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## Original Research

# Long-term Leucine Supplementation Improves Metabolic But Not Molecular Responses in the Skeletal Muscle of Trained Rats Submitted to Exhaustive Exercise

Gustavo Barbosa dos Santos, PhD, André Gustavo de Oliveira, PhD, Maria Cristina Cintra Gomes Marcondes, PhD, Miguel Arcanjo Areas, PhD

*Department of Structural and Functional Biology, Institute of Biology, University of Campinas (UNICAMP), Campinas, São Paulo, BRAZIL (G.B.d.S., A.G.d.O., M.C.C.G.M., M.A.A.) and Sport Science Department, Faculty of Physical Education, Metropolitan College of Campinas (Metrocamp), Campinas, São Paulo, BRAZIL (G.B.d.S.)*

**Key words:** leucine, endurance performance, skeletal muscle

**Aim:** Although there is some evidence of an ergogenic effect of leucine supplementation on acute response to exercise, there is a paucity of information on whether long-term leucine supplementation influences the adaptive response to chronic endurance training and performance. The main aim of our study was to assess the role of long-term leucine supplementation on molecular and metabolic response in skeletal muscle of trained rats after an exhaustion test.

**Methods:** Twenty-four male Wistar rats were randomly allocated into 4 groups. Two of them (control and trained groups) received a balanced control diet (18% protein) and the other 2 (control leucine and trained leucine groups) received a leucine-rich diet (15% protein with 3% leucine) for 6 weeks. The trained groups were submitted to 1 hour of swimming exercise, 5 d/wk for 6 weeks. Three days after the exercise training period, trained groups were submitted to swimming exercise until exhaustion and muscle metabolic and molecular parameters were assessed.

**Results:** Endurance training increased citrate synthase activity significantly, whereas exercise until exhaustion increased cytokine levels and led to a lack of activation of phosphorylation of the signaling intermediates assessed. Long-term leucine supplementation enhanced muscle glycogen level in trained rats and citrate synthase activity in sedentary ones. However, it failed to enhance endurance performance of trained rats submitted to an exhaustion test and did not prevent exercise-induced reduction in Akt and mTOR activation.

**Conclusion:** Long-term leucine supplementation can enhance citrate synthase activity by itself in sedentary individuals and glycogen content when combined with exercise; however, it does not improve endurance performance or prevent Akt and mTOR exercise-induced inhibition.

## INTRODUCTION

Chronic adaptation of skeletal muscle to high-intensity endurance exercise is strongly dependent on nutrition [1]. Emerging evidence indicates a role for dietary protein and amino acids in mitigating skeletal muscle damage and increasing muscle protein turnover to promote adaptive remodeling [2]. It has been

demonstrated that branched chain amino acids, particularly leucine, can reduce prolonged exercise-induced muscle damage and accelerate the recovery process, which could lead to improvement in endurance performance [3]. It not only provides substrates for gluconeogenesis but also can supply tricarboxylic acid cycle with different anaplerotic substrates [4]. These effects are especially important for competitive endurance athletes, because they often

Address correspondence to: G. B. Santos, Comissão do Programa de Pós-Graduação em Biologia Funcional e Molecular, Instituto de Biologia, Caixa Postal 6109, Av. Bertrand Russell, Bloco O, Universidade Estadual de Campinas-UNICAMP, CEP-13083-865, Campinas SP, BRAZIL. E-mail: gubsantos@hotmail.com

Abbreviations: Akt = protein kinase B, AMPK = AMP-activated protein kinase  $\alpha$ , CS = citrate synthase, IL-6 = interleukin-6, mTOR = mammalian target of rapamycin, p-AMPK = AMPK phosphorylation, PGC-1 $\alpha$  = peroxisome proliferator-activated receptor-gamma coactivator, TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .

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train or compete intensely on a daily basis, sometimes until physical exhaustion, generating a constant state of tissue catabolism and muscle wasting.

Although some evidence for an ergogenic effect of leucine supplementation on acute response to exercise, mainly regarding protein synthesis [5] and muscle recovery [6,7], has been reported, there is a paucity of information on whether long-term leucine supplementation influences the adaptive response to chronic endurance training and performance.

The aim of this study was to investigate the role of long-term leucine supplementation on metabolic and molecular responses in skeletal muscle of trained adult rats submitted to exercise until exhaustion. We hypothesized that leucine supplementation could prevent or mitigate strenuous endurance exercise-induced muscle damage and fatigue, enhancing endurance training-induced muscle adaptation and contributing to improve endurance exercise performance.

## **MATERIAL AND METHODS**

### **Animals and Diets**

Twenty-four male Wistar rats (12 weeks old, weighing  $351 \pm 28.9$  g) were obtained from the animal facilities of the University of Campinas (São Paulo, Brazil) and equally divided into 4 groups. They were housed in collective cages at  $22\text{--}24^\circ\text{C}$  on a 12-hour light-and-dark cycle, with free access to tap water and food. The semipurified isocaloric diets were a normal protein (C), containing 18% protein [8], or leucine (L), containing 15% protein plus 3% of L-leucine. Approximately 70% carbohydrate (sucrose, dextrin, and starch), 7% fat (soybean oil), and 5% fiber (purified microcellulose) were added to the diets. Vitamin and mineral mix, as well as cystine and choline, supplemented the diets. The control diet had 1.6% of L-leucine, and a leucine-rich diet contained 4.6% L-leucine, according to a previous study from our group [9]. A leucine-supplemented diet has led to a significant increase in plasma leucine concentration in fetuses from tumor-bearing pregnant mice [10] and adult rats (data not shown). Two groups were fed the control diet—sedentary control (C) and trained (T)—and 2 other groups were fed the leucine-rich diet—control-leucine supplemented (CL) and trained-leucine supplemented (TL). All experimental procedures employed were in accordance with the Ethics Committee on Animal Experimentation of Unicamp (CEEA/IB/UNICAMP, protocol 2888-1).

### **Training Protocol**

The T and TL groups were submitted to the swimming protocol adapted from Barbosa dos Santos et al. [11], 5 d/wk for 6 weeks, in a water tank ( $90 \times 70 \times 70$  cm and water temperature at  $31 \pm 1^\circ\text{C}$ ), with approximately 400 L of water. All of the rats were adapted to the water during the first week of the

experiment. The adaptation process consisted of keeping the animals in shallow water, initially for 20 minutes and then progressively increasing 10 min/d and 10 cm water/d for 5 days. Exercise sessions began with 60 min/d at the second experimental week, carrying constant loads (added to the tail) of 20 g (approximately 6% of initial body weight) initially for 10 minutes of these 60 minutes, increasing by 10 more minutes each week, until it reached 60 minutes of loaded swimming training in the sixth and last weeks of the experiment. Three days after the exercise training period, rats were submitted to an exhaustion test in order to assess endurance performance. The animals swam carrying the same load until exhaustion. Exhaustion time was determined from the beginning of swimming to the point at which rats failed to return to the water surface within 10 seconds. Animals were sacrificed under anesthesia (ketamine and xylazine, 90 mg/kg/body weight and 45 mg/kg/body weight, respectively, i.p.) between 3 and 4 hours after exercise bout, in order to reach cytokine peak after swimming exhaustion test [12,13]. Body weights were assessed weekly and muscle tissue samples were weighed after the experimental period.

### **Glycogen Content**

Gastrocnemius muscles were quickly removed, frozen immediately in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until further analysis. Muscle glycogen content was estimated colorimetrically based on the method described by Lo et al. [14]. The absorbance was read on a plate CHAMELEON V Multilabel Microplate Reader (Hidex, Turku, Finland) at 620 nm.

### **Citrate Synthase Activity**

For citrate synthase analyses  $\approx 30$  mg of gastrocnemius muscle was homogenized in ice cold extraction buffer (175 mM KCl, 2 mM EDTA, pH 7.4) and centrifuged at  $16,000 \times g$  for 20 minutes at  $4^\circ\text{C}$ . An aliquot of supernatant was combined with the reaction mixture containing 0.1 M Tris, pH 8.3, 1 mM DTNB, and 3 mM acetyl-CoA. The reaction was initiated by adding 10 mM oxaloacetic acid to the extract. The absorbance was spectrophotometrically measured at 412 nm in 30-second intervals for 5 minutes using a Dynex MRX plate reader controlled through personal computer software (Revelation, Dynatech Laboratories, El Paso, TX), as previously described [15]. All samples were tested for linearity up to 5 minutes of reaction and values were normalized by protein concentration [16].

### **Western Blot**

Muscle samples ( $40 \mu\text{g}$ ) were homogenized and protein concentration was measured using a colorimetric method [16]. The proteins were revealed using primary antibodies against  $\alpha$ -tubulin (1:20,000), phospho-mTOR<sup>Ser2448</sup> (1:1000).

Phospho-Akt<sup>Thr308</sup> (1:1000), phospho-AMPK- $\alpha$ <sup>Thr172</sup> (1:1000; Cell Signaling, Danvers, MA), proteasome subunits 20S, 19S (1:1000; Enzo, Exeter, UK), and secondary anti-mouse, anti-rabbit, and anti-goat antibodies (1:10,000, Cell Signaling) after reaction with a chemiluminescent reagent (Thermo Fisher Scientific, Waltham, MA) were added and band volume was captured using an Alliance Captura 2.7 (UVItec, Cambridge, UK) and quantified using UVI band-1D (UVItec).

### Inflammatory Biomarkers

Blood samples were taken from the heart by ventricle puncture. Serum was separated by centrifugation at  $1000 \times g$  for 10 minutes at 4°C and stored at -80°C. The analysis of serum inflammatory markers (tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], interleukin [IL]-6) was determined using beads coupled with capture antibodies specific for each protein of interest as specified by the manufacturer Millipore (Merck Millipore Corporation, Darmstadt, Germany). The analysis was carried out on Xponent software used with the Luminex 200 (Luminex Corporation, Austin, TX) equipment, following the manufacturer's technical procedures.

### Statistical Analysis

Data are expressed as mean  $\pm$  SD. The data were analyzed statistically by one-way analysis of variance followed by Tukey's test to establish differences between groups. We used Prism software (Graphpad Software Inc., San Diego, CA). The results were considered significant when  $p < 0.05$ .

## RESULTS

### Functional Parameters

There were no differences in relative gastrocnemius weight between groups ( $p = 0.551$ ). Leucine supplementation, regardless of whether rats were submitted to endurance training, significantly increased body weight gain even when compared to their respective control groups (C vs CL,  $p = 0.009$ ; T vs TL,  $P = 0.004$ ). Leucine supplementation did not enhance exercise performance (T vs TL,  $p = 0.55$ ) when compared with exercise only (Table 1).

**Table 1.** Body Weight, Muscle Weight, and Time to Exhaustion<sup>a</sup>

	C	CL	T	TL
<b>Body weight gain (g)</b>	76.40 $\pm$ 11.52a	102.2 $\pm$ 10.57b	64.17 $\pm$ 13.89a	92.17 $\pm$ 11.99b
<b>Gastrocnemius (g/100 g)</b>	6.9 $\pm$ 0.43	6.2 $\pm$ 1.6	6.8 $\pm$ 0.84	5.6 $\pm$ 0.44
<b>Time to exhaustion (minutes)</b>	—	—	208.9 $\pm$ 19.5	195.3 $\pm$ 9.8

<sup>a</sup>Data are expressed as means  $\pm$  SD ( $n = 6$ ). Different letters indicate significant differences between groups ( $p < 0.05$ ).

### Muscle Metabolic Parameters

Leucine supplementation significantly increased citrate synthase (CS) activity when compared to sedentary groups (C vs CL,  $p = 0.005$ ), but this outcome was overcome when leucine supplementation was combined with the exercise protocol (CL vs TL,  $p = 0.002$ ; Fig. 1A). Glycogen content was significantly elevated ( $p = 0.012$ ) only in the trained leucine-supplemented group (TL), when compared to all experimental groups (Fig. 1B). AMPK phosphorylation (p-AMPK) was significantly enhanced ( $p = 0.001$ ) in trained groups compared to sedentary groups (Fig. 1C).

### Muscle Structural Parameters—Protein Synthesis

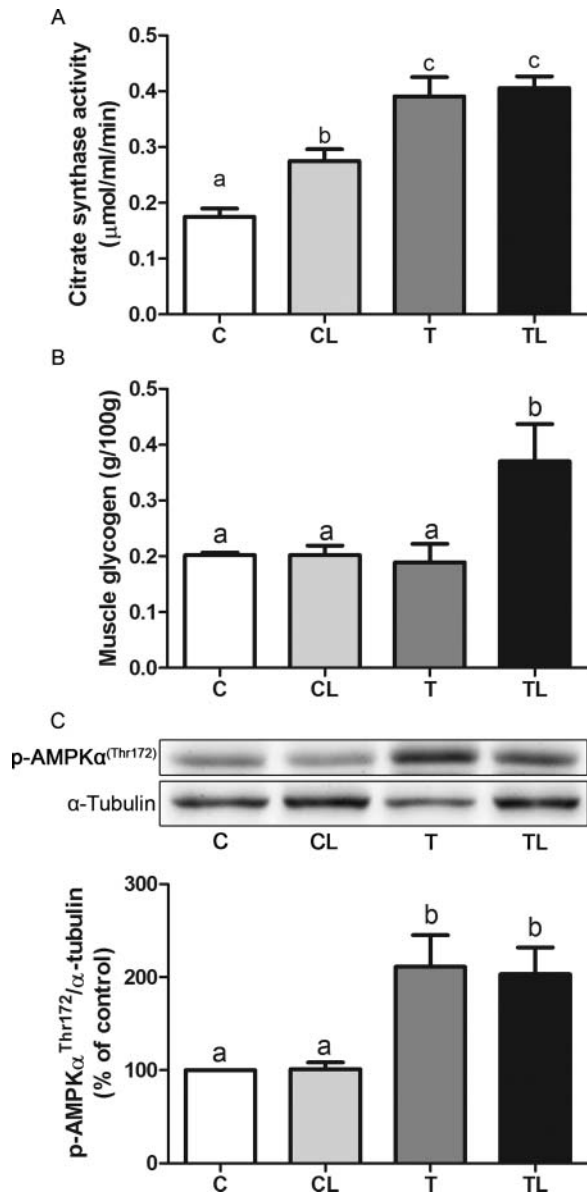
Activation of Akt was inhibited ( $p = 0.0004$ ) in trained groups (T and TL) compared to sedentary groups (C and CL) after exercise until exhaustion (Fig. 2B). A very similar pattern was found in mTOR activation; that is, exercise until exhaustion significantly decreased ( $p = 0.0004$ ) mTOR phosphorylation when compared to sedentary groups and leucine was not able to prevent this reduction (Fig. 2C).

### Exercise-Induced Systemic Inflammation

IL-6 levels were significantly elevated ( $p = 0.005$ ) in trained groups compared to control groups (Fig. 3A). TNF- $\alpha$  levels did not differ significantly ( $p = 0.569$ ) between groups (Fig. 3B).

## DISCUSSION

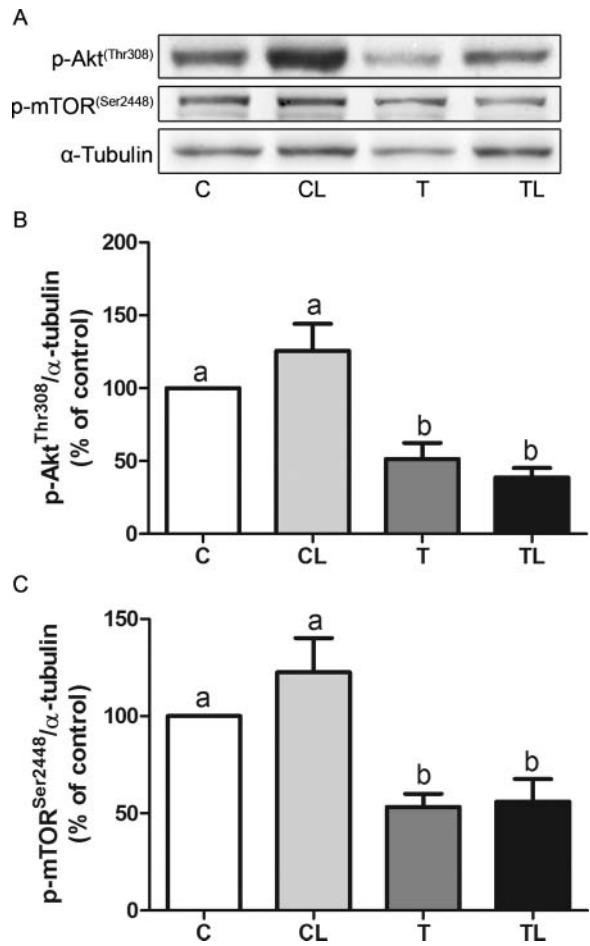
Although it is generally accepted that leucine supplementation can mitigate endurance exercise-induced skeletal muscle damage and fatigue, while also improving recovery and muscle performance [5], recent studies have challenged this belief. They show no beneficial role of leucine supplementation on performance or demonstrate a worsened metabolic response [6,17]. Here we report that leucine supplementation, combined with endurance training, failed to improve time to exhaustion when compared to endurance training only. Furthermore, leucine supplementation in endurance-trained rats was not better than only exercising in improving oxidative capacity and molecular response and failed to mitigate exercise-induced inflammatory. On the other hand, endurance exercise training



**Fig. 1.** Effects of leucine supplementation and endurance training on (A) citrate synthase activity ( $\mu\text{mol/ml/min}$ ), (B) gastrocnemius glycogen content (g/100 g), (C) representative blot for AMPK phosphorylation, and (D) AMPK activation. Experimental groups were divided as follows: sedentary control (C), control-supplemented (CL), trained (T), and trained-supplemented (TL). Data are expressed as means  $\pm$  SD ( $n = 6$ ). Different letters indicate significant differences between groups ( $p < 0.05$ ).

combined with leucine supplementation significantly enhanced glycogen content when compared to leucine supplementation only or exercise only.

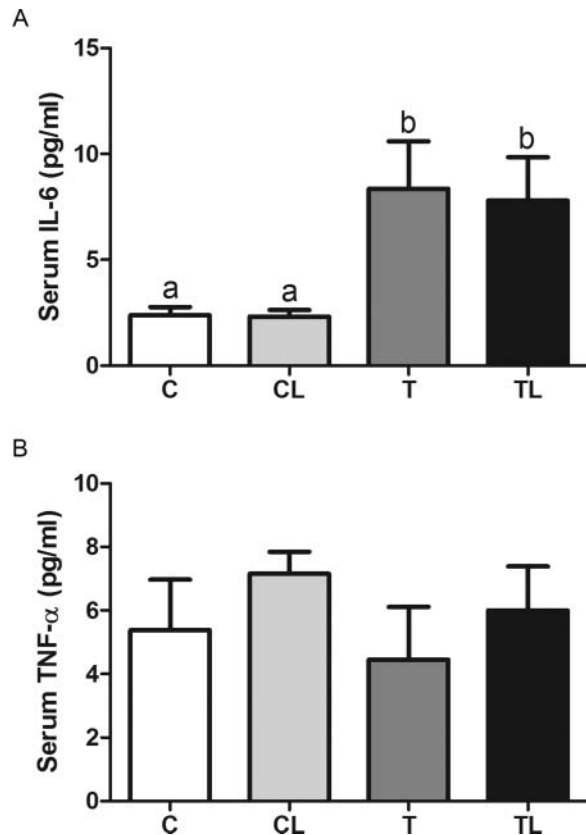
Despite promising current evidence that using leucine supplementation chronically boosts endurance performance, our data suggest that it does not seem to be the case in an exercise-until-exhaustion scenario. Therefore, the primary novel finding of the present study was that leucine supplementation, combined with endurance training, does not enhance exercise until



**Fig. 2.** Effects of leucine supplementation and endurance training on protein synthesis signaling: (A) representative blot for Akt and mTOR phosphorylation, (B) Akt activation, and (C) mTOR activation. Experimental groups were divided as follows: sedentary control (C), control-supplemented (CL), trained (T), and trained-supplemented (TL). Data are expressed as means  $\pm$  SD ( $n = 6$ ). Different letters indicate significant differences between groups ( $p < 0.05$ ).

exhaustion performance nor does it prevent exercise-induced increased inflammation response or decreased molecular signaling of protein synthesis.

Interestingly, leucine supplementation did not mitigate p-AMPK response until exhaustive exercise. Because AMPK reflects the energy status of the cell, the great metabolic stress induced by a bout of exercise until exhaustion was expected to increase p-AMPK level, and leucine supplementation could mitigate this metabolic stress and, therefore, AMPK activation. Our hypothesis was partly correct, because exercise bout did enhance p-AMPK levels and glycogen content was significantly higher (approximately 95%) in the TL group compared to the T group, suggesting lower metabolic stress or at least a higher energy availability. Thus, exercise-induced fatigue did not occur due to energy substrate depletion in this case. However, the reason why this lower metabolic stress (i.e., normal muscle glycogen) in the TL group did not lead to a lower



**Fig. 3.** Effects of leucine supplementation and endurance training on inflammatory response biomarkers: (A) IL-6 content and (B) TNF- $\alpha$  content. Experimental groups were divided as follows: sedentary control (C), control-supplemented (CL), trained (T), and trained-supplemented (TL). Data are expressed as means  $\pm$  SD ( $n = 6$ ). Different letters indicate significant differences between groups ( $p < 0.05$ ).

p-AMPK concentration remains to be clarified, considering that muscle glycogen seems to be an important factor in the regulation of muscle AMPK activity. In addition, studies have shown that glycogen can be a powerful negative controller of AMPK [18]. That calcium signaling may also have a role in activating AMPK in muscle during exercise has been proposed [18]. It would be plausible to speculate that some other factors (e.g., calcium signaling) can potentially overrule muscle glycogen depletion and stimulate AMPK phosphorylation during exercise until exhaustion, even without the occurrence of significant metabolic stress.

Because leucine also functions as a regulator of translation initiation of protein synthesis, a modulator of the insulin signal cascade, and a nitrogen donor for muscle production of alanine and glutamine [19], we hypothesized that long-term leucine supplementation could at least mitigate exhaustive endurance exercise-induced reduction Akt and mTOR phosphorylation. However, protein synthesis signaling was significantly inhibited after exercise until exhaustion and leucine supplementation failed to prevent it. Both Akt and mTOR phosphorylation were significantly reduced in exercise groups (T and TL).

Contrary to our findings, Mascher et al. [20] demonstrated increased skeletal muscle Akt and mTOR phosphorylation after 1 hour of endurance training. However, these results are also highly intensity and time-point dependent. In the present study, exercise was done until exhaustion, with mean exercise duration of 3 hours and 22 minutes ( $\pm 10$  minutes), which could have led to the different protein synthesis signaling response. Exhaustive endurance exercise seems to inhibit muscle protein synthesis, with the magnitude of the depression related to the intensity and duration of the activity. It has been established that skeletal muscle protein synthesis decreases by about 30% during contractile activity [21] and protein turnover remains negative after endurance exercise until adequate dietary protein and energy are available for recovery [19].

In our study, after exercise until exhaustion, rats remained in a fasted state, in order to assess the long-term effect of leucine supplementation. It has been proposed that ingestion of leucine directly after endurance exercise stimulated protein synthesis in the recovery period [22], which could contribute to improve endurance exercise performance. It has been previously demonstrated that leucine ingestion, along with carbohydrates, after prolonged endurance exercise enhances muscle glycogen content, insulin level, and muscle protein synthesis [23]. A recent study has also demonstrated increased mTOR phosphorylation after endurance exercise and leucine supplementation [5]. Different from our study, the 3 aforementioned studies assessed protein synthesis acutely; that is, right after leucine ingestion. Nonetheless, though it would be tempting to conclude that acute responses within specific markers may provide insight into chronic adaptations, extrapolation of the acute response to potential adaptive responses after a single bout of unfamiliar exercise should be cautiously interpreted [24]. Thereby, our data suggest that leucine-induced enhancement on protein synthesis is rather acute, and chronic supplementation has no effect on protein synthesis after a bout of exercise until exhaustion.

Concerning endurance performance, it is hard to explain why long-term leucine supplementation did not enhance exercise performance, because many metabolic parameters were improved. Surprisingly, leucine supplementation significantly enhanced CS activity. It was recently demonstrated [17] that leucine can stimulate PGC-1 $\alpha$  activation, which would explain enhanced CS activity. In the nucleus, PGC-1 $\alpha$  binds to and activates a host of transcription factors to collectively induce upregulation of a variety of proteins involved in the transport and oxidation of glucose and fatty acids. In the mitochondria, PGC-1 $\alpha$  modulates the activity of mitochondrial transcription factor. In this way, PGC-1 $\alpha$  is therefore recognized as a major point of control in regulating both nuclear and mitochondrial DNA expression [25]. The aforementioned study also showed enhanced CS activity after 12 weeks of leucine supplementation. However, different from our study, this outcome was still present in the trained leucine-supplemented group. It may have occurred due to the different exercise intensities between

these studies: Whereas we used high-intensity endurance exercise followed by a bout of exercise until exhaustion, they used low-intensity exercise. Thus, leucine seems to enhance CS activity, but this outcome can be overcome by exercising if the intensity is high enough. Oddly, in this prior study, increased CS activity in the sedentary leucine-supplemented group did not lead to improvement in exercise performance ( $VO_{2max}$  increase). Similarly, in our study, time to exhaustion was not significantly different between trained groups (Table 1), demonstrating that long-term leucine supplementation did not enhance endurance performance of the animals submitted to this exercise protocol. This result corroborates those of another study [26] that also did not find significant differences in the time to exhaustion in adult male Wistar rats submitted to a swimming exhaustion test after 6 weeks of branched chain amino acids supplementation. These authors pointed out that a significant decrease in glycemia during exercise until exhaustion could explain fatigue and, therefore, performance. Our results suggest that this seems to be unlikely. Though we have not assessed glycemia in our study, both trained groups presented normal levels of muscle glycogen after exercise until exhaustion when compared to control groups. In addition, the TL group presented a significant enhancement in muscle glycogen compared to all experimental groups. Thus, when combined with endurance exercise, long-term leucine supplementation has beneficial effects on substrate availability. Furthermore, it has been demonstrated that higher preexercise muscle glycogen concentration can delay fatigue [27]. Therefore, these results suggest that fatigue may have occurred due to any cause other than substrate availability in our experimental conditions. Chronic branched chain amino acids (BCAA) supplementation influences the activity of the hepatic branched-chain  $\alpha$ -ketoacid dehydrogenase (BCKAD) complex, promoting a lower utilization of hepatic glycogen. Moreover, it has been suggested that dietary BCAA increases BCAA catabolism and thus the oxidation of BCAA inhibits the activity of pyruvate dehydrogenase, which is located at the connection point between the glycolytic pathway and citric acid cycle, a mechanism that favors the deviation of pyruvate to the formation of alanine, which, in turn, acts as a precursor in hepatic gluconeogenesis [26]. Hence, the role of long-term leucine supplementation on oxidative metabolism and endurance performance should be further investigated. It has been proposed that exercise-induced ammonia accumulation could generate fatigue due to excess cerebral ammonia and that the inability of peripheral organs to detoxify it [26] should be considered.

In order to evaluate inflammation response due to exercise until exhaustion, we assessed IL-6 and TNF- $\alpha$  levels. Both trained groups presented significant increases in IL-6 level. It is important to keep in mind that, although mostly regarded as a pro-inflammatory cytokine, IL-6 has anti-inflammatory properties [28,29]. The exercise-induced increase in plasma IL-6 leads to increased circulating levels of well-known anti-inflammatory cytokines. Moreover, both exercise and IL-6 infusion suppress TNF- $\alpha$  production in humans [29]. In our case, there was no difference in TNF- $\alpha$  levels between the groups. This

may have occurred because TNF- $\alpha$  is only stimulated by very intense exercise [29]. Despite its long duration, exercise until exhaustion does not impose great intensity. In addition, there were no eccentric muscle contractions because it was performed in water. Other studies using exercise protocol with eccentric contractions could help to elucidate the role of muscle damage on fatigue and exhaustion mechanisms. Moreover, IL-6 has an important metabolic function during exercise and may represent a link between skeletal muscle and organs such as the liver and the adipose tissue. Studies have clearly demonstrated that contracting muscles, without any muscle damage, can induce a marked elevation in plasma IL-6 [28,30]. IL-6 production is modulated by the carbohydrate availability in skeletal muscles, suggesting that IL-6 acts as an “energy sensor” [29, 3]. IL-6 was shown to enhance AMPK activity in both skeletal muscle and adipose tissue [31], which has occurred in the present study.

In conclusion, although leucine-mediated enhanced muscle glycogen levels in trained rats and citrate synthase activity in sedentary ones, it failed to enhance endurance performance of trained rats submitted to an exhaustion test. Furthermore, it did not prevent exercise-induced reduction in protein synthesis signaling. These results suggest that nutrient-mediated acute molecular and metabolic effects that may support homeostatic restoration and adaptive remodeling of skeletal muscle to endurance exercise do not necessarily occur in long-term training. Further studies are necessary in order to better understand the mechanisms of fatigue, possibly using other types of exercise and exercise duration to conclude why these molecular and metabolic effects did not lead to enhancement in endurance performance.

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