducing tumour growth, acted indirectly improving liver lipid metabolism, since it is clear that the degree of cachexia-associated inflammation is not correlated with tumour size (Bachmann et al. 2008).

Taken together, the present results provide evidence that L-carnitine supplementation favourably acts on hepatic lipid metabolism of cachectic animals, through modulation of enzymatic activity of CPT I and MTP gene expression. Moreover, L-carnitine supplementation allowed normal weight gain in tumour-bearing animals and markedly inhibited tumour growth. We acknowledge that the protocol adopted in this study does not closely resemble the clinical setting in which L-carnitine supplementation is generally started, e.g. after tumour appearance. However, it is important to note that L-carnitine supplementation in control rats did not enhance hepatic lipid metabolism, neither increased the activity/expression of key genes. We therefore believe that pre-treatment with L-carnitine had minimal effects on the examined metabolic pathways. Nevertheless, we chose to start the supplementation protocol before the injection of the tumour cells, because the Walker 256 tumour induces severe cachexia within a markedly short period and animals usually die spontaneously between the 14th and 15th day after inoculation. Therefore, we feared that we would not to be able to increase L-carnitine plasma and tissue content rapidly enough as to perceive its effects if we started both

protocols on the same day. The fact that we found reduced enteral absorption of the amine in TB also contributed to this view. A group of rats initiating supplementation on the same day of tumour injection showed increased MTP gene expression, but no significant modification of the other studied parameters, as expected. Thus, although we do recognise that in patients, supplementation will start after diagnosis, we may affirm that the short interval in this model renders it impossible to mimic precisely what occurs in human treatment. Nevertheless, it is possible to state that L-carnitine supplementation, offered for an interval that allows the appearance of significant differences in its plasma and tissue concentration and enables regulation of gene expression, will improve lipid metabolism in the liver.

In summary, although further experimental and clinical studies are needed, these data support the potential therapeutic use of L-carnitine in the treatment of cancer cachexia by providing the mechanistic rationale.

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