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Endurance training blocks uncoupling protein 1 up-regulation in brown adipose tissue while increasing uncoupling protein 3 in the muscle tissue of rats fed with a high-sugar diet

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ABSTRACT

The mitochondrial uncoupling proteins (UCPs) of interscapular brown adipose tissue (iBAT) and of muscles play important roles in energy balance. For instance, the expression of UCP1 and UCP3 are modulated by free fatty acid gradients induced by high-sugar diets and acute exercise that is dependent on sympathetic stimulation. However, the effects of endurance training in animals fed with high-sugar diets are unknown. This study aims to evaluate the long-term effects of diet and exercise on UCP1 and UCP3 levels and energy balance efficiency. Rats fed with standard or high-sugar (HSD) diets were simultaneously subjected to running training over an 8-week period. After the training period, the rats were decapitated, and the iBAT and gastrocnemius muscle tissues were removed for evaluation of the β_3 -receptor, *Ucp1*, and *Ucp3* mRNA and protein expression, which were analyzed by quantitative reverse transcriptase polymerase chain reaction and Western blot, respectively. Groups fed with an HSD displayed a higher adiposity index and iBAT weight ($P < .05$), whereas exhibited an up-regulation of *Ucp1* mRNA and protein levels ($P < .05$). Training increased β_3 -receptor mRNA in iBAT and reduced the *Ucp3* mRNA in muscle tissues. In association with an HSD, training restored the increasing β_3 -receptor mRNA and greatly up-regulated the levels of *Ucp3* mRNA. Therefore, training blocked the HSD-induced up-regulation of UCP1 expression in iBAT, whereas it up-regulated the expression of *Ucp3* mRNA in muscle. These results suggest that training enhances the relationship between *Ucp1/Ucp3* mRNA levels, which could result in higher energy efficiency, but not when HSD-induced elevated sympathetic activity is maintained.

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Abbreviations: HSD, high-sugar diet; iBAT, interscapular brown adipose tissue; RGD, rat genome database; S-HSD, sedentary–high-sugar diet; S-STD, sedentary–standard diet; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; T-HSD, trained–high-sugar diet; T-STD, trained–standard diet; UCP1, uncoupling protein 1; UCP3, uncoupling protein 3.

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1. Introduction

The sympathetic nervous system stimulates brown adipose tissue (BAT) via noradrenergic nerve endings, inducing non-shivering thermogenesis [1]. Noradrenaline binds to β_3 , α_1 , and α_2 -adrenergic receptors, triggering downstream signaling events that impinge on lipolysis, thermogenesis, apoptosis, and gene expression [2]. In this way, the activation of the β_3 receptor in BAT initiates a cascade of metabolic events that culminate in the activation of UCP1, an uncoupling protein (UCP) that is found in the mitochondria and that is responsible for the translocation of protons through the respiratory chain and consequently for heat production [1]. In addition, prolonged β -adrenergic stimulation of BAT results in sustained thermogenic activity by increasing UCP1 expression and mitochondrial number, as well as by causing hyperplasia of BAT [1,3,4].

The thermogenic activity of the interscapular brown adipose tissue (iBAT) modulates energy expenditure in mammals, thereby contributing to the maintenance of energy balance [5,6]. Obesity, cold exposure and metabolic hormones, such as leptin and thyroid hormones, can induce the expression of UCP1, resulting in the hyperplasia of BAT and non-shivering thermogenesis [3,5,6]. These observations suggest an important role for UCP1 in energy balance during situations of altered metabolism [6–8].

Ingestion of nutrients increases energy expenditure above basal metabolic rates [9]. Indeed, diet-induced thermogenesis stimulates the activation of BAT, which increases its tissue mass [10,11], in an apparent physiological effort to restrain weight gain and obesity [6]. Highly palatable diets, as well as diets with high levels of sugar, are used as a method to increase BAT and consequent UCP1 expression [2], as high-sugar diets have previously been shown to increase the metabolic and hyperplastic rates of BAT, as well as plasma levels of triacylglycerol and free fatty acids [12].

At the end of the last decade, UCP3, a homolog of UCP1, was discovered and found to be related to the regulation of energy expenditure in skeletal muscle. As UCP3 expression appears to be regulated by the same mechanisms as other mitochondrial constituents, energy intake and fatty acids metabolism also appear to contribute to its increased expression [13]. Furthermore, because energy expenditure, as well as energy intake, has an important impact on the overall energy balance, it is conceivable that UCP3 plays a role in regulating metabolic rate and weight control. Studies that have reported reduced weight gain and resting metabolic rates have also found simultaneous decreases in UCP3 mRNA expression and protein levels in muscle [14,15].

Ucp3 mRNA expression is transiently up-regulated after acute exercise, an effect that is largely attributable to increased free fatty acids levels after exercise [16]. The increased levels of free fatty acids are proposed to activate an AMP-dependent protein kinase that regulates UCP3 expression during acute exercise [17]. After acute exercise, free fatty acids are released from adipose tissue, which can account for the relatively transient increase in UCP3 expression.

However, during physical training, UCP3 is rapidly down-regulated. Studies have suggested that this down-regulation is related with energy efficiency. The reduction of UCP3 associ-

ated with training might be responsible for enhanced energy efficiency, as concomitant negative effects on mechanical efficiency and oxygen consumption have been observed [18].

Taking these data into account, we hypothesize that a high-sugar diet will increase UCP1 and UCP3 mRNA levels due to elevated sympathetic activation and increased serum levels of free fatty acids, which might contribute to altered energy efficiency. In addition, physical training might restore the effects induced by a high-sugar diet upon induction of increased serum levels of free fatty acids. Our understanding of the combinatorial effects of diet and exercise in long-term energy balance remains limited and warrants further investigation. Thus, we sought to analyze the influence of physical training associated with a high-sugar diet on the expression of UCP1 and UCP3.

2.0. Methods and materials

2.1. Animals

All of the experimental procedures were approved by the Ethics Committee of the Federal University of Minas Gerais for the Care and Use of Laboratory Animals (protocol 192/08) and were conducted in accordance with the regulations described in the Committee's Guiding Principles Manual.

Four-week old weaned male Wistar rats were housed in individual cages under controlled light (0500–1900 hours) and temperature ($24.0 \pm 2.0^\circ\text{C}$) conditions with water and rat chow provided ad libitum. Before the beginning of any experimental procedures, the animals were randomly divided into the following four groups: (1) sedentary rats fed with a standard chow diet (S-STD, sedentary–standard diet; $n = 6$), (2) trained rats fed with a standard chow diet (T-STD, trained–standard diet; $n = 6$), (3) sedentary rats fed with a high-sugar diet (S-HSD, sedentary–high-sugar diet; $n = 6$), and (4) trained rats fed with a high-sugar diet (T-HSD, trained–high-sugar diet; $n = 6$).

2.2. Diet

The animals were fed during an 8-week period with a high-sugar diet (S-HSD and T-HSD groups), consisting of 33% standard chow (Nuvilab CR1; Nuvital, Brazil), 33% condensed milk and 7% sucrose by weight (the remainder is water). The control groups were fed only STD. The ingredient and nutrient composition of the diets is shown in Table 1

The body weight and energy intake were measured once a week during the observation period. At the termination of the study, the energy intake was computed by multiplying the weekly food intake by the energy density of the STD (12.22 kJ) and the HSD (13.31 kJ) groups.

2.3. Exercise training

All of the animals were first conditioned to exercise on a motor-driven treadmill (Gaustec, Contagem, Brazil) by running at a speed of 10 m min^{-1} at 5% inclination for 5 minutes per day for the 5 consecutive days before exercise training.

Next, the rats were subjected to a workload running test that increased from an initial velocity of 10 m min^{-1} (5%

Table 1 – Ingredient and nutrient composition of diets fed to rats for an 8-week period

Ingredient or nutrient	Dietary treatments g/kg	
	Standard chow ^b	High-sugar ^a
Carbohydrate (% energy)	57.5	68
Moisture	125	270
Protein	220	95.7
Lipid	40	57.8
Ash	90	29.7
Fiber	70	23.1
Calcium ^b	12	960.96
Phosphorus ^b	0.008	2.64
Vitamin A ^b	0.0026	0.001
Vitamin B ₁ ^b	0.005	0.02
Vitamin B ₂ ^b	0.006	0.02
Vitamin B ₆ ^b	0.007	0.02
Vitamin B ₁₂ ^b	2.2×10 ⁻⁵	0.007
Niacin ^b	0.06	0.02
Folic acid ^b	0.001	3.3×10 ⁻⁴
Biotin ^b	5×10 ⁻⁵	1.65×10 ⁻⁵
Choline ^b	1.9	0.627
Iron ^b	0.05	0.0165
Sodium ^b	2.7	1.188
Magnesium ^b	6×10 ⁻⁸	1.98×10 ⁻⁸
Zinc ^b	0.06	0.0198
Copper ^b	0.01	0.003
Selenium ^b	5×10 ⁻⁵	1.65×10 ⁻⁵
Protein (% energy)	30	16

HS diet (g/kg): rat chow NUVILAB-CR (33%); sweetened condensed milk (33%); granulated sugar (7%) and water.

^a Adapted by [12].

^b Based on the manufacturer's information.

inclination) at increments of 1 m min⁻¹ every 3 minutes until fatigue [19] to determine the maximal performance prior to exercise training. Fatigue was defined as the point at which the animals were no longer able to maintain pace with the treadmill for at least 10 s [20]. During the first test, the performance evaluated by exercise time to fatigue did not differ among experimental groups. This test was repeated at the end of the eighth week of training to assess improvement in running performance. Exercise time to fatigue and workload were considered indices of performance. Workload (W) was calculated as $W = [\text{body weight (kg)}] \times [\text{time to fatigue}] \times [\text{treadmill speed (m min}^{-1})] \times [\sin \theta (\text{treadmill inclination})]$ [21].

After the first incremental workload, the rats were subjected to the exercise training protocol, which consisted of daily running sessions with gradual increases of intensity. The protocol started at 10 m min⁻¹ during a 30-minute period and was increased until the rats were able to run at 25 m min⁻¹ (5% inclination) for 60 m min⁻¹. The protocol was adapted from a previous study [22], and the achievement of this exercise intensity and duration ensured that significant endurance training adaptation had been produced. Exercise training was conducted over an 8-week period, a time frame in which stabilization of food intake and reduced UCP3 expression in muscle has been previously reported [23,24]. To ensure that all of the animals were subjected to consistent handling procedures, the S-STD and S-HSD groups were subjected to running exercises for 2 minutes following the same physical training schedule. All running procedures were performed

between 8 and 11 AM at an ambient temperature of 23°C ± 1°C to reduce environmental interference on the physical performance of the animals [25,26].

2.4. Euthanasia

Twenty-four hours after completion of the physical training protocol, the animals were decapitated, and iBAT and gastrocnemius muscles were dissected, weighed, frozen in liquid nitrogen, and stored at -80°C until further analysis. The adiposity index was used to evaluate the development of obesity induced by HSD and was calculated as $100 \times (\text{sum of fat pad weights})/(\text{body weight})$ [27]. To calculate the fat pad weights, epididymal, retroperitoneal, inguinal white adipose tissues were used.

2.5. Enzyme assay

The citrate synthase activity in soleus muscle tissues was measured as biomarker of oxidative metabolism using the Citrate Synthase Assay kit (Sigma-Aldrich). Tissue samples were homogenized in a solution of 50 mM Tris-HCl, 1 mM EDTA, and 0.01 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.4). Homogenization was performed using a Polytron homogenizer on the highest speed setting for 30 s. Homogenates were then centrifuged at 725×g for 10 minutes at 4°C. The supernatant was decanted, and citrate synthase activity was assayed according to the manufacturer's protocol.

2.6. Total RNA preparation and expression analysis of Ucp1, Ucp3, and β₃-receptor by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA from iBAT and gastrocnemius muscle tissues were obtained using a combination of the Trizol reagent (Invitrogen, São Paulo, Brazil) and chloroform (Sigma-Aldrich) for extraction and then purified using the SV total RNA Isolation System (Promega, Belo Horizonte, Brazil) according to the manufacturer's protocol. The RNA was quantified using a NanoVue, followed by electrophoresis and analysis on a 1.2% agarose formamide-Tris-Borate-EDTA gel. The RNA preparation was treated with RNase-free DNase I in 3 different rounds of decreasing enzyme concentrations (RQ1 DNase; Promega). The extracted RNA was measured at a wavelength of 260 nm, where the 260/280 wavelength ratio was indicative of purity and the 260/230 wavelength ratio was indicative of contamination. Ratios above 1.8 were considered acceptable for the quantification of gene expression [28].

One microgram of total RNA was reverse-transcribed using random primers from the high-capacity reverse transcriptase PCR system (Applied Biosystems) according to the manufacturer's protocol. The complementary DNAs (cDNAs) encoding for Ucp1, Ucp3, β₃-receptor, and rRNA 18S were obtained by PCR amplification using specific primers. The efficiency of DNase I treatment was evaluated by PCR amplification of the cDNA reaction mix without the addition of the reverse transcriptase (Multi Scribe Reverse Transcriptase). Primers were designed using the Gene Runner Software and sequences deposited in Rat Genome Database (RGD) as follows (upstream and downstream, respectively): Ucp1 [RGD: NM 012682]

(forward 5'-CAAAGTCCGCCTCAGATC-3', reverse 5'-TGGTGATGGTCCCTAAGAC-3'); *Ucp3* [RGD: NM_013167.2] (forward 5'-CCCAAAGGAACGGACCACTC-3', reverse 5'-GGGTTGAGCACAGGTCAGT-3'); β 3-receptor (*Adrb3*) [RGD: NM_013108] (forward 5'-TGGTGATGGTCCCTAAGAC-3', reverse 5'-CACGCCGAGACTACAGAC-3'), and *rRNA 18S* [RGD: X01117.1] (forward 5'-GTAAGTGGGGTGCATAAG-3', reverse 5'-CCATCCAATCGGTAGTAGC 3'), used as endogenous control. Reverse-transcribed cDNA samples were used as templates for PCR amplification using the SYBR Green PCR Master Mix (Applied Biosystems) and Applied Biosystems ABI 7300. The efficiency for each pair of primers was evaluated according to the protocol developed by the Applied Biosystems application (cDNA dilutions were 1:10, 1:100 and 1:1000). For the investigated transcripts, 3 biological replicates were performed, and their gene expression was normalized against the *rRNA 18S* transcript according to the $2^{-\Delta C_t}$ method [29] using the Applied Biosystems ABI 7300 software. The relative expression ($2^{-\Delta \Delta C_t}$ method) was measured using the S-STD group as the calibrator sample, where its expression was considered as the 1 \times control index for comparison with other genes.

2.7. Western blot analysis for UCP1

Determination of UCP1 protein was performed by Western blotting using a rabbit polyclonal antibody against UCP1 (AB1426, Millipore Corporation, Billerica, MA, USA). One hundred milligrams of iBAT was homogenized with a Polytron homogenizer in lysis buffer (50 mM Tris-HCl pH 6.8, 1 mM EDTA, 1% NP40, 150 mM NaCl, 2 mM DTT and 1 mM of the following proteases inhibitors: PMSF, Tosyllysine chloromethylketone (TLCK), and Tosyl phenylalanyl chloromethyl ketone (TPCK)). After centrifugation at 725 \times g for 30 minutes, the concentration of soluble proteins was determined with a QuantiPro BCA Assay Kit (Sigma-Aldrich). Fifty micrograms of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide gel) and electrotransferred to polyvinylidene difluoride membranes at 25 V for 2 h at 4°C. After 16 h of incubation in blocking solution, the membranes were washed and incubated with primary antibodies (1:500). The results were visualized by BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) color development substrate (Promega) using HRP-conjugated anti-rabbit IgG antibodies (Sigma Aldrich). To normalize the blots, we used the total protein levels obtained on 12% SDS-PAGE replicate gels [30]. Relative expression levels were obtained by densitometric analysis of bands using Quantity One (Bio-Rad) software.

2.8. Statistical analyses

Statistical analyses were performed using Graph Pad Prism version 5.0 software package (Irvine, CA, USA). The sample size had considered the minimum difference between means and standard deviation of the errors, with a power of 0.9 and significance level (α) of .05. We chose the highest estimated size to assess our outcomes ($n = 6$). Normalization of the data was verified using the Shapiro-Wilk test. The data are reported as the mean \pm SD. Differences between groups were evaluated using 2-way analysis of variance (ANOVA)

followed by the Bonferroni test. $P < .05$ was considered statistically significant.

3. Results

3.1. Effects of diet and endurance training on energy intake and body weight

Energy intake increased over time and stabilized starting from the sixth week across all groups. The alimentary patterns did not differ among groups, even those fed with an HSD (Fig. 1, top).

Consistently, body weight progressively increased during the 8 weeks for all 4 groups, without any differences among them (Fig. 1, bottom). However, the adiposity index was approximately 76% (Table 2; $P < .05$) higher in the S-HSD animals compared to S-STD animals. Endurance training reduced the adiposity index in animals fed with a standard

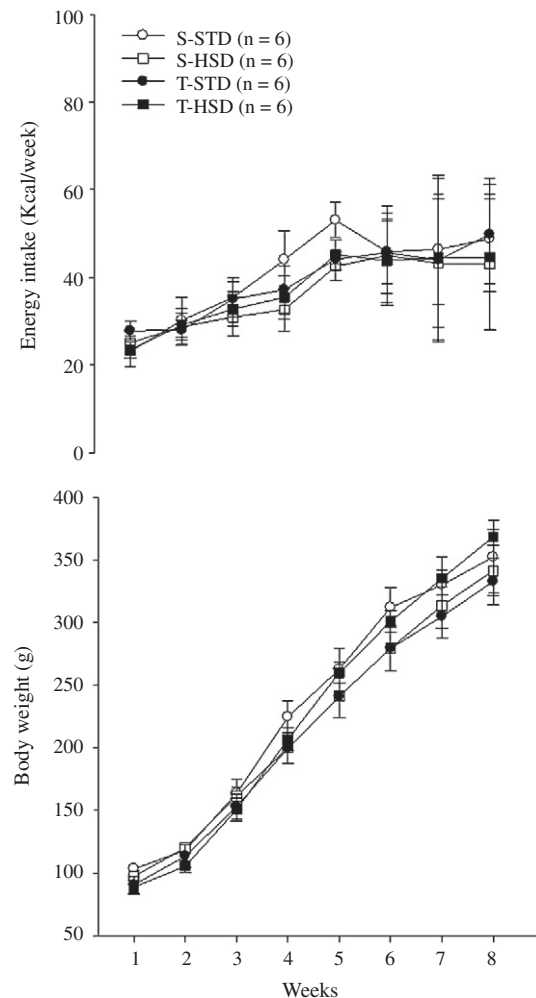


Fig. 1 – Energy intake (top) and body weight (bottom) of rats fed either a standard or high-sugar diet and subjected to exercise training over an 8-week period. The data are expressed as means \pm SD. The analysis of the effects of diet and/or training was performed by two-way ANOVA (Bonferroni text) and $P < .05$ was considered statistically significant.

Table 2 – Adiposity index and exercise intensity in sedentary and trained groups of rats

	S-STD group	S-HSD group	T-STD group	T-HSD group
Adiposity index	2.8 ± 0.3	4.6 ± 0.63*	1.5 ± 0.23*	4.3 ± 0.46 [†]
Workload	48.4 ± 5	45.6 ± 4.7	86.2 ± 11*	77.5 ± 11.5 [‡]
CS activity ($\mu\text{mol mL}^{-1} \text{min}^{-1}$)	8.8 ± 1.96	11.3 ± 1.74	14.9 ± 1.32*	18.4 ± 1.13 [‡]

The data are expressed as the means ± S.D. (n = 6). All comparisons were performed by 2-way ANOVA (Bonferroni test). $P < .05$ were considered statistically significant. *Statistical differences compared to S-STD group; [†]Statistical differences compared to T-STD group; [‡]Statistical differences compared to S-HSD group. CS, citrate synthase.

diet but was insufficient to revert its increasing in T-HSD rats (Table 2).

3.2. Effects of diet and endurance training on performance and citrate synthase activity

The running workload was improved in trained rats (T-STD and T-HSD) compared to sedentary groups (S-STD and S-HSD). The running workload was measured during the incremental exercise tests until fatigue at the end of endurance training (Table 2; $P < .05$). Similarly, the exercise training increased the citrate synthase activity in the soleus muscle tissue of trained rats ($P < .001$), regardless of diet, compared to sedentary rats (Table 2).

3.3. Effects of diet and endurance training on iBAT: $\beta 3$ -receptor and UCP1 expression

As shown in Fig. 2A, an HSD directly influences iBAT weight ($P < .001$), inducing an increase of approximately 65% in the

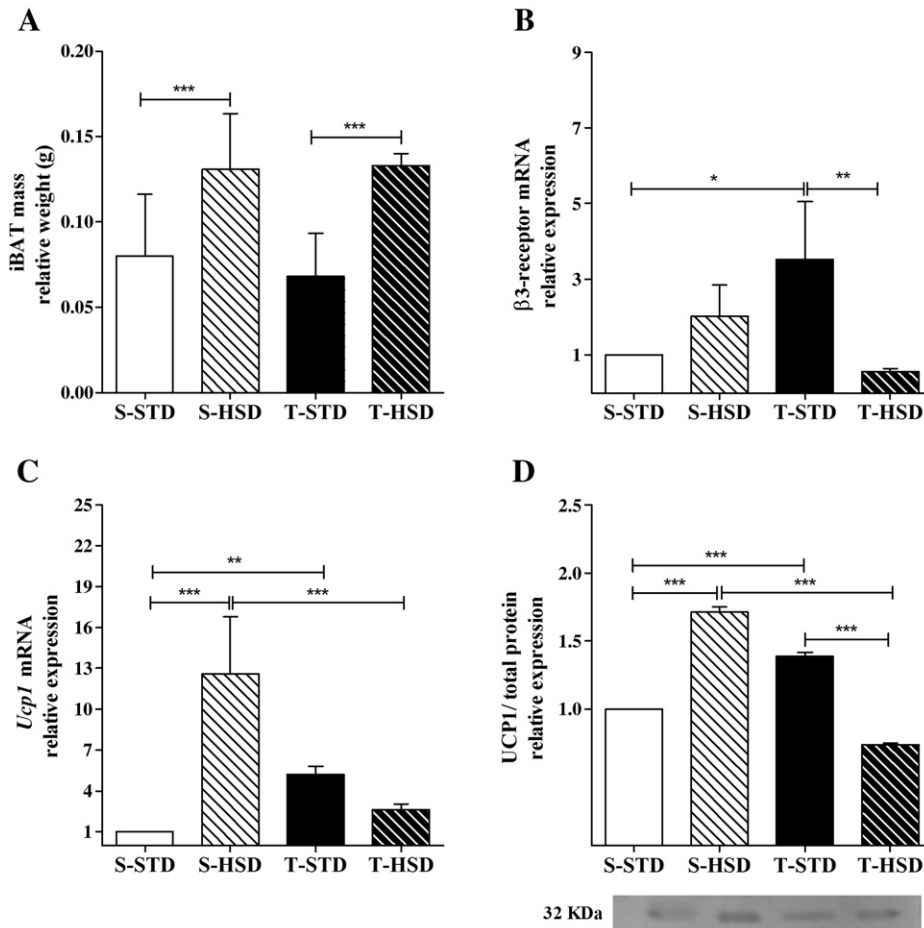


Fig. 2 – Effect of a high-sugar diet and exercise training over an 8-week period on iBAT, $\beta 3$ -receptor mRNA levels, and UCP1 expression. (A) Relative iBAT weights. Data are expressed as means ± SD. (B) $\beta 3$ -receptor, (C) UCP1 mRNA levels, and (D) UCP1/protein. The gene expression profiles were evaluated in the S-STD, S-HSD, T-STD, and T-HSD groups by the $2^{-\Delta\Delta Cq}$ method. rRNA18S was used as reference gene and the S-STD group was used as a calibration sample, where its expression was considered at 1× control index for comparison (B) Western blot anti-UCP1. Fifty micrograms of crude extract was analyzed by 12% SDS-PAGE followed by immunoblotting analysis using an anti-UCBP1 antibody. Densitometry was performed using Quantity One Software (Bio-Rad). The effects of diet and/or training were performed by 2-way ANOVA (Bonferroni test) and $P < .05$ were considered statistically significant. * $P < .05$, ** $P < .01$, and * $P < .001$.**

S-HSD group compared to the S-STD group. Although endurance training resulted in a decreased adiposity index (Table 2), it did not elicit any differences in iBAT of animals fed with a standard diet. However, exercise training did not reverse the increased iBAT weight when rats were fed with an HSD (Fig. 2A).

The relative expression of *Ucp1* mRNA in iBAT was directly affected by diet ($P < .001$), training ($P < .01$) and the interaction between both treatments ($P < .001$) (Fig. 2C). Compared to the S-STD group, the HSD and the exercise up regulated *Ucp1* mRNA expression by approximately 12- and 5-fold in the S-HSD and T-STD groups, respectively. Conversely, compared to the S-HSD group, the transcription of *Ucp1* was down-regulated by 7.2-fold in exercised rats of the T-HSD group.

We performed immunoblot analysis to verify whether the elevated levels of *Ucp1* mRNA were reflected at the protein level. As shown in Fig. 2D, the HSD increased UCP1 levels (~50%) and the combination of diet and exercise down regulated the protein levels, corroborating our observations of *Ucp1* transcription (Fig. 2C).

One mechanism involved in the regulation of UCP1 expression is the link between noradrenaline and β_3 -receptors and the activation of their downstream pathways. Next, we analyzed the β_3 -receptor mRNA levels in iBAT to assess their role in the processes induced by the experimental treatments, and we found that the transcription of the β_3 -receptor in iBAT was directly affected by the combination of diet and exercise ($P < .01$). The β_3 -receptor mRNA levels were approximately 3.5-fold higher in the T-STD group compared to S-STD group, and the rats in the T-HSD group had transcript levels that were approximately 6-fold lower than those in the T-STD group (Fig. 2B).

3.4. Effects of diet and endurance training on the *Ucp3* mRNA levels in gastrocnemius muscle tissue

The tissue-specific expression pattern of each UCP is distinct, which might reflect separate thermoregulatory roles for the

UCPs. Therefore, we analyzed the *Ucp3* mRNA expression levels in gastrocnemius muscle tissues in order to assess its contribution to thermogenesis in our model. Compared to the S-STD group, exercise training down-regulated the relative expression of *Ucp3* in gastrocnemius muscle tissues (Fig. 3A). Furthermore, the relative expression of *Ucp3* in the gastrocnemius muscle tissues of the T-HSD group was up-regulated by approximately 4.5-fold compared to the T-STD group and 2.5-fold compared to the S-HSD group (Fig. 3A). The ratio between *Ucp1/Ucp3* mRNAs is shown in Fig. 3B and illustrates that exercise training contributed substantially to HSD-induced adaptations of energy expenditure.

4. Discussion

The main finding of this study is that endurance training of rats fed with a standard diet improved energy efficiency, which was associated with increased ratios of *Ucp1/Ucp3* mRNA. However, when the rats were fed with a high-sugar diet, the stimulus of training was insufficient to reduce the adiposity index and iBAT weight. In addition, the combination of training and HSD blocked the increase of β_3 -receptor and UCP1 expression in iBAT observed in the S-HSD rats and up-regulated the transcription of *Ucp3* in muscle.

Previous studies using the same HSD found a robust increase in energy intake [12] and, consequently, in weight gain [12,31–33], consistent with the obesity-inducing effects of the HSD. In all of these studies, the HSD was administered over long time frames and was observed to alter energy intake and body weight as early as 6 to 8 weeks. In addition, when an HSD was provide to weaning rats, the onset of alterations in weight gain were delayed, suggesting a perturbation in animal growth [12]. Although such responses of increased energy intake and body weight were not observed in our study, the administration of an HSD induced elevated adiposity indices and hyperplasia of iBAT, indicating that dietary stimulus was effective in inducing obesity and elevated sympathetic

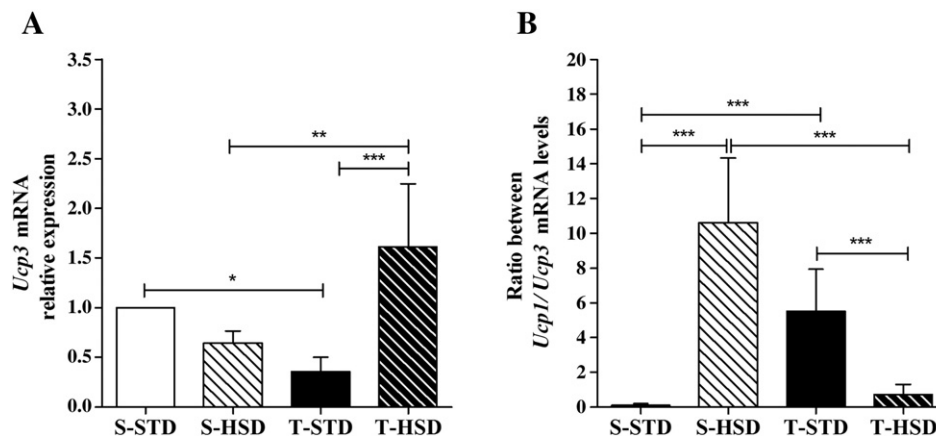


Fig. 3 – Effect of a high-sugar diet and exercise training over an 8-week period on *Ucp3* mRNA levels in gastrocnemius muscle (A) and ratio of *Ucp1/Ucp3* mRNA (B). (A) The gene expression was evaluated in S-STD, S-HSD, T-STD, and T-HSD groups by the $2^{-\Delta\Delta Cq}$ method. The reference gene *rRNA18S* was used as the calibration sample for the S-STD group, where its expression was considered as the 1x control index for comparison. The effect of diet and/or training was performed by 2-way ANOVA (Bonferroni test) and significance level was accepted at $P < .05$. (B) The ratio of *Ucp1/Ucp3* mRNA was obtained with the $2^{-\Delta Cq}$ values for each group. * $P < .05$, ** $P < .01$ and * $P < .001$.**

activation. De Lima et al [12] had previously found enlarged white and brown adipose pads and an increased resting metabolic rate associated with serum triacylglycerol and free fatty acid levels. Notably, these factors influence the expression of UCP1 and UCP3 [30,34,35]. The iBAT levels of UCP1 have been previously shown to correlate positively with an increased noradrenaline response and negatively with metabolic efficiency [36], suggesting a response that attenuates the development of obesity.

In this study, we observed the up-regulation of UCP1 expression in response to an HSD, although the intensity of the effects differed at the mRNA and protein levels. The regulation of UCP1 levels has been proposed to involve the β 3-receptor/CREB (cAMP response element-binding protein), as well as further influence from UCP1 mRNA stability and from the delay caused by translation [36]. This temporal delay is a consequence of the relatively slow turnover of UCP1 protein in its native environment [37] and includes the synthesis of new mitochondria, and thus, a difference between mRNA and protein levels may occur without an observable physiological significance. This notion is consistent with our experimental system, as our rats were not fasted prior to euthanasia, which has been suggested to promote acute decreases in *Ucp1* mRNA levels that are not reflected by changes at the protein level [36].

The HSD also induced the up-regulation of β 3-receptor mRNA levels, and preliminary experiments have shown that an HSD increased total catecholamine levels in iBAT (unpublished observations). These findings suggest that an additional mechanism of adrenergic stimulation must be involved in UCP1 regulation [11,38] because elevated β 3-receptor mRNA levels might indicate reduced sympathetic activation [39] or might indicate receptor desensitization by increased levels of catecholamine. Therefore, another mechanism that might regulate UCP1 in response to an HSD in iBAT includes serum leptin [11,38].

Diet-induced enlarges in fat pads produce increasing levels of leptin, which signal to suppress food intake and to increase heat production by UCP1. Although energy intake remained constant in our study, previous studies have shown that plasma leptin concentrations increase proportionately with the expansion of adipose tissue [12]. Thus, we speculate that increased plasma leptin levels contribute to regulate energy efficiency as an inefficacious response to prevent weight gain. Consistent with this notion, rats fed with an HSD were previously found not to exhibit altered serum glucose concentrations but rather to exhibit hyperinsulinemia [12,32], which also modulates UCP1 expression [40,41].

UCP3 expression has been reported to increase in obese rats [42,43] because of elevated circulating levels of fatty acids [43]. Although an HSD augmented the adiposity index, free fatty acids were unchanged (unpublished observations). This result could explain the lack of variation in *Ucp3* mRNA levels of muscle tissues. This result indicates that UCP3 levels in gastrocnemius muscle tissue play a physiological role in the mitochondrial processing of fatty acids, which is likely accompanied by regulation of energy efficiency. UCP3 levels have previously been shown to exhibit tissue-specific differences in expression [42], suggesting that the response could be modified in other tissues, such as iBAT or heart.

Improving energy efficiency is one the adaptive responses already that has already been described during exercise training. The activity of mitochondrial enzymes is used to confirm oxidative adaptation induced by training in skeletal muscle [44]. The endurance training protocol used in this study increased citrate synthase activity and workload in trained rats. Thus, compared to untrained animals, the maximum energy consumption was elevated in trained animals, contributing to higher fatty acid metabolism with the same absolute power during physical activity. In addition, the analysis of the workload confirmed the effectiveness of the training protocol, owing to the increased maximal velocity and exercise time until fatigue exhibited during the incrementally increasing workload running test. These values indicate increased physical performance and endurance before fatigue [45–47].

Endurance training can reduce the adiposity index without altering body weight. Adipose pads might be replaced by lean muscle mass, accounting for the unchanged body weight. Furthermore, iBAT was not altered in the T-STD group. Notably, exercise can itself augment the metabolism and heat-generating process, which might result in a decreased requirement for iBAT recruitment and UCP1 levels [48]. Moreover, the effects of exercise are transient, and the exact time elapsed between the end of training period and the measurement of brown fat-related parameters might determine whether an effect is observed [49]. Conversely, exercise training marginally increases serum fatty acid levels, which might restrain the atrophy of iBAT and reduction of UCP1. Consecutive sympathetic stimulations and the consequent elevation in serum fatty acid levels induced by the exercise sections of the training protocol might induce iBAT hypertrophy and up-regulation of the UCP1 and UCP3 levels. The lack of an effect on iBAT weight coupled with the up-regulation of the β 3-receptor suggest that exercise does not lead to chronic sympathetic stimulation in this tissue or regulation of the iBAT lipolysis and thermogenesis [35,39,50]. In addition, UCP1 was up-regulated, contributing to triglyceride turnover induced by endurance training. However, the increase in UCP1 activity is also associated with sympathetic tonus, and this uncoupling activity affects the efficiency of energy use. A decrease in metabolic efficiency, which increases the metabolic rate, might result in a decreased propensity to gain weight. Therefore, alterations in the UCP activities might have important effects on energy balance [51].

Previous studies have found that aside from UCP1, UCP3 levels are associated with changes in the adiposity index [52] and significantly contribute to thermogenesis and adaptations of energy expenditure. In this regard, endurance training resulted in significantly reduced UCP3 expression levels, which were negatively correlated with aerobic capacity [53]. This finding was corroborated by the denervation of the gastrocnemius muscles, which was associated with increased *Ucp3* mRNA levels [54]. Furthermore, the electric stimulation of inactive muscles has been reported to block the increase in UCP3 expression [55]. Our results show that the *Ucp3* mRNA levels were reduced in the T-STD group and were elevated after training, suggesting a role of fatty acid metabolism. Furthermore, UCP3 expression is altered in parallel with changes in energy efficiency, consequently increasing heat generation [56]. Considering the relation between adipose

pads and lean mass, we speculate that after exercise training, energy efficiency is increased and is better correlated with the ratio of *Ucp1/Ucp3* mRNA, as a functional representation of the replacement of the type of body tissues and changes in metabolism.

When the rats were administered an HSD, endurance training was insufficient to reverse the increase in the adiposity index or iBAT weight. However, the increased expression of β_3 -receptor and UCP1 was blunted in the T-HSD group. These data suggest that there is a subsequent modulation of iBAT weight because the tissue mass was still higher. Intriguingly, the UCP1 levels were not maintained or increased in parallel with the increased iBAT weight and presumably increased sympathetic activity, suggesting that the increased metabolic efficiency in trained rats is associated predominantly with decreases in UCP1 expression rather than with morphological alterations.

Considering the coregulatory effects between UCP homologues, such as the up-regulation of UCP3 in skeletal muscle and the concomitant down-regulation of UCP1 in iBAT [43,57], our results suggest that combined with exercise, an HSD increases the levels of *Ucp3* in muscle by facilitating the uptake and metabolism of lipids as fuel substrates from circulating free fatty acids [43,58], resulting in a down-regulation of UCP1 in iBAT, possibly by mechanisms that suppress iBAT thermogenesis and that accelerate fat deposition. Nonetheless, further experiments are warranted to evaluate the molecular mechanism underlying this process.

Whether sympathetic stimulation by an HSD is attributed to the endurance training, and the resulting impaired modulation of energy efficiency, remains unclear. Nonetheless, this possibility could be illustrated by the block of the increase in the ratio of *Ucp1/Ucp3* mRNA observed in the T-HSD rats.

Taken together, our findings suggest that training increases the ratio between *Ucp1/Ucp3* expression, which can mediate the induction of higher energy efficiency, but not when the HSD-induced elevated sympathetic activity is maintained. The relationship between *Ucp1* and *Ucp3* mRNAs might be responsible for the maintenance of body balance, allowing for changes in body composition, without change in body weight.

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