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Abstract

We examined the capacity of high-intensity intermittent training (HI-IT) to facilitate the delivery of lipids to enzymes responsible for oxidation, a task performed by the carnitine palmitoyl transferase (CPT) system in the rat gastrocnemius muscle. Male adult Wistar rats (160-250 g) were randomly distributed into 3 groups: sedentary (Sed, N = 5), HI-IT (N = 10), and moderate-intensity continuous training (MI-CT, N = 10). The trained groups were exercised for 8 weeks with a 10% (HI-IT) and a 5% (MI-CT) overload. The HI-IT group presented 11.8% decreased weight gain compared to the Sed group. The maximal activities of CPT-I, CPT-II, and citrate synthase were all increased in the HI-IT group compared to the Sed group (P < 0.01), as also was gene expression, measured by RT-PCR, of fatty acid binding protein (FABP; P < 0.01) and lipoprotein lipase (LPL; P < 0.05). Lactate dehydrogenase also presented a higher maximal activity (nmol·min^{-1}·mg protein^{-1}) in HI-IT (around 83%). We suggest that 8 weeks of HI-IT enhance mitochondrial lipid transport capacity thus facilitating the oxidation process in the gastrocnemius muscle. This adaptation may also be associated with the decrease in weight gain observed in the animals and was concomitant to a higher gene expression of both FABP and LPL in HI-IT, suggesting that intermittent exercise is a "time-efficient" strategy inducing metabolic adaptation.

Key words: High-intensity intermittent training; Lipid metabolism; Carnitine palmitoyl transferase

Introduction

High-intensity intermittent training (HI-IT) is characterized by repeated bouts of brief near-maximal or maximal intensity work, interspersed with periods of recovery (rest or low-intensity work) (1) and is known to be a potent stimulus leading to metabolic adaptation in skeletal muscle (2). A wide range of adaptations has been described for carbohydrate metabolism in response to HI-IT, including increased glycogen content (3), and increased lactate transport capacity (4). In contrast, HI-IT is generally thought to be less effective on the capacity of muscle to deliver lipids to mitochondria (3).

Few studies have described the alterations of muscle fat metabolism during intermittent exercise, despite the fact that most collective sports are of an intermittent nature, as reviewed by Boutcher in 2011 (5). Moreover, intermittent physical activity is associated with a reduced risk of morbidity and mortality from cardiovascular diseases, and cumulative short bouts of repetitive exercise are thought to mimic the effects of longer bouts of exercise (5). Since fat metabolism disorders are closely associated with the development of cardiovascular diseases, it becomes imperative to better understand the metabolic response to HI-IT.

There is recent evidence that HI-IT may indeed induce adaptations in lipid metabolism and may represent a time-efficient strategy to induce muscle adaptation similar to that elicited by continuous endurance training (6). However, the wide variations in HI-IT exercise protocols adopted in the literature make it difficult to identify the effect of this type of exercise upon specific aspects of lipid metabolism. We compared the adaptations in lipid metabolism-associated proteins in HI-IT with those resulting from moderate-intensity continuous training (MI-CT) in an animal model. A higher rate of fatty acid utilization by skeletal muscle may be achieved by the enhancement of maximal activity of the carnitine palmitoyltransferase (CPT) complex, which is considered to be the key regulatory step in the transport of long-chain fatty acids (7).

Thus, the objective of the present study was to investigate the response of the CPT complex to HI-IT and to compare it to the response elicited by MI-CT in sedentary (Sed) control animals. The gene expression of some important muscle proteins associated with muscle lipid oxidative capacity, including peroxisome proliferator-activated receptor α and β (PPARα), fatty acid binding protein (FABP) and lipoprotein lipase (LPL) was examined.

Material and Methods

Animals

Male adult Wistar rats (160-250 g) obtained from Instituto de Ciências Biomédicas, Universidade de São Paulo, were maintained on a 12-h light/12-h dark cycle (lights on at 7:00 am) under controlled temperature conditions (23 ± 1°C), receiving water and food (commercial chow, Nuvilab®, Nuvital, Brazil) *ad libitum*. Animals were randomly divided into the following groups: Sed (N = 5), HI-IT (N = 10) and MI-CT (N = 10). The animals were euthanized by decapitation 24 h after the last exercise bout. The Ethics Committee for Animal Research of Instituto de Ciências Biomédicas, USP, approved the procedures used (protocol

#148/2001), which were carried out in accordance with the ethical principles stated by the Brazilian College of Animal Experimentation.

Experimental design

Weight gain, muscle lipid content, plasma lactate, and the activity of the proteins related to mitochondrial long-chain fatty acids transport (CPT-I and -II), oxidation (citrate synthase) and glycolysis (lactate dehydrogenase), as well as the mRNA expression of CPT-I, CPT-II, FABP, LPL, PPARα, PPARβ, and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1-α) were assessed in the gastrocnemius of trained rats submitted to HI-IT. The results were compared to those of the Sed group and the MI-CT group.

All training protocols were performed in a swimming system consisting of 10 individual PVC chambers of 100 x 60 x 60 cm, with a water temperature of 31 ± 1°C for 8 weeks, after 2 weeks of adaptation, 5 days a week. The swimming training model was chosen over treadmill running because it induces higher recruitment of muscle fibers from the gastrocnemius rather than from the soleus, as previously described (8). Increasing weight loads attached to the tail were adopted so as to reach a maximum of 10% of total body weight for the HI-IT protocol and 5% of total body weight for the MI-CT protocol. For HI-IT, the intensity of effort corresponded to ~100% VO$_2$ max (8) and the lactate threshold was achieved with extra loads of 5-6% of total body weight (9). For MI-CT, the exercise intensity corresponded to ~65% VO$_2$ max. This intensity of effort was assured by constant evaluation of the adaptations to the overweight percentage related to body mass (10). A complete description of the two training protocols is provided in Table 1.

Analytical procedures

Body weight was measured every week, always at the same time of day. Plasma lactate concentration was measured with a lactate analyzer (YSI 1500 Sport, YSI

Table 1. High-intensity intermittent training (HI-IT) and moderate-intensity continuous training (MI-CT) protocols.

HI-IT					MI-CT					
Week*	Bouts	Activity and rest duration	Overload	Total time	Day/week	Overload	Time	Day/week	Overload	Time
3rd week	11	1 min:1 min	10% BW	22 min	1st day	0% BW	30 min	7th day	3% BW	50 min
4th week	12	1 min:1 min	10% BW	24 min	2nd day	0% BW	40 min	8th day	3% BW	60 min
5th week	13	1 min:1 min	10% BW	26 min	3rd day	1% BW	30 min	9th day	4% BW	60 min
6th week	14	1 min:1 min	10% BW	28 min	4th day	1% BW	40 min	10th day	5% BW	60 min
7th week	15	1 min:1 min	10% BW	30 min	5th day	2% BW	40 min	3rd to	5% BW	60 min
8th week	15	1 min:1 min	10% BW	30 min	6th day	2% BW	50 min	8th week	(5 times/week)	

*HI-IT was preceded by 2 weeks of an adaptation protocol. Overload was progressively increased until reaching 10 bouts and 10% of body weight (BW).

Life Sciences, USA) on the last day of training before the beginning of exercise and after the 5th and last bout of exercise (HI-IT) and at 0 min, 30th min and after the training session (MI-CT), as described by Gobatto et al. (9). Plasma lactate concentration is reported as means ± SEM in µmol/mL plasma.

Citrate synthase activity was measured as described by Zammit et al. (11). Muscles were homogenized (1:20, v/v) in a buffer consisting of 25 mM Tris-HCl and 1 mM EDTA, pH 7.4. After centrifugation for 1 min at 2000 rpm the supernatant was collected and 80 µL was added to 890 µL of the assay mixture, pH 8.0, which consisted of 50 mM Tris-HCl, 1 mM EDTA, 0.05% Triton X-100, 0.2 mM DTNB, 0.5 mM oxaloacetate, and 0.1 mM acetyl-CoA. Enzyme activity, determined at 412 nm with a Hitachi Spectrophotometer (USA), is reported as means ± SEM in $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$.

Lactate dehydrogenase activity was evaluated as described by Zammit et al. (11). The assay medium consisted of 120 mM Tris, 3.4 mM NADH, 1 µM antimiacin, 1% Triton X-100, and deionized water, pH 7.4. Muscle homogenates were added to the assay mixture and the reaction was started with the addition of 2 mM pyruvate and monitored at 340 nm for 10 min, at 25°C. Lactate dehydrogenase activity is reported as means ± SEM in $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$.

Muscle total fat content

The gastrocnemius muscle was removed and treated as described by Stansbie et al. (12). Duplicate samples were digested with 30% KOH (w/v) for 15 min, absolute ethanol was added and the preparation was incubated for 2 h at 70°C. The free fatty acids from the lipid fraction were extracted 3 times with petroleum ether and, after evaporation, the mass of the lipid present in the sample was assessed.

CPT-I and CPT-II activity

To isolate the mitochondria the muscles were minced with scissors and homogenized 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 1000 *g* for 12 min. The supernatant was then centrifuged twice at 8500 *g* for 15 min (13). The isolated mitochondria were suspended in a buffer consisting of 0.15 mM KCl and 5 mM Tris-HCl, pH 7.5, centrifuged at 10,000 *g* for 15 min, resuspended in 10 mM phosphate buffer, pH 7.5, frozen in liquid nitrogen, and thawed. Samples were then ultracentrifuged at 100,000 *g* for 1 h. 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 in order 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 a modification of the method described by Seelaender et al. (13). The assay medium consisted of 60 mM KCl, 40 mM mannitol, 20 mM HEPES, 0.15 mM EGTA, 1.5 mM KCN, 0.5% fat-free bovine serum albumin, 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 (blank). 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 7% perchloric acid, and the acylcarnitine formed was extracted with n-buthanol. CPT activity is reported as $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ in the isolated enzyme fraction.

Reverse transcription and real-time polymerase chain reaction (PCR)

Total RNA was obtained from aliquots of 100 mg gastrocnemius muscle by Trizol reagent extraction according to manufacturer instructions. RNA concentration was determined spectrophotometrically (Beckman DU 640, USA). The reverse transcriptase and real-time PCR method was used for the estimation of the concentration of the mRNA of CPT-I and CPT-II, FABP, PPAR-α, PPAR-β, PGC1-α, and LPL. 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 10X reaction buffer (100 mM Tris-HCl, 500 mM KCl and 150 mM $MgCl_2$ in nuclease-free water; Invitrogen, USA).

In a final volume of 25 µL, 5 µL cDNA was mixed with 2X SYBR Green PCR master mix (Applied Biosystems, UK) and primers (Invitrogen, Brazil). The primer sequences are presented in Table 2. Quantitative real-time PCR was carried out with an ABI 7300 Real-Time PCR Systems

Table 2. Real-time PCR primer sequences.

Gene	Sense (5'-3')	Antisense (5'-3')	Accession No.
CPT-I	CCGAGCTCAGTGAGGACCTA	ATCTGTTTGAGGGCTTCGTG	NM_031559
CPT-II	GAGCCCCTAGTAGGCCCTTA	AGGCTTCTGTGCATTGAGGT	NM_0129301
FABP	ACCTCATTGCCACCATGAAC	CTTCCCTTCATGCACGATTT	NM_0125561
LPL	GAACCTGGCCACATCATTTC	CAGCAAAACCTTTGTGGTGA	NM-0125981
PGC1-α	TTGCCCAGATCTTCCTGAAC	TGAGGACCGCTAGCAAGTTT	NM_176075.2
PPARα	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA	NM_0131961
PPARβ	GAGGACAAACCCACGGTAAA	GGCCACTTCCTCTTTCTCCT	NM_013141.2

CPT-I and CPT-II = carnitine palmitoyl transferase I and II, respectively; FABP = fatty acid binding protein; LPL = lipoprotein lipase; PGC1-α = peroxisome proliferator-activated receptor-γ coactivator-1α; PPARα and PPARβ = peroxisome proliferator-activated receptor α and β, respectively.

(Applied Biosystems). mRNA levels were determined by a cycle threshold (C_t) value comparison method already performed and described by our group (14). 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 Δ-ΔC_t value. The expression of each gene was then evaluated by $2^{-(\Delta\text{-}\Delta Ct)}$.

Sample protein content was assessed by the method of Lowry et al. (15). Results are reported as means ± SEM.

Statistical analysis

Data analysis was performed using a commercial statistical package from Sigma Stat (version 3.1, Sigma Stat, SYSTAT, USA). Data are reported as means + SEM. Statistical analyses were performed using one-way ANOVA. The Tukey *post hoc* test was used in the event of a significant ($P < 0.05$) *F* ratio.

Results

During the last 4 weeks of the training HI-IT rats and rats submitted to MI-CT showed lower weight gain when compared to Sed ($P < 0.05$; Figure 1). Muscle fat content and mitochondrial protein content did not differ among groups ($P > 0.05$). Citrate synthase activity was significantly higher in both HI-IT and MI-CT compared to Sed and in MI-CT compared to HI-IT. The enzymatic activity of lactate dehydrogenase was significantly higher only in HI-IT compared to Sed (Table 3).

The activity of CPT-I in the gastrocnemius muscle of MI-CT and HI-IT was enhanced ~25 and ~28%, respectively, in comparison to Sed ($P < 0.05$; Figure 2). However, maximal CPT-II activity was increased solely in HI-IT compared to the Sed group ($P < 0.05$). Surprisingly, only rats training under the HI-IT protocol showed a significant increase in mRNA expression of the lipid-metabolism related proteins FABP and LPL compared to Sed (Table 4).

Plasma very low-density lipoprotein (VLDL), cholesterol, and triglyceride (TG) concentrations were all significantly lower in the HI-IT group than in the Sed group, while only plasma cholesterol levels were significantly lower in MI-CT.

Moreover, plasma VLDL concentration was decreased significantly in HI-IT compared to MI-CT ($P < 0.05$). Plasma insulin levels were similar among groups ($P > 0.05$; Table 5).

Discussion

The major finding of the present study was that HI-IT resulted in the enhancement of maximal activity of the CPT complex, and a higher gene expression of proteins related to fatty acid uptake and transport. The results demonstrate that HI-IT stimulated the adaptation of steps leading to lipid oxidation.

The main component of the system is thought to be CPT-I (11). Continuous exercise training has been reported to induce enhancement of CPT-I activity and expression in humans (16). Accordingly, CPT-I maximal activity was increased in the present MI-CT protocol. Corroborating our hypothesis that high-intensity training also induces such an adaptation, the maximal gastrocnemius activities of both CPT-I and -II were increased after 8 weeks of high-intensity intermittent training.
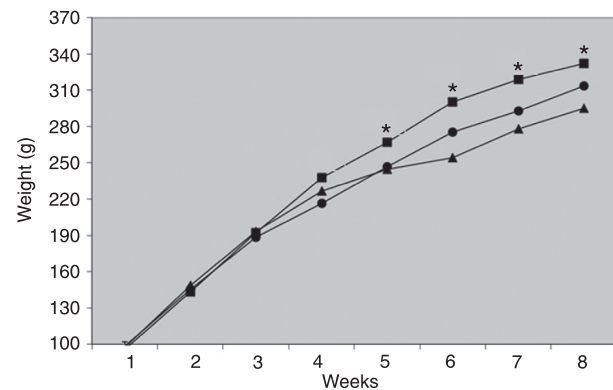

Figure 1. Variation in weight during the 8-week training protocols of sedentary (Sed; N = 5, squares) rats and of rats subjected to moderate-intensity continuous training (MI-CT; N = 10, circles) and high-intensity intermittent training (HI-IT; N = 10, triangles). Data are reported as means ± SEM. *P < 0.05 Sed compared to MI-CT and HI-IT (one-way ANOVA).

Table 3. Fat content, mitochondrial protein content, and citrate synthase and lactate dehydrogenase maximal enzymatic activities in the gastrocnemius muscle.

	Fat content (mg fat/g tissue; N = 6)	Mitochondrial protein content (mg/mL; N = 8)	Citrate synthase (nmol·min^{-1}·mg protein^{-1}; N = 6)	Lactate dehydrogenase (nmol·min^{-1}·mg protein^{-1}; N = 7)
Sed	15.04 ± 1.39	1.44 ± 0.10	327.1 ± 38.30	1184.35 ± 195.47
MI-CT	16.20 ± 1.12	1.24 ± 0.16	640.5 ± 40.9*	1699.17 ± 349.65
HI-IT	15.24 ± 1.02	1.10 ± 0.06	423.8 ± 50.3**	2166.96 ± 205.63***

Data are reported as means ± SEM. Sed = sedentary; MI-CT = moderate-intensity continuous training; HI-IT = high-intensity intermittent training. *P < 0.001 *vs* Sed and P < 0.01 *vs* HI-IT; **P < 0.01 *vs* Sed; ***P < 0.05 *vs* Sed (one-way ANOVA).
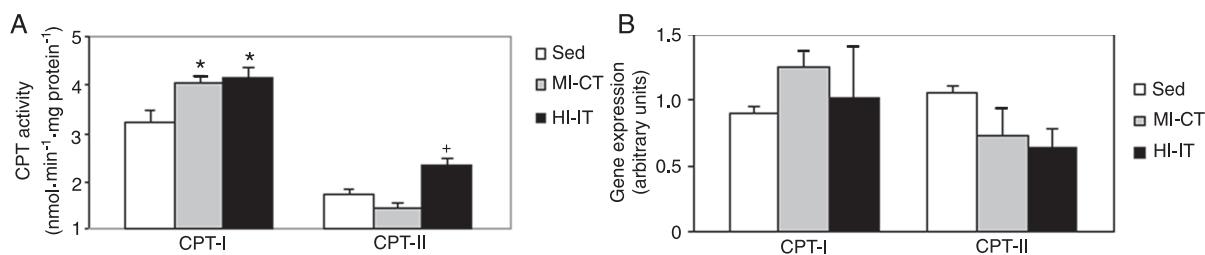
Figure 2. Carnitine palmitoyl transferase (CPT) activity (*A*) and gene expression (*B*) analysis in the isolated fraction of gastrocnemius muscle of sedentary (Sed) rats and of rats subjected to moderate-intensity continuous training (MI-CT) and high-intensity intermittent training (HI-IT). Data are reported as means ± SEM (N = 9 for maximal activity and N = 5 for gene expression). *P < 0.05 *vs* Sed; $^+$P < 0.01 *vs* Sed and MI-CT (one-way ANOVA).

Since there were no detectable differences in mitochondrial protein content or in CPT-I and -II gene expression in the gastrocnemius muscle, the increase in maximal CPT-I and -II activity in the HI-IT protocol most likely reflects an enhanced catalytic capacity of these two enzymes. It is noteworthy that 16 h of accumulated HI-IT for 8 weeks (including adaptation protocol and resting intervals) promoted similar adaptations in the maximal activity of CPT-I and -II to those elicited by 37.6 h of the MI-CT protocol.

Citrate synthase activity is one of the most commonly used markers of muscle oxidative capacity because it occurs in a constant proportion with other mitochondrial enzymes (17). In agreement with the CPT-I and -II results, citrate synthase activity was enhanced in both HI-IT and MI-CT protocols. Previous studies adopting very short sprints (<10 s) failed to observe an increase in citrate synthase activity (18). In contrast, most studies, including ours, report increases in citrate synthase activity in high-intensity training, when sprint bouts lasting 15-30 s in humans (4,19-22) and rats (3) are adopted. In fact, the results of citrate synthase activity in the present HI-IT protocol are similar to those reported following 2-6 weeks of HI-IT in humans (6,22,23). However, citrate synthase activity was less increased in the HI-IT protocol than in the MI-CT protocol. This might be due to the type of fiber recruitment induced by HI-IT (mainly short twitch contraction fibers type II) (24).

MI-CT is known to increase intramuscular triacylglycerol, while data related to HI-IT are controversial. Our results demonstrate that total muscle fat content was not altered by any of the present training protocols. However, gene expression of LPL, which is the enzyme responsible for rendering the fatty acid in lipoproteins available to the muscle, was increased in HI-IT. This may be taken as additional support of the interpretation that lipid metabolism is increased in muscle after HI-IT. This interpretation is further corroborated by the finding that plasma levels of VLDL, cholesterol, and TG were lower after the HI-IT protocol compared to the values for sedentary animals (25,26).

Several studies have examined muscle metabolic and/or performance adaptations to intermittent versus continuous training (27-29). However, in all cases the total volume

Table 4. Ratio of gene expression of molecular markers of lipid metabolism in the gastrocnemius muscle/GADPH by RT-PCR.

	Sed	MI-CT	HI-IT
FABP	0.78 ± 0.15	0.21 ± 0.08	1.60 ± 0.18*
LPL	1.18 ± 0.44	2.09 ± 0.64	4.37 ± 0.89*
PPARα	1.12 ± 0.11	1.04 ± 0.24	0.65 ± 0.27
PPARβ	0.95 ± 0.25	0.56 ± 0.04	1.03 ± 0.29
PGC1-α	1.35 ± 0.2	0.94 ± 0.39	1.37 ± 0.35

Data are reported as means ± SEM of the ratio gene/GAPDH. Sed = sedentary; MI-CT = moderate-intensity continuous training; HI-IT = high-intensity intermittent training; FABP = fatty acid binding protein; LPL = lipoprotein lipase; PPARα/β = peroxisome proliferator-activated receptor α and β, respectively; PGC1-α = peroxisome proliferator-activated receptor-γ coactivator-1α. *P < 0.05 *vs* Sed (one-way ANOVA).

Table 5. Plasma concentrations of very low-density lipoprotein-triglycerides (VLDL-TG), TG, cholesterol, and insulin after 8 weeks of training.

	Sed	MI-CT	HI-IT
VLDL-TG (mM)	0.18 ± 0.01	0.17 ± 0.01	0.14 ± 0.01**
TG (mM)	0.90 ± 0.05	0.84 ± 0.03	0.69 ± 0.05*
Cholesterol (mM)	2.20 ± 0.1	1.70 ± 0.10*	1.80 ± 0.01*
Insulin (μU/mL)	5.59 ± 0.30	4.81 ± 0.26	4.71 ± 0.25

Data are reported as means ± SEM. Sed = sedentary; MI-CT = moderate-intensity continuous training; HI-IT = high-intensity intermittent training. *P < 0.05 *vs* Sed; **P < 0.05 *vs* Sed and MI-CT (one-way ANOVA).

of work was similar for all groups. In agreement with the results observed in humans by Gibala et al. (6), our data indicate that, despite the difference in volume, 16 h of HI-IT (including resting intervals) and 37.6 h of MI-CT promote lower weight gain compared to sedentary animals, indicating that HI-IT may be a "time-efficient" strategy to promote weight loss. Although the cause of such efficiency of HI-IT

is unknown, several factors may be proposed. It is expected that a higher effort intensity should recruit more fibers (30), especially of the fast twitch kind (31), increased recruitment of stabilizing muscles and increased work of the heart and respiratory muscles (32), hence resulting in higher energy consumption rates compared to submaximal-intensity exercise. It is not surprising, therefore, that high-intensity intermittent exercise has been considered a good option for weight reduction both in rodent models (33) and in human obesity (34).

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