Reversion of hepatic steatosis by exercise training in obese mice: The role of sterol regulatory element-binding protein-1c

Dennys E. Cintra a, Eduardo R. Ropelle a, Marcelo F. Vitto b, Thais F. Luciano b, Daniela R. Souza b, Julia Engelmann b, Scherolin O. Marques b, Fabio S. Lira b, Ricardo A. de Pinho b, Jose R. Pauli a, Cláudio T. De Souza b,*

a Faculty of Applied Sciences, University of Campinas, UNICAMP, Limeira, SP, Brazil
b Laboratório de Fisiologia e Bioquímica do Exercício, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil

A B S T R A C T

Aim: The dysregulation of regulatory element-binding protein-1c (SREBP-1c) is associated with hepatic steatosis. However, effects of exercise on SREBP-1c protein level in liver have not been investigated. Thus, in this study we investigated if reversion of the hepatic steatosis-induced by exercise training is related with levels of SREBP-1c.

Main methods: Mice were divided into two groups: control lean mice (CT), fed on standard rodent chow, and obese mice (HF), fed on a high-fat diet for 2 months. After this period obese mice were divided in two groups: obese mice and obese mice submitted to exercise (HF + EXE). The HF + EXE group performed a running program of 50 min per day, 5 days per week, for 8 weeks. Forty-eight hours after the last exercise session, biochemical, immunoblotting, histology and immunohistochemistry analyses were performed.

Key findings: Livers of HF mice showed increased SREBP-1c, FAS (Fatty Acid Synthase), SCD1 (Stearoyl-CoA Desaturase1) and CPT1 (Carnitine Palmitoyl Transferase1) protein levels (3.4, 5.0, 2.6 and 2.9 times, respectively), though ACC (Acetyl-CoA Carboxilase) phosphorylation dropped 4.2 times. In livers of HF + EXE, levels of SREBP-1c, FAS, SCD1 and CPT1 decreased 2.1, 1.9, 1.8, and 2.7 times, respectively), while ACC phosphorylation increased 3.0 times. Lower SREBP-1c protein levels after exercise were confirmed also by immunohistochemistry. Total liver lipids content was higher in HF (2.2 times) when compared to CT, and exercise training reduced it significantly (1.7 times).

Significance: Our study allows concluding that the reduction in SREBP-1c protein levels is associated with steatosis reversion induced by exercise training.

Crown Copyright © 2012 Published by Elsevier Inc. All rights reserved.

Introduction

The liver plays a central role in whole body lipid metabolism. Therefore, this regulation affects whole-body lipid composition and may contribute to the onset and progression of several chronic diseases, including atherosclerosis, diabetes, and obesity (for a review see Jump, 2011). Since dysregulations in hepatic lipid synthesis have been associated with several diseases, a comprehensive understanding of lipogenesis regulation seems crucial in both physiology and physiopathology studies.

The mechanism of triacylglycerol synthesis in liver has been intensely studied. In line with this, the sterol regulatory element binding proteins (SREBPs), which are master transcription factors which regulate lipid metabolism, was discovered (Wang et al., 1993). The SREBP family has been established as a group of transcription factors regulating transcription of genes involved in cholesterol and fatty acid synthesis. These proteins are inserted into the endoplasmic reticulum (ER) membrane with both the amino and carboxylic acid domains facing the cytosolic side of the membrane. In sterol deficient cells, proteolytic cleavage of SREBPs (125 kDa) occurs, which makes them release their N-terminal mature and active forms (68 kDa) and enabling them to enter the nucleus, where they bind to the sterol regulatory response element (SRE) and/or E-box sequences and activate genes involved in cholesterol, triglyceride, and fatty acid bio-synthesis (Brown and Goldstein, 1997; Nothdurft et al., 2000; Nothdurft et al., 1999). A number of studies have reported that SREBP-1c levels are high in alcoholic or non-alcoholic fatty liver disease (for a review see Ahmed and Byrne, 2007; Yang et al., 2010; You and Crabb, 2004), and fatty liver was attenuated in mice deficient in SREBP-1 (Yahagi et al., 2002). Furthermore, SREBP-1c plays a pivotal role in the dietary regulation of most hepatic lipogenic genes, like fatty acid synthase (FAS),
acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase 1 (SCD1) (Brown and Goldstein, 1997; Nothdurft et al., 2000, 1999).

The ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, which is a key molecule in the control of intracellular fatty acid metabolism (Abu-Elheiga et al., 1995; Ruderman et al., 1999). Results suggest that ACC is involved in regulating fat oxidation in the liver and that the use of antisense oligonucleotide (anti-ACC) technique reverses hepatic steatosis in high-fat fed rats (Savage et al., 2006). ACC is a major isozyme that supplies malonyl-CoA to FAS, the multifunctional enzyme catalyzing the de novo synthesis of fatty acid in many tissues via subsequent nutritional and hormonal regulation (Abu-Elheiga et al., 1995; Ruderman et al., 1999). SREBP-1c also plays pivotal regulation of SCD1. SCD1 is a central lipogenic enzyme that catalyzes the delta-9-cis desaturation of saturated fatty acyl-CoA used in the biosynthesis of monounsaturated fatty acids (MUFAs). Recent studies with a mouse model with SCD1 targeted disruption gene have provided evidence that SCD1 plays an important role in lipid homeostasis and lipoprotein metabolism (Cohen et al., 2002; Miyazaki et al., 2000, 2007).

According to what is presented above, the liver plays a crucial role in fat metabolism. In addition, physical exercise has been demonstrated to increase metabolism in general, especially fat liver metabolism (Johnson et al., 2009; Schultz et al., 2012; Yasari et al., 2010). Physical exercise, one of most extreme metabolic forms of stress experienced, leads to activation/inactivation of the many enzymes in the liver that are associated as fatty acid oxidation and triglyceride formation (Johnson et al., 2009; Marques et al., 2010; Rector et al., 2008a; Schultz et al., 2012; Yasari et al., 2010). On the other hand, low aerobic fitness, by itself or combined with overnutrition, is the underlying cause of NAFLD (for a review see Rector et al., 2011), and cessation of physical activity alters fat oxidation capacity in the liver (Rector et al., 2008b). The reversion of fatty liver induced by exercise has been poorly investigated. In an elegant work on the subject, Rector et al. (2008a) indicated that exercise has a beneficial effect upon hepatic lipid metabolism. That said, the next step is the identification of the molecular mechanisms responsible for these exercise-induced alterations. In addition, effects of exercise on SREBP-1c transcriptional factor in liver have not been investigated. In this scenario, this study investigates the involvement of SREBP-1c in decreasing hepatic lipid content after exercise training.

**Material and methods**

**Animals and diet**

Four-week-old male Swiss mice were obtained from our breeding colony (UNESC) and kept housed in individual cages under a 12-h artificial light–dark cycle. The investigation followed the University guidelines for the use of animals in experimental studies. After the acclimatization period (3 days), the animals were randomly divided into two groups: lean control mice (CT), fed on standard rodent chow, and obese mice, fed on a high-fat diet for 2 months, characterizing diet-induced obesity (HF). Eight weeks of fat-rich diet feeding were defined as the time by which all mice should have developed obesity (De Souza et al., 2005). After this period, obese mice were divided in two groups: mice fed on high-fat diet and non-trained (HF) and mice fed on high-fat diet and submitted to exercise training (HF + EXE).

**Exercise protocol and proceeding**

The group of mice trained was habituated on a nine-channel motor-drive treadmill at a speed of 10 m/min for 10 min/day for one week, to reduce stress to the new environment. The mice did not receive any electric stimulus to run, but manual stimulation was used. The exercise group performed a running program of 50 min per day (1.0 km/h), 5 days per week, for 8 weeks (Fernando et al., 1993). The non-trained mice were placed on the switched-off treadmill for the same 8 weeks. Forty-eight hours after the last exercise session, one set of mice (n = 8) was randomly chosen and anesthetized for hormone and biochemical analysis. Another set of mice was decapitated for immunoblotting and total hepatic lipids (n = 6) and histology and immunohistochemistry (n = 6). The mice used for hormone and biochemical analysis were anesthetized with an intraperitoneal (i.p) injection of ketamine chlorohydrate (50 mg/kg; Ketalar; Parke-Davis, Ann Arbor, MI) and xylazine (20 mg/kg; Rompun; Bayer, Leverkusen), and decapitated.

**Hormone and biochemical measurements**

After a 6-h fasting period, serum insulin was determined using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit (Crystal Chem Inc., Chicago, IL), and blood glucose was measured by glucometer (Advantage, Boehringer Mannheim, Irvine, CA). Total liver fat was extracted. For this, 4.0 mL chloroform/methanol 2:1 (v/v) was added to each 1 g of liver sample, vortexed well and incubated at room temperature for 5 min. Then, additional volumes of 1.25 mL chloroform and 1.25 mL deionized H2O were added, and finally, following vigorous homogenized for 3 min, samples were centrifuged at 1000 rpm for 5 min at room temperature to obtain a two-phase system: aqueous top and organic bottom phases from which lipids were obtained.

**Protein analysis by immunoblotting**

Forty-eight hours after the last session of the exercise protocol mice were decapitated and liver fragments were homogenized in extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride and 0.1 mg of aprotinin/ml) at 4 °C with a Polytron MR 2100 (Kinematica, Switzerland). The extracts were centrifuged at 11,000 rpm and 4 °C in an Eppendorf centrifuge 5804R (Eppendorf AG, Hamburg, Germany) for 40 min to remove insoluble material. The supernatants of these tissues were used for protein quantification (Bradford, 1976) and proteins were denatured by boiling in Laemmli (Laemmli, 1977) sample buffer containing 100 mM DTT. After, 0.2 mg of protein extracts obtained from each sample were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-ACC, anti-CPT1, anti-FAS, anti-SREBP-1c, anti-SCD1, and anti-β-actin purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and anti-phospho [Ser79]ACC purchased from Cell Signaling Technology (Beverly, MA, USA). The original membrane was stripped and rebotted with β-actin loading protein. Chemiluminescent detection was performed with horseradish peroxidase-conjugate secondary antibodies. Autoradiographs of membranes were taken for visualization of protein bands. The results of blots are presented as direct comparisons of bands in autoradiographs and quantified by densitometry using the Scion Image software.

**Histology**

Hydrated, 5.0-μm sections of paraformaldehyde-fixed, paraffin-embedded liver fragments of four mice were stained with haematoxylin/eosin and photo-documented using an Olympus BX60 microscope.

**Immunohistochemistry**

Livers obtained from four mice were examined to determine the expression and tissue distribution of SREBP-1c protein, as previously described (Araujo et al., 2004). Hydrated, 5.0-μm sections of paraformaldehyde-fixed, paraffin-embedded tissue were transferred...
and coated with poly-L-lysine and fixed. The sections were washed twice in 0.01 M phosphate-buffered saline (PBS), pH 7.4 and incubated in PBS with 1% bovine serum albumin (BSA, w/v) for 30 min to block nonspecific background. Upon BSA removal, the sections were treated with primary antibody anti SREBP-1c (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:100 dilution in PBS/BSA overnight at 4 °C. After washing in PBS, slides were covered with a 1:200 dilution of FITC-conjugated donkey anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in PBS, for 120 min. The sections were subsequently washed in PBS and incubated for 10 min with DAPI (Vectashield, Vector Laboratory Inc., USA) prepared according to manufacturer’s instructions. Photo-documentation of results was performed using a Leica FW 4500 B microscope. Represent the positive SREBP-1c cells number quantification of HF and HF + EXE groups were performed.

Statistical analysis

The results were expressed as the means ± SEM. Differences between the groups were evaluated using one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. A probability of less than 0.05 was considered significant. The software used for analysis of the data was the Statistical Package for the Social Sciences (SPSS) version 17.0 for Windows.

Results

Physiological and metabolic parameters

Table 1 shows comparative data for the CT, HF and HF + EXE groups. The HF mice were heavier, presented higher levels of epididymal fat, fasting serum insulin, and fasting glucose than age matched controls. Significant variations were found in body weight, epididymal fat, serum insulin and blood glucose of HF mice after exercise training, when compared to HF non-trained group. No difference was observed in food intake in the three groups (data not shown).

Exercise training reduces SREBP-1c, FAS and SCD1 protein levels in liver of HF mice

The effects of exercise training on transcription factor SREBP-1c and the protein levels of the enzymes (FAS and SCD1) controlled by it were examined in livers of CT, HF and HF + EXE. SREBP-1c levels increased 3.4 times in livers of HF mice, when compared to CT group (Fig. 1A). In HF + EXE, SREBP-1c was reduced by 2.1 times, when compared with the HF group (Fig. 1A). High-fat diet induced an increase in FAS protein levels. In the livers from HF mice, FAS protein levels increased 5.0 times, when compared with CT (Fig. 1B). In the liver of HF + EXE mice, FAS dropped by a factor of 1.9, when compared with the HF group. The high-fat diet increased SCD1 protein levels by 2.6 times, and exercise led to a 1.8-fold drop in the parameter (Fig. 1C). As SREBP-1c antibody recognizes the membrane-bound and nuclear forms, we performed long-run SDS-PAGES with 0.2 mg protein samples. As illustrated in Fig. 1A, the ~125 kDa (membrane-bound form) and ~68 kDa (nuclear form) bands detected in total liver protein extract indicates, respectively, were the precursor and mature forms of SREBP-1c protein. The levels of precursor as well as the mature forms of SREBP-1c were higher when the mice were submitted to high-fat diet (Fig. 1A – long-run SDS-PAGE). On the other hand, the protein levels of the two forms of SREBP-1c were reduced after exercise protocol in these obese mice (Fig. 1A – long-run SDS-PAGE). The membrane was stripped and immunoblotted with anti-β-actin as loading protein. Neither treatment changed β-actin protein levels.

Exercise training increases ACC phosphorylation and CPT1 protein levels in liver of HF mice

The high-fat diet reduces the phosphorylation of ACC (4.2 times), when compared to the CT group (Fig. 2A). Exercise training increases ACC phosphorylation by a factor of 3.0, when compared to the HF group (Fig. 2A). CPT1 protein levels decreased 2.9 times in livers of HF mice, when compared with the CT group (Fig. 2B). The exercise training adopted here increased the level of CPT1 by 2.7 times, when compared to the HF group, demonstrating a similar alteration in ACC phosphorylation. Exercise did not lead to any difference in ACC total protein levels (Fig. 2A – lower panel). Fig. 2B, lower panel, shows the membrane stripped and immunoblotted with anti-β-actin as loading protein.

Exercise training reduces total hepatic lipids in HF mice

Total lipid extraction and histological analysis was performed. Fig. 3B shows lipid deposition in liver slices. The CT group showed clear and conserved parenchyma. Fat drops in hepatocyte of the HF group were larger, characterizing a steatotic hepatic state, with considering fat deposits predominant in the perivenular zone and spreading to the external areas of the lobule. On the other hand, exercise training markedly reduces it. Similarly, total liver lipid content was higher in animals of the HF group (2.2 times) than in animals of CT (Fig. 3A). On the other hand, exercise training reduced (1.7 times) significantly the total liver lipids content.

SREBP-1c levels were also evaluated by immunofluorescence staining. Comparative evaluation of the pattern of staining suggested that livers of obese mice submitted to exercise training expressed lower levels of SREBP-1c, when compared with HF group (Fig. 4A). Immunohistochemistry quantification showed a percentage change in SREBP-1c positive cells number of HF + EXE 50% lower compared with HF group (Fig. 4B). The results are in accordance with those observed in Fig. 1A.

Discussion

Hepatic steatosis is a secondary, but not less important, characteristic of cardiometabolic risk. Effective changes in lifestyle emerge as important strategies to reduce liver fat. In the present study, we evaluated the effects of exercise training on hepatic lipid metabolism in obese mice. We found that exercise training reduced the SREBP-1c, FAS and SCD1 protein expression in the liver of obese mice; in addition, it was observed that exercise training increased ACC phosphorylation and CPT1 expression in this tissue. In parallel, exercise reduced the total hepatic lipids and reversed the hepatic steatosis in obese mice.

Physical activity should be strongly promoted for the management of fatty liver; however, it is not clear whether exercise benefits in liver depend on body weight loss. Our results demonstrate that the exercise training protocol was sufficient to reduce the total body and epididymal weight in obese mice. In addition, exercise reduced blood glucose and plasma insulin levels. In this regard, Johnson and colleagues provide direct experimental evidence, demonstrating that regular aerobic exercise reduces hepatic lipids in obesity, even in the absence of body weight reduction (Johnson et al., 2009). However, the molecular mechanism by which exercise modulates the lipid liver metabolism remains unclear. It was demonstrated that daily physical activity attenuates hepatic steatosis and NAFLD in an
obese rodent model, and suggested that this effect is likely mediated, in part, through enhancement of hepatic fatty acid oxidation and reductions in key protein intermediates of fatty acid synthesis (Rector et al., 2008a). However, no investigation has been made on SREBP-1c protein levels in HF mice submitted to physical exercise. Our study allows concluding that hepatic SREBP-1c, FAS and SCD1 protein levels are significantly lower, at the same time that ACC phosphorylation increased in obese mice after exercise training. This was associated with reversion of hepatic steatosis. Conversely, Ikeda and co-authors showed that swimming and treadmill exercise training in mice up-regulated expression of lipogenic genes in skeletal muscle, such as ACC, SCD1, and acyl-CoA diacylglycerolacyltransferase (Ikeda et al., 2002). These discrepancies between results obtained in studies using muscle and liver may be due to the well-defined differences in metabolism between these tissues. Higher lipid content was observed in the skeletal muscle of endurance trained human subjects, in

Fig. 1. Protein levels of SREBP-1c, FAS and SCD1. Liver extracts were immunobotted with anti-SREBP-1c (A), anti-FAS (B), anti-SCD1 (C). Long-run SDS gel electrophoresis of representative changes in SREBP-1c precursor (125 kDa) and mature (68 kDa) forms after exercise protocol (A). The membrane was stripped and immunobotted with anti-β-actin antibody and used as loaded protein (lower panels in figures A, B and C). The results of scanning densitometry are expressed as arbitrary units. Bars represent means±S.E.M. of n=6 mice. *p<0.05, HF versus CT group and *p<0.05, HF+EXE mice versus HF group.

Fig. 2. ACC phosphorylation and CPT-1 protein level. Samples were blotted (IB) with (A) anti-p-ACC (upper panel) and anti-ACC for total protein level (lower panel) and (B) anti-CPT1 (upper panel) and anti-β-actin for loaded protein (lower panel). The results of scanning densitometry are expressed as arbitrary units. Bars represent means±S.E.M. of n=6 mice. *p<0.05, HF versus CT and *p<0.05, HF+EXE mice versus HF group.
comparison to that of untrained subjects (Goodpaster et al., 2001). In addition, several studies reported an increase of SREBP-1c protein levels in skeletal muscle after exercise training in rodents (Ikeda et al., 2002; Nadeau et al., 2006). On the other hand, several lines of evidence demonstrate that exercise increases the activity of enzymes involved in lipid oxidation in the hepatic tissue (Camacho et al., 2006; Takekoshi et al., 2006). In accordance with these previous studies, our results demonstrate that exercise increased the ACC phosphorylation and reduced the SREBP-1c expression in the liver of obese mice.

SREBP-1c is a transcription factor implicated in lipid metabolism through its regulation of FAS, ACC, and SCD1 (Brown and Goldstein, 1997; Nohturfft et al., 2000, 1999). Our results demonstrated an association between transcription factor SREBP-1c and FAS and SCD1 protein levels (Fig. 1), ACC phosphorylation (Fig. 2), and total lipids and histology (Fig. 3) in liver of HF mice and in HF mice submitted to exercise training. Thus, these data suggest that exercise training reduces SREBP-1c protein levels and that this reduction could be responsible for the modulation of lipogenic enzymes. Interestingly, exercise training inhibited the membrane-bound (125 kDa) as well as the nuclear/mature (68 kDa) forms of the protein (Fig. 1A – long-run SDS-PAGE). These results are important, since they demonstrate that the decline in the membrane-bound form does not reflect increased cleavage and, thus, no abundance of nuclear SREBP-1c. An effect on nuclear form could explain the incomplete accordance between the levels of SREBP-1c measured and the abundance of its transcriptional targets after the exercise protocol.

**Fig. 3.** Lipids total content and haematoxylin/eosin staining. Haematoxylin/eosin staining of 5.0-μm sections of livers (n=4) (A). Total hepatic lipids content is expressed as mg/100 g tissue (B). In B, bars represent means ± S.E.M. of n = 6 mice. *p<0.05, HF versus CT group and #p<0.05, HF + EXE versus HF group.

**Fig. 4.** Representative and quantification SREBP-1c expression staining. (A) Signal detection and image acquisition were performed in fluorescence microscope. White arrows in merge depict positive hepatocytes; 200× amplification. Photographs are representative of six distinct experiments (n=6). (B) Immunohistochemistry quantification (percentage change in SREBP-1c positive cells number of HF) *p<0.05, HF + EXE versus HF group.
The importance of the SREBP-1c in fat metabolism in liver has been studied. To better clarify its role, some techniques were performed to achieve direct or indirect inhibition or up-regulation of SREBP-1c, such as liver-specific SREBP-1 knockout mice (Yahagi et al., 2002), mice germ line knockedout for SREBP-1a and 1c (Shimano et al., 1997) and SREBP-1c (Liang et al., 2002), adenosine-activated overexpression (Wang et al., 2010) or recent study performed by our group (Frederico et al., 2011). In an elegant study, Knebel and colleagues generated mice with liver-specific over-expression of mature human SREBP-1c under control of the albumin promoter and a liver-specific enhancer (alb-SREBP-1c) to analyze systemic perturbations caused by this distinct alteration. SREBP-1c targets specific genes and causes key enzymes in lipid metabolism to be upregulated. Thus, the alb-SREBP-1c mice developed hepatic lipid accumulation featuring a fatty liver by the age of 24 weeks under normocaloric nutrition (Knebel et al., 2012). All these studies showed that SREBP-1c plays a role in the increased rates of fatty acid biosynthesis and TG levels in the liver by controlling important enzymes of fatty acid synthesis, such as FAS. As shown in Fig. 1, exercise reduced SREBP-1c and FAS protein levels by similar magnitudes, suggesting that direct inhibition of transcription factor SREBP-1c is crucial for determination of FAS protein levels; however, the mechanism by which exercise reduces SREBP-1c and FAS expression remains unclear and deserves further investigations.

In rats with NAFLD, suppression of ACC with a single ASO significantly reduces hepatic malonyl-CoA levels in vivo, lower hepatic lipids and increased hepatic fat oxidation (Savage et al., 2006). In the present study, we observed higher ACC phosphorylation in the group of animals submitted to exercise training protocol when compared with the high-fat feeding groups, which demonstrates lower activity. In addition, SCD1 protein levels were changed with exercise. In the present study SCD1 protein level was reduced, possibly because of a reduction in subcellular levels, as malonyl-CoA and/or minor transcription by SREBP-1c. In the present study SCD1-knockout mice also showed similar results (Gutiérrez-Juárez et al., 2006).

Conclusions

In summary, we demonstrated that exercise training was effective to reduce the SREBP-1c, FAS and SCD1 protein expression in the liver of obese mice; in addition, it was observed that exercise increased ACC phosphorylation and CPT1 protein levels in this tissue; concomitantly, it was accompanied by the reversion of hepatic steatosis. Collectively, our data suggest that exercise training could be an excellent non-pharmacological treatment to fatty liver diseases.

Conflict of interest statement

None.

Acknowledgments

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and University Southern of Santa Catarina (UNESC).

References


