Exercise Training Reduces PGE_2 Levels and Induces Recovery from Steatosis in Tumor-bearing Rats

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Key words
- cachexia
- exercise training
- CPT system
- PGE_2 levels

Abstract

The effects of endurance training on PGE_2 levels and upon the maximal activity of hepatic carnitine palmitoyltransferase (CPT) system were studied in rats bearing the Walker 256 carciosarcoma. Animals were randomly assigned to a sedentary control (SC), sedentary tumor-bearing (ST), exercised control (EC), and as an exercised tumor-bearing (ET) group. Trained rats ran on a treadmill (60% VO_2 max) for 60 min/day, 5 days/week, for 8 weeks. We examined the mRNA expression (RT-PCR) and maximal activity (radioassay) of the carnitine palmitoyltransferase system enzymes (CPT I and CPT II), as well as the gene expression of fatty-acid-binding protein (L-FABP) in the liver. PGE_2 content was measured in the serum, in tumor cells, and in the liver (ELISA). CPT I and CPT II maximal activity were decreased ($p<0.01$) in ST when compared with SC. In contrast, serum PGE_2 was increased ($p<0.05$) in cachectic animals as compared with SC. In the liver, PGE_2 content was also increased ($p<0.05$) when compared with SC. Endurance training restored maximal CPT I and CPT II activity in the tumor-bearing animals ($p<0.0001$). Exercise training induced PGE_2 levels to return to control values in the liver of tumor-bearing training rats ($p<0.05$) and decreased the eicosanoid content in the tumor ($p<0.01$). In conclusion, endurance training was capable of reestablishing liver carnitine palmitoyltransferase (CPT) system activity associated with decreased PGE_2 levels in cachectic tumor-bearing animals, preventing steatosis.

Introduction

Cancer cachexia is a paraneoplastic syndrome, which is associated with poor prognosis and decreased survival. Among its symptoms there is marked disruption of lipid metabolism. We have shown [1–3] that liver fatty acid oxidation capacity and ketone body production are decreased in tumor-bearing rats, while hepatocyte oleate incorporation is increased and VLDL lipid fraction composition is different from that of nontumor-bearing animals. Progression of cancer cachexia is associated with humoral changes, including increase in local prostaglandin E_2 (PGE_2) production by Kupffer cells and high circulating levels of the eicosanoid [4]. All these modifications result in liver steatosis.

The main step in the control of long-chain fatty acid (LCFA) oxidation and ketone body synthesis comprises the entry of the substrate into mitochondria, which requires the contribution of a membrane-associated transport complex, involving 2 enzymes, CPT I and II, CPT II and a transporter, carnitine-acylcarnitine translocase (CACT) [5,6]. The maximal activity of both enzymes was found to be decreased [3] in the liver of cachectic Walker 256 tumor-bearing rats. In vivo treatment of cachectic animals with indomethacin was shown [1] to prevent the decreased CPT system transport capacity.

Cyclooxygenase inhibitors attenuate cachexia both in patients and animal models [7]. Pharmacological therapy may nevertheless induce side effects, and NSAID (nonsteroidal anti-inflammatory drugs), in particular, have been associated with several deleterious symptoms [8].

Exercise, on the other hand, presents itself as a noncollateral effect inducing strategy in the treatment of chronic inflammatory diseases [9,10]. Previous studies by others [10,11] and by our own group [9] showed that moderate exercise training is able to prevent fat accumulation in the liver (hepatic steatosis). The mechanisms underlying this effect are related with changes in hepatocyte lipid metabolism towards normalization of lipid uptake, and enhancement of lipid oxidation as well as VLDL-TAG secretion regulation.

received 28.03.2010
accepted 29.09.2010

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DOI http://dx.doi.org/10.1055/s-0030-1267949
Published online: 2010
Horm Metab Res

ISSN 0018-5043

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We therefore hypothesized that the chronic exercise-induced changes in liver lipid metabolism could be associated with an eventual effect on PGE_2 levels. Since PGE_2 production is clearly regulated by TNF-α (tumor necrosis factor alpha), a cytokine whose concentration is markedly increased both locally and systemically in cachexia [12], we also addressed the effect of the training protocol on TNF-α expression.

Materials and Methods

The Biomedical Sciences Institute/USP Ethical Committee for animal research approved all of the adopted procedures, which were carried out in accordance with the ethical principles stated by the Brazilian College of Animal Experimentation, protocol No. 041/2005.

Animals

Male adult Wistar rats (150–200 g), obtained from the Institute of Biomedical Sciences, University of São Paulo, were maintained under a 12-h light/12-h dark cycle (lights on at 06:00 h), and controlled temperature conditions (23 ± 1 °C), receiving water and food (commercial chow, Nuvilab1, Nuvital, Brazil) ad libitum.

Experimental design

The rats were randomly assigned to either a sedentary or an exercised group. The animals were divided into 4 groups: sedentary control (SC, n = 10), sedentary tumor-bearing (ST, n = 10) or, exercised control (EC, n = 8), and exercised tumor-bearing (ET, n = 8). The trained groups ran for 2 weeks in order to adapt to the treadmill. After that period, they were submitted to the 8-week training protocol (described below). At the end of the 6th week of training, Walker 256 tumor cells were inoculated in ST and ET. After the fourteenth day, all the animals were killed by decapitation, without anesthesia.

Training protocol

The rats were submitted, as described by Lira et al. [9], to a pretraining period of 2 weeks, during which they ran progressively from 15 to 60 min at 10 m/min. During the following training period of 8 weeks (training 5 d/week), animals exercised on a motorized treadmill (Enlaup, São Paulo, Brazil) at 24 °C and 80% humidity. Running velocity was increased to 20 m/min in the last 2 weeks, and intensity maintained between 60 and 65% VO_2 max, as determined periodically in an Oxymax Columbus System (Columbus Instruments, Columbus, OH, USA). The sensor response time was 4 s at 80 ml/min flow; the repeatability was ± 0.01% O_2 at constant temperature and pressure, and the drifty was lower than 0.01% O_2/h. Due to the ready adaptation of the animals to the treadmill, and also due to the fact that exercise sessions were performed during the period of activity of the animals, no reinforcement was required. After a resting period of 24 h from the last workout session, animals were sacrificed.

VO_2 max determination

VO_2 max was determined by having each rat perform a maximal exercise test adapted from Lira et al. [9] The parameters were measured using the Oxymax gas analyzing system for small animals (Columbus Instruments). The test was always carried out after a 1-day recovery period. A baseline measurement was taken before the beginning of the training protocol. The volume of the supplied air was 4.5 l/min. The gas analyzer was calibrated with a reference gas mixture before each test. The VO_2 max test protocol involved stepwise increasing of the treadmill speed as follows: after a 15-min period of acclimation, the treadmill was started at 10 m/min, and the speed was incrementally increased 5 m/min every 3 min until the rat reached exhaustion. Exhaustion was defined as spending time on the shocker plate without attempting to reengage the treadmill within 15 s. The highest VO_2 max measured at each workload was taken as a measure of each rat's running economy (VO_2 submax) for that workload, and at the last step, as VO_2 max.

Tumor cell inoculation

Walker-256 carcinosarcoma (2×10^7 cells/rat) cells in 1 ml PBS solution were injected subcutaneously, into the right flank of the rats, according to Seelaender et al. [2] All experiments were carried out on the fourteenth day following tumor cell injection. After sacrifice, the tumor was dissected and weighed.

Analysis of gene expression

Total RNA was obtained from aliquots of 100 mg of liver of the rats by TRIzol® reagent extraction, as previously described [13]. RNA concentration was determined spectrophotometrically (Beckman DU 640, Fullerton, CA, USA). A 33 µl assay mix containing 3 µg RNA, 10 U placental RNAse inhibitor, 2 µl oligo (dt), 2 µl dNTP (10 nmol), 2 µl dithiothreitole, 10 U Moloney-murine leukemia virus reverse transcriptase (Invitrogen, USA), and 4 µl 10× reaction buffer (100 mM TRIS–HCl pH 8.3, 500 mM KCl, 150 mM $MgCl_2$ in nuclease-free water) was used to produce cDNA. The RT-mixture was incubated at 80 °C for 3 min, followed by 21 °C for 10 min, 42 °C for 30 min, and then 99 °C for 10 min. The obtained product (2 µl) was fractionated in 1% agarose and ethidium bromide gel to assess the quality of the reaction. The primers were designed with regard to the published Genebank sequences.

Real-time PCR

Primer sets for rat proteins above were designed using Primer Express software v2.0 (Applied Biosystems, Foster City, CA, USA). The results for mRNA concentrations are expressed as ratio over GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which was amplified as house keeping gene. For each sample, PCR was performed in duplicate in a 25 µl reaction volume of 5–20 ng of cDNA, 12.5 µl Syber Green Master Mix (Applied Biosystems), and 200 nM of each primer (**Table 1**). PCR analyses were carried out using the following cycle of parameters: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Fluorescence was quantified and analyses of amplification plots were performed with the AB 7300 Sequence Detector System (Applied Biosystems). Results were expressed using the comparative cycle threshold (Ct) method as described by the manufacturer.

Table 1 Primer sequences for real-time PCR mRNA analyses

Target mRNA	PCR Primer sequence 5′→3′
GAPDH	Fwd: ACATCATCCCTGCATCCACT Rev: GGGAGTTGCTGTTGAAGTCA
CPT I	Fwd: CCAAGCTGTGGCCTTCCAGT Rev: GGACGCCGCTCACAATGTTC
CPT II	Fwd: GATAAGCAGAATAAGCACACC Rev: GGAGGAACAAAGCGAATGAGT
FABP	Fwd: TTCTCCGGCAAGTACCAAGTG Rev: CCCAGTGTCATGGTATTGGTG

Measurements of carnitine palmitoyltransferases I and II activities

To isolate the mitochondria [14], the livers were minced with scissors and homogenized manually in isolation buffer (mannitol 220 mM, sucrose 70 mM, Hepes 2 mM, EDTA 0.1 mM, pH 7.4). The homogenate was filtered and centrifuged twice at 1 000 × *g* (12 min). The supernatant was then centrifuged twice at 8 500 × *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 and treated with Tween 20 (1% w/v) and stirred on an ice-bath for 30 min in order to separate CPT I (membrane bound) from CPT II [15]. Another ultracentrifugation followed, after which the fractions containing CPT I (pellet) and CPT II (supernatant) were obtained. CPT activity was measured with the method [14], which was modified by Woeltje et al. [16] 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 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, as described previously Kolodziej et al. [17] CPT activity is expressed as nmol/min per mg of protein in the isolated enzyme fraction.

Protein content analysis

Samples protein content for both maximal carnitine palmitoyltransferase activity and for immunoassays (ELISA) was determined by the method of Bradford et al. [18]

Measurement of PGE_2 and TNF-α concentration

Blood was collected and serum samples were separated after allowing blood to clot on ice. Serum was frozen at −80 °C and stored for analysis. Prostaglandin E_2 was quantified using immunoassays commercial kits (Cayman Chemical Company, Ann Arbor, MI, USA).

Tissue samples (liver and solid tumor) were carefully rinsed in ice-cold 0.9% saline to remove any blood contaminants. Samples were minced in 1 ml/100 mg tissue of ice-cold RIPA buffer (PBS, pH 7.4, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 100 µg/ml PMSF, 30 µl aprotinin, 100 mM $NaVO_4$) and homogenized with a Polytron homogenizer. The homogenate was centrifuged at 4 °C for 20 min at 15 000 × *g*. Clear supernatants were used. Protein concentration was determined by the classical method of Bradford [18]. PGE_2 was determined in tissue extracts samples using specific enzyme immunoassays (Cayman Chemical Company, Ann Arbor, MI, USA), following the manufacturer's instructions. Quantitative assessment of TNF-α protein was carried out with ELISA (DuoSet ELISA, R & D Systems, MN, USA). For TNF-α (DY510), assay sensitivity was found to be 5.0 pg/ml in the range of 31.2–2 000 pg/ml.

Statistical analysis

Data are expressed as means ± SEM. A primary observation indicated that the results showed a normal distribution. Post-training measurements were analyzed by 2-way ANOVA, 2 × 2 design, where the data were partitioned into main effects (sedentary vs. exercise group effects, A; and tumor-bearing vs. control group effects, B). The interaction effects consisted of A × B. When a significant F-value was found by 2-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 obtained data for sedentary tumor and exercised tumor groups were compared using the *t*-student test. The level of significance of at least $p < 0.05$ was chosen for all statistical comparisons.

Results

Cachexia showed no effect upon CPT I, CPT II, and FABP gene expression in the liver (**Table 2**). The maximal activity of CPT I and II decreased (27%; $p < 0.05$ and 10-fold respectively; $p < 0.001$) in ST when compared to SC (**Table 3**). In contrast, PGE_2 levels in ST were increased in the serum (32%; $p < 0.05$), when compared with SC (**Fig. 1**). PGE_2 levels were also increased in the liver in ST (1-fold; $p < 0.05$), when compared with SC (**Fig. 2**). Endurance training decreased FABP liver gene expression in EC (43%; $p < 0.05$) when compared with SC, but an increase in FABP gene expression (1.2-fold; $p < 0.01$) was observed in ET when compared with EC (**Table 2**). CPT II gene expression in the liver increased in ET (39%; $p < 0.05$ and 54%; $p < 0.01$, respectively), when compared with ST and EC. **Table 3** shows that CPT I maximal activity increased in EC and ET (1.7-fold and 3-fold; $p < 0.001$, respectively), when compared with SC and ST. CPT II maximal activity was augmented in EC and ET

Table 2 CPT I, CPT II, and FABP mRNA in liver

	SC	ST	EC	ET
CPT I	0.94 ± 0.12	0.88 ± 0.18	0.81 ± 0.06	1.12 ± 0.02
CPT II	1.81 ± 0.24	1.31 ± 0.17	1.46 ± 0.23	2.03 ± 0.05$^{\&\#}$
FABP	1.11 ± 0.07	0.91 ± 0.07	0.63 ± 0.08*	1.38 ± 0.13$^{\&\#}$

Results are expressed as mean value ± SEM arbitrary units

SC: Sedentary control; ST: Sedentary tumor; EC: Exercise control; ET: Exercise tumor

* Indicates significant difference ($p < 0.05$) from SC; $^{\&}$ indicates significant difference ($p < 0.05$) from ST, and $^{\#}$ indicates significant difference ($p < 0.05$) from EC

Table 3 Maximal activity of carnitine palmitoyltransferase I and II in the liver of the 4 studied groups (nmol/min/mg protein)

	SC	ST	EC	ET
CPT I	3.66 ± 0.38	2.66 ± 0.30*	9.96 ± 1.02*	10.61 ± 1.62$^{\&}$
CPT II	1.68 ± 0.16	0.16 ± 0.10*	14.79 ± 2.74*	9.25 ± 1.12$^{\&}$

Results are expressed as mean value ± SEM

SC: Sedentary control; ST: Sedentary tumor; EC: Exercise control; ET: Exercise tumor

* Indicates significant difference ($p < 0.01$) from SC and $^{\&}$ indicates significant difference ($p < 0.0001$) from ST

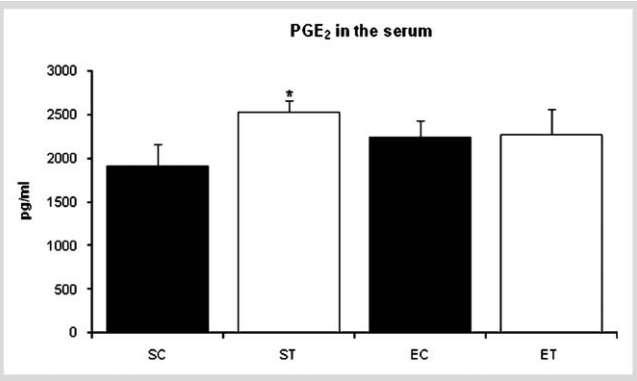

Fig. 1 PGE_2 serum levels of the studied groups. Data are mean SEM. * Indicates significant difference ($p<0.05$) from SC.

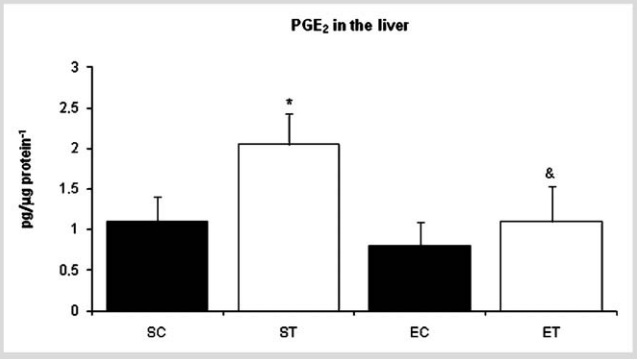

Fig. 2 PGE_2 levels in the liver of the studied groups. Data are mean SEM. * Indicates significant difference ($p<0.05$) from SC; & indicates significant difference ($p<0.05$) from ST.

Table 4 Tumor parameters

	ST	ET
Weight (g)	17.2 ± 2.1	1.90 ± 0.30*
PGE_2 (pg/µg protein^{-1})	4.40 ± 0.74	2.20 ± 0.67*
TNF-α (pg/µg protein^{-1})	3.67 ± 0.86	3.00 ± 0.56

Results are expressed as mean value ± SEM
ST: Sedentary tumor; ET: Exercise tumor
* $p<0.01$ significantly different from ST

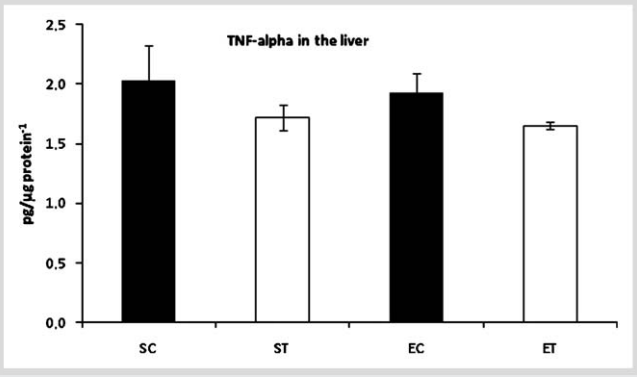

Fig. 3 TNF-α levels in the liver of the studied groups. Data are mean SEM. No differences were found.

(8-fold and 56-fold; $p<0.001$, respectively), in relation to SC and ST. ◦ **Fig. 1** shows no change in PGE_2 serum levels in tumor-bearing exercised animals when compared with ST. However, in the trained groups PGE_2 levels decreased in the liver (44%; $p<0.05$) and in the tumor (90%; $p<0.05$) when compared with the sedentary counterparts (◦ **Fig. 2**, ◦ **Table 4**).

TNF-α protein expression in the liver and tumor was neither modified by cancer cachexia nor by exercise training (◦ **Fig. 3**, ◦ **Table 4**). 8 weeks of endurance training decreased tumor weight in ET (90%; $p<0.01$), when compared with ST (◦ **Table 4**).

Discussion

Cancer cachexia is a chronic inflammatory syndrome characterized by "metabolic chaos". The liver due to its central role in intermediary metabolism, was the focus of the present study. We and others have shown [3,19] that lipid metabolism is markedly disrupted in this organ and the reported changes greatly contribute for the aggravation of cachexia, affecting the organism as a whole.

The chronic endurance exercise protocol herein adopted was able to fully reestablish hepatic LCFA oxidation capacity. The maximal activities of CPT I and II are decreased in cachexia, yet no changes regarding mRNA expression were found [3]. Endurance training affected both the catalytic capacity of the CPT system, the key step in the regulation of LCFA oxidation and the mRNA expression of CPT II [20].

Therefore, it is possible to affirm that the effect of training caused lipid oxidation capacity to be even higher in the trained tumor-bearing group than in control animals. Increased lipid oxidation capacity is associated with the reduction of re-esterification and TAG accumulation in the hepatocyte cytoplasm [3,21].

Indeed, we have shown before [9] that chronic exercise is able to prevent cachexia-related steatosis, as well as to normalize VLDL assembly and secretion in cachexia and in rats submitted to high-fat diet [11]. The mechanisms underlying these effects were, however, not discussed before.

We presently suggest that the decrease in PGE_2 induced by the exercise protocol plays a role in the reestablishment of the oxidative capacity and therefore renders the hepatocyte capable of performing more precise control of LCFA fate. It is clear that tumor-bearing animals present increased PGE_2 levels in plasma [4]. In previous studies of our group we showed that a) indomethacin treatment reverted the detrimental effect of cachexia on CPT II activity in the liver [5,6], b) intracellular lypolisis in inhibited by PGE_2 in the hepatocye [22]; c) Kupffer cells of cachectic rats actively produce PGE_2 [5,6] and are more metabolic active [6]; and d) Kupffer cell cachexia-related response is dependent on intracellular Ca^{2+} concentration and is mediated through α-adrenergic receptors [5].

Enomoto et al. [23] demonstrated that liver fat accumulation is enhanced by PGE_2 both in control and ethanol treatment conditions. The authors conclude that the effect of PGE_2 on this parameter is mediated through EP2/EP4 receptors and is dependent on cAMP. Calin et al. [7] suggested that nitric oxide (NO) activation many act in concert with cyclooxygenase (COX), since theirs effects are similar.

There is growing consensus [24,25] that regular aerobic physical activity may be effective for reducing chronic inflammation. Our own efforts show that, for instance, regular exercise induces a shift in TNF-α/IL-10 ratio [25], favoring the establishment of an anti-inflammatory milieu, even in healthy animals.

While in human peripheral blood mononuclear cells COX-2 expression and NF-κB DNA binding (a major transcription factor involved in the regulation of the expression of various inflammatory markers, including COX-2) are increased after a single bout of high intensity exercise, Hewitt et al. [26] report that moderate intensity exercise decreased PGE_2 production by 5-fold.

Our results show a 44% decrease of PGE_2 content in the liver, suggesting that the protocol diminished Kupffer cell PGE_2 production, which was nevertheless not presently examined.

The tumor tissue is capable of great PGE_2 production [27]. Therefore, it is a potential contributor to serum PGE_2. A 90% reduction of PGE_2 content was found in the tumor. It is known [28] that PGE_2 mediates several of the effects of TNF-α. However, we were not able to detect changes in the expression of this cytokine. We may not, nevertheless, discard an eventual contribution of modified TNF-α content in the plasma upon the liver, since we did not measure TNF-α protein content.

Although the reported modification of PGE_2 concentration could underlie the results concerning CPT activity, other regulatory pathways may be involved. Nevertheless, it is possible to affirm that the effect of exercise is directly associated with long-term regulation, since the experiment for assessment of CPT maximal activity was carried out in vitro, and variation of CPT I physiological inhibitor (malonyl CoA) concentration are therefore discarded. Still it is also possible that exercise modulates yet this other regulatory aspect, which has not been presently addressed. Cha et al. [29] also reported increased liver CPT system after aerobic exercise activity.

The literature lacks consistent information regarding the effect of chronic exercise in the liver as most of the studies examined only the skeletal muscle [30].

We intended this way, to provide with these results further insight into hepatic response to physical exercise. Changes concerning liver fatty acid-binding protein (FABP), for instance, have not been comprehensively studied. This protein is associated with intracellular transport of LCFA resulting in increased re-esterification, and was decreased in exercised control animals not having tumor in relation to the sedentary counterparts, possibly indicating decreased incorporation into intracellular TAG. However, in the tumor-bearing animals the effect of exercise was the opposite.

We suggest that in the latter reestablishment of VLDL secretion promoted by training induces higher FABP expression leading to increased incorporation of TAG into cytoplasmatic pools and consequent enhanced incorporation into nascent VLDL particles.

It is also possible that other cytokines are involved in the response, since we [25] and others [24] reported marked changes in different inflammation markers induced by training. Still, the effect on PGE_2 presents itself as a major contribution to the results.

8 weeks of endurance training decreased tumor weight (sedentary 17.2 g vs. trained 1.94 g) in rats [9,25]. The mechanism by which endurance training inhibits tumor growth is unknown, although it is clear that immune system function is enhanced by moderate intensity exercise training leading to improved antitumor resistance [31].

In conclusion, the endurance training protocol herein adopted was efficient in promoting the reestablishment of normal liver lipid metabolism during cancer cachexia, with a pronounced effect on CPT system. To the best of our knowledge, this is the first report describing the effect of moderate intensity endurance training based upon this parameter. We suggest that these results are related with the effect of moderate intensity endurance training in modulation of PGE_2 concentration, which is more evident in the tumor-bearing animals.

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