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ORIGINAL ARTICLE

Leucine supplementation does not affect protein turnover and impairs the beneficial effects of endurance training on glucose homeostasis in healthy mice

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Abstract Endurance exercise training as well as leucine supplementation modulates glucose homeostasis and protein turnover in mammals. Here, we analyze whether leucine supplementation alters the effects of endurance exercise on these parameters in healthy mice. Mice were distributed into sedentary (C) and exercise (T) groups. The exercise group performed a 12-week swimming protocol. Half of the C and T mice, designated as the CL and TL groups, were supplemented with leucine (1.5 % dissolved in the drinking water) throughout the experiment. As well known, endurance exercise training reduced body weight and the retroperitoneal fat pad, increased soleus mass, increased VO_{2max}, decreased muscle proteolysis, and ameliorated peripheral insulin sensitivity. Leucine supplementation had no effect on any of these parameters and worsened glucose tolerance in both CL and TL mice. In the soleus muscle of the T group, AS-160$^{Thr\text{-}642}$ (AKT substrate of 160 kDa) and AMPK$^{Thr\text{-}172}$ (AMP-Activated Protein Kinase) phosphorylation was increased by exercise in both basal and insulin-stimulated conditions, but it was reduced in TL mice with insulin stimulation compared with the T group. Akt phosphorylation was not affected by exercise but was lower in the CL group compared with the other groups. Leucine supplementation increased mTOR phosphorylation at basal conditions, whereas exercise reduced it in the presence of insulin, despite no alterations in protein synthesis. In trained groups, the total FoxO3a protein content and the mRNA for the specific isoforms E2 and E3 ligases were reduced. In conclusion, leucine supplementation did not potentiate the effects of endurance training on protein turnover, and it also reduced its positive effects on glucose homeostasis.

Keywords Endurance exercise · Leucine supplementation · Skeletal muscle adaptations

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Introduction

It is well known that endurance exercise training induces several adaptations in tissues throughout the body. In skeletal muscle, endurance exercise increases free fatty acid uptake and utilization, increases glucose uptake and favors the switching of muscle fiber type (Lira et al. 2010; Maarbjerg et al. 2011; O'Neill 2013). This modulation occurs, at least in part, through the activation of AMP-activated

protein kinase (AMPK), which acts as an energy sensor that increases cellular catabolism and decreases anabolic processes (Ruderman et al. 2013).

AMPK plays a role in protein turnover by inducing a decrease in protein synthesis (Chan and Dyck 2005; Hardie 2011). AMPK activates the TSC1/TSC2 protein complex, leading to the inhibition of mammalian target of rapamycin (mTOR) which is the main regulator of protein synthesis in skeletal muscle (Nader 2006).

The role of AMPK in protein degradation of skeletal muscle is unclear. AMPK activates the ubiquitin-proteasome system (Tong et al. 2009), which is the major protein degradation pathway in skeletal muscle (Bodine et al. 2001). Paradoxically, AMPK activates PGC1-α, a transcriptional co-activator that decreases the expression of genes related to the ubiquitin-proteasome system (Sandri et al. 2006).

In contrast, leucine activates mTOR directly by an amino acid-sensing cascade involving RAG GTPases (Jewell and Guan 2013), or indirectly by inhibition of AMPK (Saha et al. 2010). In this sense, the effects of leucine supplementation on AMPK and mTOR pathways are in opposition to the effects of endurance exercise training. Interestingly, both endurance exercise training and leucine supplementation decrease the expression of genes of the ubiquitin-proteasome system (UPS) in sarcopenic models, whereas their effects on protein turnover in healthy animals remain unclear (Salomão et al. 2010).

Moreover, both AMPK and mTOR pathways also control glucose homeostasis. Endurance exercise training activates AMPK, which improves glucose uptake in skeletal muscle (Hardie 2011; Maarbjerg et al. 2011; O'Neill 2013), whereas leucine supplementation impairs insulin signaling via mTOR/p70S6K activation, leading to inhibition of IRS1 through Ser^{307} phosphorylation (Krebs et al. 2007; Saha et al. 2010). In addition, leucine might reduce the activity of AMPK, thereby impairing glucose uptake (Saha et al. 2010).

Data on the effects of leucine supplementation as well as endurance exercise on glucose homeostasis are present in the literature. However, the interaction between both is not yet fully explored. Because they decrease the expression of genes of the ubiquitin-proteasome pathway in sarcopenic models (Salomão et al. 2010), we analyzed here the effects of endurance exercise training plus leucine supplementation on protein turnover and glucose homeostasis in healthy mice.

Methods

Ethical approval

This study was conducted in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). The protocol was approved by the Institutional Committee for Ethics in Animal Research of the State University of Campinas (Unicamp), permit number 1984-1. All surgery was performed using a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) for anesthesia, and all efforts were made to minimize animal suffering. Mice were killed by decapitation after CO_2 inhalation in a chamber.

Animal care and husbandry

The study was performed using female Swiss mice obtained from the breeding colony at Unicamp. The mice were randomly assigned into the following experimental groups ($n = 16$ mice in each group): (a) the control (C) group was fed commercial chow (Purina-São Paulo, Brazil) from weaning (21 days) to 120 days; (b) the trained (T) group was fed commercial chow (Purina) from weaning (21 days) to 120 days and was swim-trained from days 30 to 120; (c) the control-supplemented (CL) group was fed commercial chow (Purina) from weaning (21 days) to 120 days, was not exercised and had leucine (1.5 %) supplemented in their drinking water; and (d) the trained-supplemented (TL) group was fed on commercial chow (Purina) from weaning (21 days) to 120 days, had leucine (1.5 %) supplemented in their drinking water and was swim-trained from days 30 to 120. Training or leucine supplementation alter neither food intake nor water consumption. Leucine intake was estimated in 2.5 g/kg/day for CL group and 2.6 g/kg/day for TL group from days 30 to 120. The mice were maintained at 22 ± 1 °C on a 12 h light–dark cycle, and their body weight was measured once per week. At the end of the experimental period, fasting mice were anesthetized in a CO_2 chamber and then killed by decapitation.

Exercise training protocol

To study the chronic adaptations caused by swim training, the mice in the trained groups were not exercised during the 48-h period preceding the experimental procedures. Exercise training consisted of 12 weeks of swimming 5 days per week with no overload attached to the body. The mice swam in collective tanks with dimensions of 50 cm × 80 cm × 120 cm and a water temperature of 30–32 °C. The initial 4 weeks of swim training consisted of a swimming adaptation period where the duration of each session was progressively increased as follows: 3 min/day in week 1, 15 min/day in week 2, 30 min/day in week 3, 45 min/day in week 4 and up to 1 h/day from week 5 to week 12.

Considering that the maximal lactate steady state (the highest intensity of exercise where there is a balance between lactate production and lactate removal)

during swimming exercise is around a load of 4.6 % of body weight (Gobatto et al. 2009), we designed an exercise protocol without load to test the effect of leucine supplementation in a program of exercise training with low intensity. The efficacy of our exercise protocol, as well as the minimum variation as the adaptations acquired by the exercise training proposed here, was validated by a maximal oxygen consumption test.

Maximal oxygen consumption (VO_{2max})

Mouse VO_{2max} was assessed in an individually sealed treadmill with a 25° incline; the treadmill was also coupled to a gas analyzer (Oxylet system, Pan Lab/Harvard Instruments, Spain). Oxygen uptake data were recorded using Metabolism software (Pan Lab/Harvard Instruments, Spain), and VO_{2max} was assumed to occur when the oxygen uptake reached a plateau despite increasing the treadmill speed. After an 8-minute warm-up, the test started at a treadmill speed of 15 cm s^{-1}. The speed was increased by 15 cm s^{-1} every 45 s until the mice were unable to maintain the effort level (Rezende et al. 2006).

Intraperitoneal glucose tolerance test

Food and supplemental leucine were withdrawn 12 h before performing the intraperitoneal glucose tolerance test (ipGTT). Mice were weighed, and a basal blood sample was taken from the tip of the tail at time 0 ($t0$). The glucose concentration was measured using an Accu-Check Advantage II (Roche). The mice subsequently received an intraperitoneal glucose solution (2 g/kg body wt). At 15, 30, 60 and 120 after injection, additional blood samples were collected, and blood glucose was measured. The area under the blood glucose curve was calculated using the trapezoidal method. Blood collected at 0, 30 and 60 min was also used for the plasma insulin measurement (ELISA kit, Millipore). The mice in the trained groups did not exercise during the 48-h period preceding the test.

Intraperitoneal insulin tolerance test

Food and leucine were withdrawn 6 h before the intraperitoneal insulin tolerance test (ipITT). Mice were injected with human insulin at 1.5 U/kg body weight (Biohulin R, Biobras, Brazil). Before $t0$ and at 4, 8, 12 and 16 min after insulin administration, blood samples were collected, and blood glucose was measured (Accu-Check Advantage II Glucometer, Roche). The glucose disappearance rate (KITT) was calculated using the formula $0.693/t_{1/2}$. Glucose $t_{1/2}$ was calculated using the slope of the least-squares analysis of blood glucose concentrations during the linear decay phase. Mice in the trained groups did not exercise during the 48-h period preceding the test.

Plasma leucine content

Plasma was separated by centrifugation at 6,000g for 15 min at 4 °C and stored at −20 °C. The plasma leucine concentration was determined using liquid chromatography as previously described (Bidlingmeyer et al. 1984).

Citrate synthase activity

After being killed, samples of the soleus muscle were quickly removed and homogenized in an extraction buffer containing Tris–HCl (0.5 mM) and EDTA (1 mM) at a pH of 7.4. The reaction was performed in a medium containing Tris/aminomethane (100 mM), DTNB (0.2 mM), acetyl-CoA (0.1 mM), and Triton X-100 (0.1 %) at a pH of 8.1. The reaction was initiated by combining 10 µL of the tissue extract and 50 µL of oxaloacetic acid (10 mM). The absorbance at 412 nm (25 °C) was spectrophotometrically measured for 5 min as previously described (SRERE 1963).

Protein synthesis

The isolated soleus muscles were incubated ex vivo in a Krebs–Ringer solution containing a pool of amino acids, as described in Scharff and Wool (1966). After a 1 h equilibration, the muscles were incubated for an additional 2 h in a similar medium containing L-[U-14C]tyrosine (0.05 Ci/mL). The specific activity of acid-soluble tyrosine was estimated as described by Waalkes and Udenfriend (1957). The rate of synthesis was calculated using the specific activity of the intracellular pool of tyrosine for each muscle, assuming that there was no label recycling during the incubation period (Tischler et al. 1982).

Protein degradation

The isolated soleus muscles were incubated ex vivo in 2 mL of Krebs–Ringer solution containing NaCl (0.12 M), $NaHCO_3$ (0.015 M), KCl (4.8 mM), $MgSO_4$ (1.21 mM), KH_2PO_4 (1.2 mM), and $CaCl_2$ (2.4 mM). Medium containing glucose (5.6 mM) and cycloheximide (0.5 mM) was equilibrated with O_2 (95 %) and CO_2 (5 %). The temperature was maintained at 37 °C, and the pH was set at 7.33. After 1 h, the medium was removed, and 2 mL of fresh Krebs–Ringer solution was added. After 1 h, the Krebs–Ringer solution was removed, and the tyrosine concentration was measured at wavelengths of 450 nm for excitation and 570 nm for emission as previously described (Waalkes and Udenfriend 1957).

Protein expression

Mice fasted for 12 h were anesthetized with a mixture of ketamine (50 mg/kg, Vetbrands, Paulínia, SP, Brazil) and xylazine (10 mg/kg, Rompun, Bayer, São Paulo, SP, Brazil) before organ harvesting. Afterwards, we removed the soleus muscle from the left hind limb, and then we administered insulin (100 μL, 10 IU) through the vena cava in the peritoneal cavity 2 min before extraction of the soleus muscle from the right hind limb. The muscles were immediately homogenized in solubilization buffer (1 % Triton X-100, 100 mM Tris (pH 7.5), consisting of 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride and 1.0 μg of aprotinin per milliliter) at 4 °C using a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). The protein concentration of each supernatant was measured using the Bradford method. Samples containing 30 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. The antibodies used to probe the blots were Akt1/2/3 (Santa Cruz, sc81434), Thr308-phospho-Akt (Santa Cruz, sc16646), AMPKα (Cell Signaling, #CS2532), Thr172-phospho-AMPKα (Cell Signaling, #CS2531), AS160 (Cell Signaling, #CS2447), Thr642-phospho-AS160 (Cell Signaling, #CS4288), mTOR (Abcam, Ab2732), Ser2448-phospho-mTOR (Abcam, Ab1093), FOXO3a (Santa Cruz, sc11351), Ser253-phospho-FOXO3a (Santa Cruz, sc101683) and GAPDH (Santa Cruz, sc166545). Then the phosphorylated proteins bands as well as their respectives internal control (GAPDH), were detected using enhanced chemiluminescence (SuperSignal West Pico; Pierce, Rockford, Ill) after 2 h of incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (diluted 1:1,000 in 5 % skim milk; Invitrogen, Carlsbad, CA, USA). The immunoreactive bands were revealed using enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA), detected using an LAS-3000 CCD camera and quantified using ImageJ software. After that, the membranes were stripped using the restore western blot stripping buffer in accord with data sheet (#21059 Thermo Scientific). Post the confirmation of stripping procedures the total protein were evaluated.

mRNA expression

Soleus muscles were homogenized in Trizol® following a phenol–chloroform RNA extraction performed according to the manufacturer's instructions. Reverse transcription was then performed on 3 μg of total RNA using reverse transcriptase and random hexamer primers. Real-time PCR reactions were performed in a total volume of 15 μL using Fast SYBR Green technology (Applied Biosystems, Foster City, CA, USA). The samples were denatured at 95 °C for 10 min, then subjected to 40 PCR cycles of 95 °C for 10 s and 60 °C for 45 s. PCR amplifications were performed in duplicate. The purity of the amplified PCR products was verified by their melting curves. The expression of the target genes was normalized against the expression levels of the housekeeping gene GAPDH. The sequences of the primers used were as follows (5′–3′):

MURF-1
forward: GACTCCTGCAGAGTGACCAAG,
reverse: CTTCTACAATGCTCTTGATGAGC;
PGC-1α
forward: GCACGCAGCCCTATTCATTG,
reverse: AGGATTTCGGTGGTGACACT;
UBE2K
forward: GCGTTCGTGTGCTCGGGTC,
reverse CTCCTCCGCCACCGCTACGA;
PSMB7
forward: TGCCGCAGGAATGCGGTCTTG,
reverse: GACCACCCCCGCGATGGTA;
UCHL1
forward GGCGCTTTGCCGACGTGCTA,
reverse: TTTCATGCTGGGCCGTGAGGG.

Statistics

The results are expressed as the mean ± SEM. Before performing a parametric two-way ANOVA, Levene's test and the Kolmogorov–Smirnov (Lilliefors) test were performed to check the data for homogeneity of variance and normality, respectively. To correct for variance heterogeneity or non-normality, the data were log transformed when necessary. Significant effects of the variable 'exercise' are represented by the symbol #, and effects of the variable 'leucine-supplementation' are represented by the symbol &. When an interaction between the two variables was detected by two-way ANOVA, the Student–Newman–Keuls post hoc test was used, and letters denote significant differences among the groups (different letters indicate significant differences). An alpha level of $p < 0.05$ was considered statistically significant.

Results

After the 5th week, exercise reduced the total body weight (Fig. 1a), retroperitoneal fat pad weight (Fig. 1b), and increased soleus muscle weight (Fig. 1c), but leucine supplementation did not affect any of these measurements.

Although leucine supplementation enhanced citrate synthase activity in both trained and control groups (Fig. 2a), the VO_{2max} was increased only by endurance training

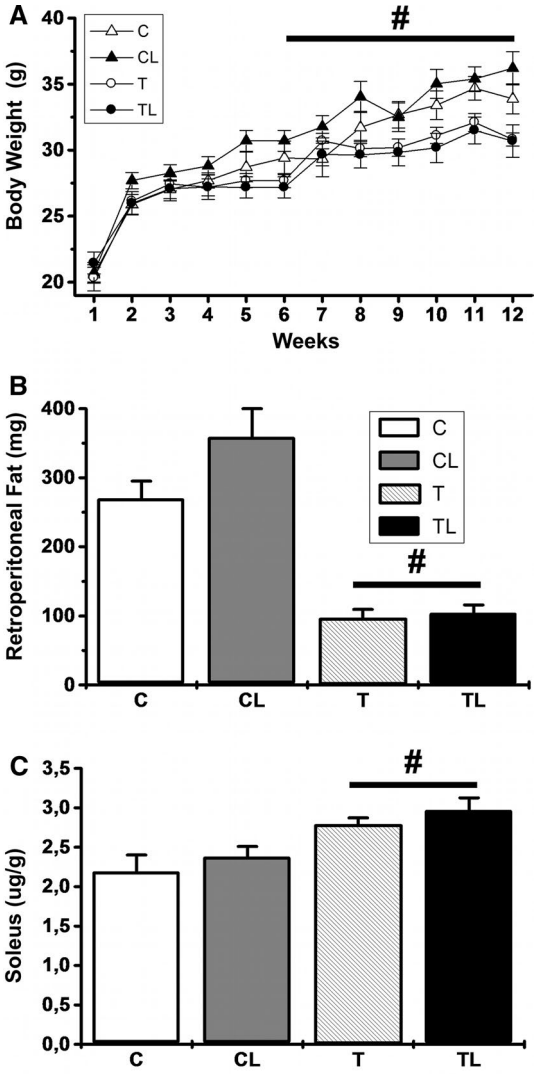

Fig. 1 Leucine supplementation did not alter body composition in any group, however, training reduced body and fat mass and increased muscle mass. Total body weight throughout the experiment (**a**), retroperitoneal fat pad weight (**b**) and relative soleus muscle mass (**c**) at the end of the experiment in swim-trained mice that were supplemented with leucine. Data are shown as the mean ± SEM. $n = 10$ for **a**, $n = 6$ for **b** and **c**. #Effect of exercise, $p < 0.05$ (two-way ANOVA)

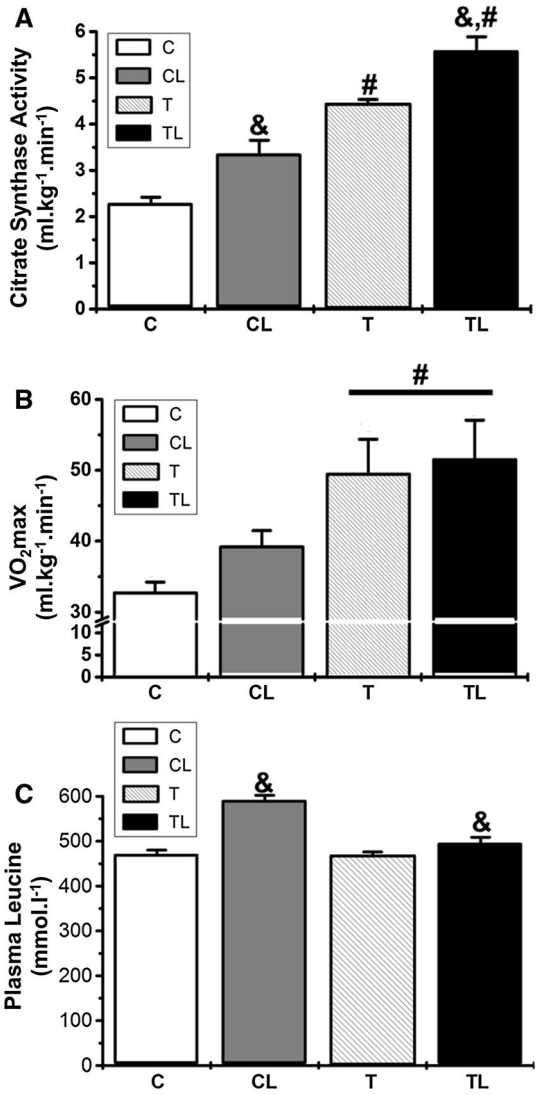

Fig. 2 Supplementation increased plasma leucine levels. Supplementation and training increased systemic and mitochondrial determinants of endurance performance and showed a sum effect on these parameters. Citrate synthase activity measured from total homogenates of the soleus skeletal muscle (**a**), maximal oxygen consumption (VO_{2max}) (**b**) and plasma leucine level (**c**) of swim-trained mice that were supplemented with leucine. Data are shown as the mean ± SEM. $n = 5$ for **a**, $n = 5$ for **b** and $n = 6$ for **c**. #Effect of exercise, $p < 0.05$; &Effect of leucine supplementation, $p < 0.05$ (Two-way ANOVA). *Different letters* indicate significant differences, $p < 0.05$ (Newman–Keuls post hoc test after two-way ANOVA)

(Fig. 2b). These observations suggest that in our model of exercise, leucine supplementation does not improve physical performance. As expected, leucine supplementation was effective to increase plasma leucine levels (Fig. 2c).

Exercise reduced protein degradation (Fig. 3a) with no effect on protein synthesis (Fig. 3b). None of these measures were affected by leucine supplementation.

Trained mice showed reduced MURF1 (Fig. 4a) and UBE2K (Fig. 4b) mRNA levels, neither of which was enhanced by leucine supplementation, and increased levels of PSMB7 (Fig. 4c) and UCHL1 (Fig. 4d), which were decreased by leucine supplementation. In addition, endurance training increased PGC1α mRNA levels, an effect that was improved by leucine supplementation (Fig. 4e).

Most of those atrogenes are regulated by FOXO3a, and we found that FOXO3a phosphorylation is increased (Fig. 5f) associated with a reduction in the total expression of FOXO3a (Fig. 5e). In every case, leucine supplementation had no additional effect.

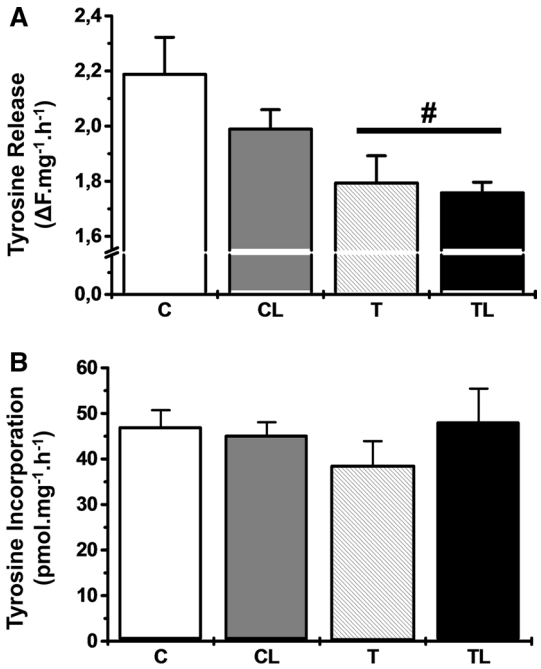

Fig. 3 Increased muscle mass was due to training reduced protein breakdown but not protein synthesis. Tyrosine release (**a**) and tyrosine incorporation (**b**) in the soleus muscle from swim-trained mice that were supplemented with leucine. Data are the mean ± SEM. $n = 6$. #Effect of exercise, $p < 0.05$ (two-way ANOVA)

FOXO3a is regulated not only by PGC1α but also by AKT. In our model, trained mice showed no difference in phosphorylation of AKT (Fig. 5a), while leucine supplementation reduced that only in insulin-stimulated control mice, an effect that was abolished by training. AKT also controls protein synthesis through mTOR, whose phosphorylation was reduced in trained mice and was unaffected by leucine supplementation (Fig. 5d). This apparent inconsistency in AKT-mTOR phosphorylation can be explained by an increase in AMPK phosphorylation in trained mice, which was inhibited by leucine supplementation (Fig. 5c). Training protocol increased AS160 phosphorylation, but leucine supplementation prevented this effect (Fig. 5b).

Similarly, trained mice showed increased insulin sensitivity as indicated by the ITT (Fig. 6a) and increased KiTT (Fig. 6b). This effect was blocked by leucine supplementation. In addition, leucine supplementation also decreased glucose tolerance in trained mice supplemented with leucine compared to trained mice (Fig. 7a, b).

Additionally, trained mice showed a change in insulin dynamics during a GTT (Fig. 7c) and a reduction in the AUC of insulin during a GTT (Fig. 7d); these results were unaffected by leucine supplementation.

Discussion

Endurance training is known to increase whole body oxygen consumption, skeletal muscle oxidative capacity, reduce body fat and maintain lean body mass. On the other hand, controversial results have been reported regarding leucine supplementation on protein turnover and glucose metabolism. In agreement with these evidences, our training protocol increased endurance performance and reduced protein breakdown. Supplementation protocol also led to higher plasma leucine content, however, increased leucine availability did not enhance performance and/or protein turnover, but, instead, showed undesirable effects on glucose metabolism.

Skeletal muscle mass results from a balance between protein synthesis and degradation. It is known that endurance exercise training increases mitochondrial but not contractile protein synthesis in the skeletal muscle (Wilkinson et al. (2008). In contrast, leucine supplementation induces contractile protein synthesis when associated with resistance exercise training, increasing mTOR phosphorylation (Dreyer et al. 2008), and it also reduces protein degradation in sarcopenic models by inhibiting the ubiquitin-proteasome system (Baptista et al. 2010). Here, we supplemented endurance-trained mice with leucine in an attempt to increase the global protein.

Contrary to what was observed by previous authors in the skeletal muscle of animal models as well as in patients with degenerative conditions such as movement restriction (Bajotto et al. 2011), cancer-induced cachexia (Peters et al. 2011) or motor neuron injury (Nicastro et al. 2011), where leucine increased protein synthesis, our results show that leucine supplementation was unable to increase protein synthesis in endurance-trained healthy mice. In agreement with previous data (Baar et al. 2006; Nader 2006), we observed that an increase in AMPK activation was associated with decreased mTOR phosphorylation, in healthy trained mice only after an insulin injection compared with control group in the same conditions. The unchanged phosphorylation of mTOR after leucine supplementation is consistent with the observation that both endurance training and leucine supplementation do not increase protein synthesis in these mice. This is an important distinction because an increase in mTOR phosphorylation with leucine supplementation has been described in animals with movement restriction (Bajotto et al. 2011), cachexia (Peters et al. 2011) or motor neuron injury (Nicastro et al. 2011).

On the other hand, endurance training reduced protein degradation, whereas leucine supplementation failed to potentiate this effect. This is in agreement with previous observations that the decrease in protein degradation induced by endurance training was not affected by leucine

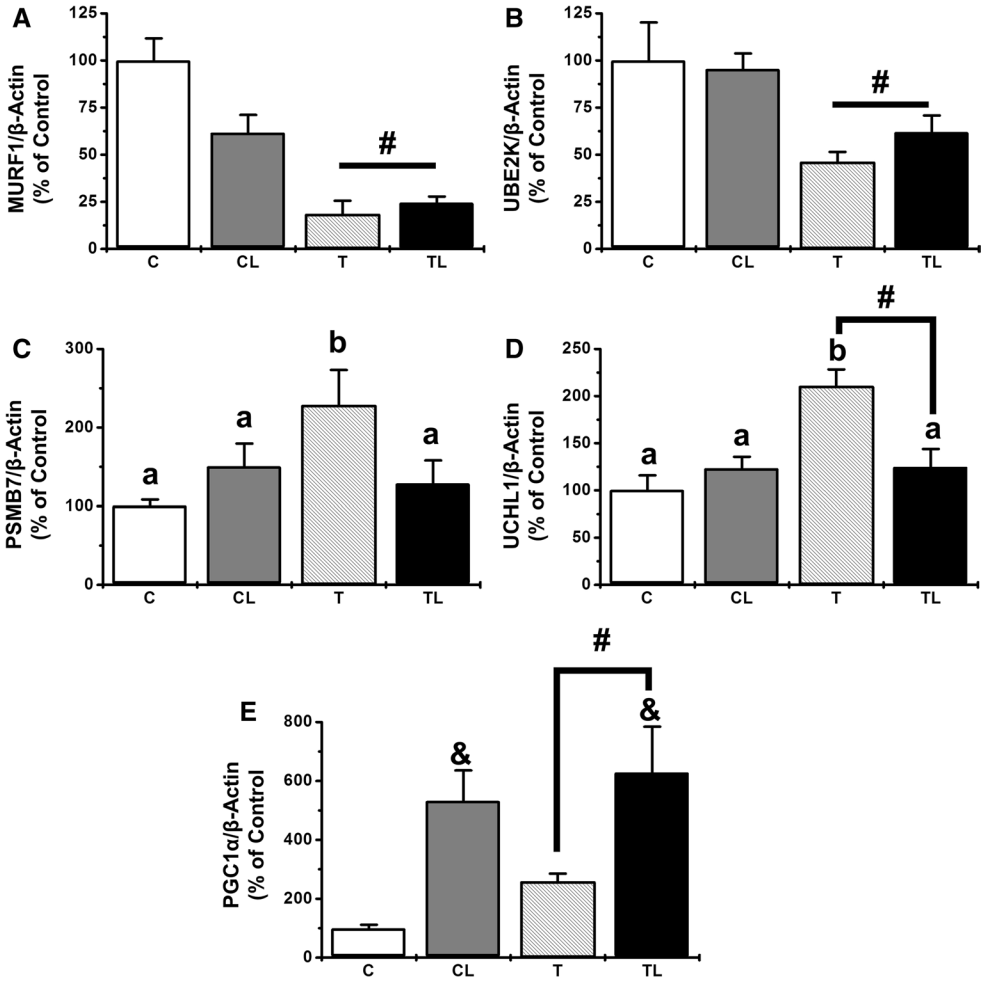

Fig. 4 Training reduced protein breakdown through its effect on ubiquitin-proteasome system components. Levels of MuRF1 (**a**), UBE2K (**b**), PMSB7 (**c**), UCHL1 (**d**) and PGC-1α (**e**) mRNAs in the muscle of swim-trained mice that were supplemented with leucine. Data are the mean ± SEM. $n = 5$. #Effect of exercise; &Effect of leucine supplementation (two-way ANOVA). *Different letters* indicate significant differences using a Newman–Keuls post hoc test after two-way ANOVA

supplementation in healthy animals (Salomão et al. 2010; Salomão and Gomes-Marcondes 2012).

The primary mechanism responsible for protein degradation in skeletal muscle is the ubiquitin-proteasome system (UPS), which, by acting on contractile and mitochondrial proteins, plays a major role in muscle atrophy (Bodine et al. 2001; Sandri et al. 2004; Baar et al. 2006). We showed that exercise reduces the expression of the mRNA of the E2 ligase, UBE2K, as well as the E3 ligase, MuRF-1, key UPS enzymes in muscle proteolysis (Bodine et al. 2001; Sandri et al. 2004; Baar et al. 2006). When ubiquitinated, proteins are recognized and may be degraded by proteasomes, releasing small peptides and amino acids. However, the rate of proteolysis relies on the activity of enzymes in the proteasome catalytic core. Unexpectedly, we reported that the PSMB7 mRNA, which encodes a protein subunit of the 20S catalytic multi-enzymatic complex of the proteasome, increased by approximately 100 % as a result of exercise. This effect was abolished by leucine in the TL group. Because exercise training decreases the expression of E3 ligase MuRF-1, we speculate that the increase in PMSB7 in the T group may be a compensatory mechanism that maintains the efficiency of the UPS; however, this outcome remains to be further investigated. In the T group, exercise training also increases the expression of UCHL-1 mRNA, which is a DUB enzyme. The exact function of UHCL1 is not yet fully understood; however, DUB enzymes regulate several cellular processes responsible for reversing the effects catalyzed by ubiquitin ligases (Kim et al. 2009; Hussain et al. 2010), and this increase might have contributed to the reduced protein degradation reported in our results.

Although AMPK activation may reduce mTOR phosphorylation, reducing protein synthesis (Nader 2006; Hardie 2011) and increasing protein degradation through activation of the ubiquitin-proteasome system (Tong et al. 2009), it can activate PGC-1α as well, a transcriptional co-activator that decreases the expression of atrogenes (Sandri et al. 2006). Therefore, we also analyzed the expression of the PGC-1α that decreases the expression of FOXO3a, a transcription factor required for the induction of MuRF-1 (Brault et al. 2010). Both exercise and leucine increase the level of PGC-1α mRNA, but the expression of E2 and E3 ligase(s) gene(s) was reduced only by exercise. Similarly,

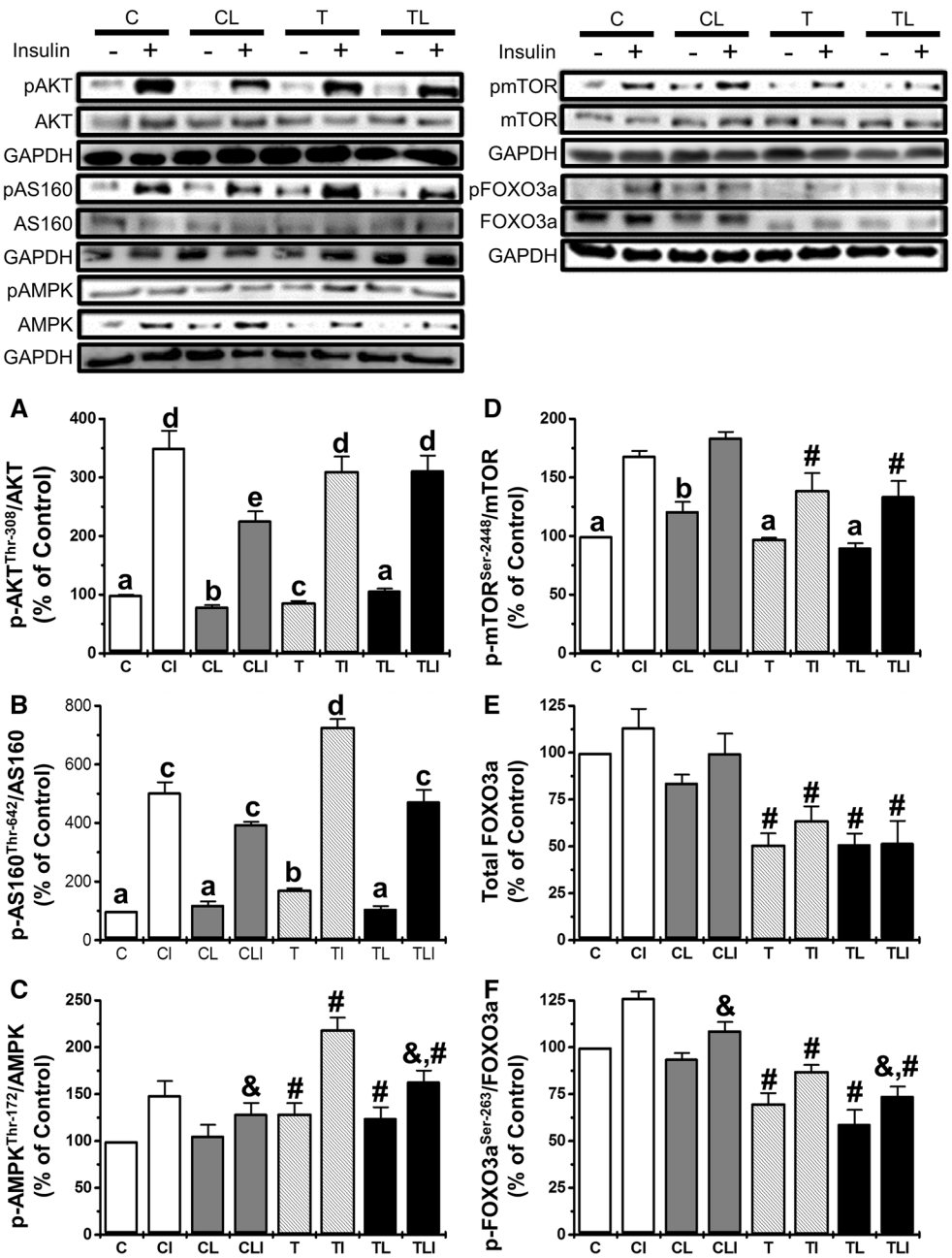

Fig. 5 Training and supplementation effects on molecular mechanisms of protein turnover and blood glucose control. The ratio levels of p-AKT Thr-308/Total AKT (**a**), p-AS160 Thr-642/Total as160 (**b**), p-AMPK Thr-172/Total AMPK (**c**), p-mTOR Ser-2448/Total mTOR (**d**), total FOXO3a (**e**) and p–p-FOXO3a Ser-263/Total FOXO3a (**f**) in the soleus muscle from swim-trained mice that were supplemented with leucine. Data are the mean ± SEM. $n = 3$–4. $^{\#}$Effect of exercise; $^{\&}$Effect of leucine supplementation (two-way ANOVA). *Different letters* indicate significant differences using a Newman–Keuls post hoc test after two-way ANOVA

only exercise reduced the total content of the FOXO3a protein, resulting in a significant decrease in muscle protein degradation. FOXO3a is also controlled by AKT, and, when phosphorylated, AKT inhibits FOXO3a translocation to the nucleus, reducing atrogene transcription (Sandri et al. 2004). The lower levels of phosphorylated AKT reported in untrained mice might explain why leucine supplementation alone was unable to reduce both protein degradation and the UPS activity, despite increased levels of PGC1-α.

Protein turnover and glucose metabolism have common signaling pathways through AKT and AMPK activation. Considering our results of reduced AKT AND AMPK phosphorylation in untrained and trained mice, respectively, as well as other studies that reported impaired insulin signaling after leucine supplementation (Krebs et al. 2007; Saha et al. 2010), we also investigated the blood glucose response in our models.

It has been well established that endurance exercise training improves glucose homeostasis due, at least in part, to increased AMPK activity in skeletal muscle (Maarbjerg et al. 2011; O'Neill 2013). Both AKT and AMPK pathways control glucose homeostasis through a common downstream protein, AS160. AKT (activated by insulin) and AMPK (activated by muscle contraction) signals converge

at AS160 to promote the translocation of GLUT4-containing vesicles to the sarcolemma, allowing the entry of glucose (Nader 2006; Maarbjerg et al. 2011; O'Neill et al. 2011). Here, we observed that insulin-induced AKT phosphorylation was similar among the C, T and TL groups. In the CL group, both basal and insulin-stimulated AKT phosphorylation were reduced compared to the other groups, indicating that in the sedentary mice, leucine supplementation impaired insulin signaling through AKT pathway, which then reduced glucose tolerance.

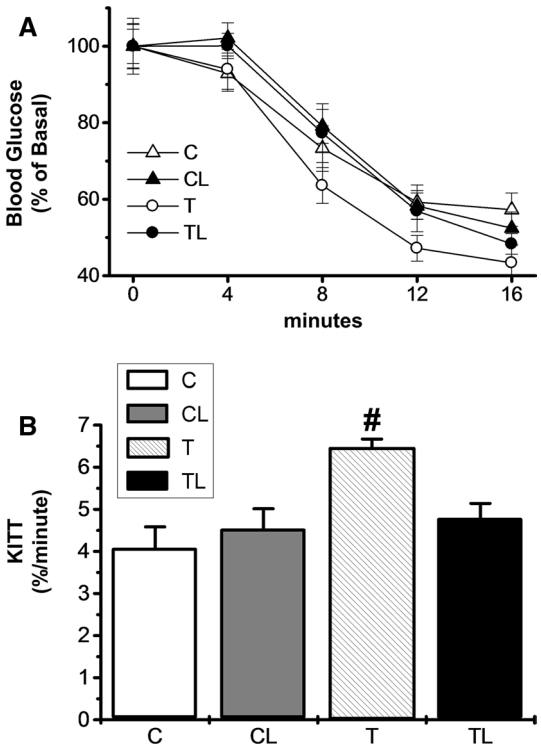

Fig. 6 Training increases insulin sensitivity and leucine supplementation counteracts this outcome. Blood glucose decay (**a**) and blood glucose disappearance index (KITT) (**b**) during an ipITT on swim-trained mice that were supplemented with leucine. Data are the mean ± SEM. $n = 8$–10. #Effect of exercise, $p < 0.05$ (two-way ANOVA)

The impairment in glucose homeostasis induced by leucine can occur via the inhibition of Pi3K/AKT (Macotela et al. 2011) and also AMPK pathway (Saha et al. 2010). Because the initial steps of the insulin-signaling pathway were not affected by exercise, other proteins, including AMPK and AS160, may be involved. In fact, exercise increased the phosphorylation of AMPK and AS160 under both basal and insulin-stimulated conditions, and this effect was significantly reduced by leucine. Our results are in agreement with those from studies showing that leucine supplementation impairs glucose uptake by reducing AMPK activity (Saha et al. 2010). Increased mTOR phosphorylation impairs insulin signaling through p70S6K activation, which negatively regulates insulin receptor substrate 1 via serine-308 phosphorylation. As an inhibitory feedback mechanism, this process senses the cell's energy status, preventing or reducing the entry of an excess of nutrients into an already energy-rich cell (Saha et al. 2010). Thus, the alterations of the AMPK-AS160 pathway may explain the impairment of glucose tolerance and the reduced insulin sensitivity induced by leucine supplementation in the trained mice.

In conclusion, leucine supplementation failed to enhance endurance performance, the protein turnover and worsened the beneficial effects of endurance exercise training on glucose homeostasis. These results suggest that leucine

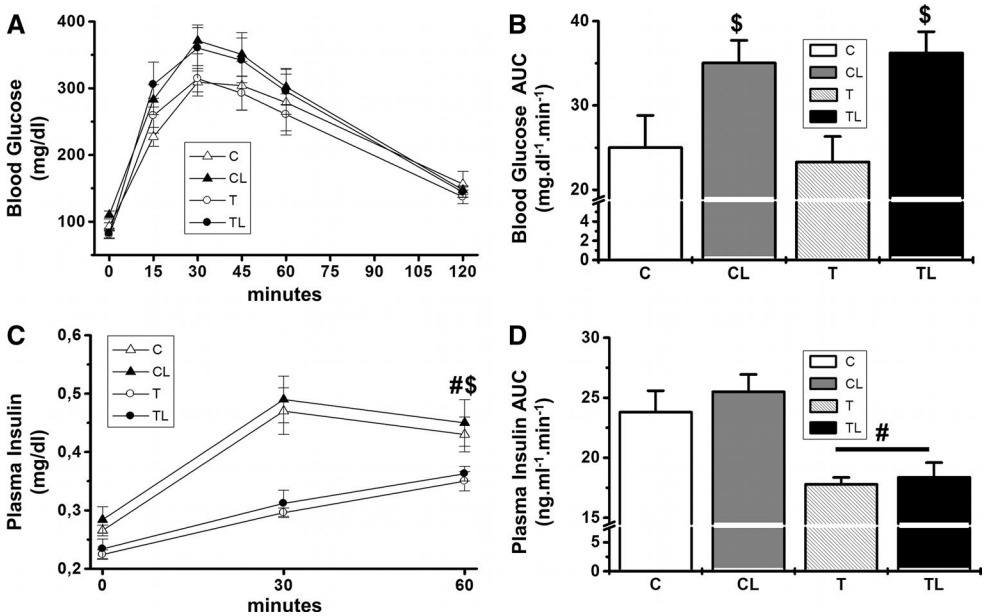

Fig. 7 Leucine supplementation decreases glucose tolerance and training increases its levels also reducing plasma insulin. Blood glucose (**a**), Area under the curve (AUC) of blood glucose (**b**), plasma insulin (**c**) and AUC of plasma insulin during an ipGTT (**d**) on swim-trained mice that were supplemented with leucine. Data are the mean ± SEM. $n = 10$ for **a** and **b**; $n = 7$ for **c** and **d**. #Effect of exercise, $p < 0.05$; &Effect of leucine supplementation, $p < 0.05$ (two-way ANOVA)

supplementation may not be recommended for healthy subjects or even for individuals with type 2 diabetes who plan to manage their metabolic disorders with a regular endurance exercise training program.

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Conflict of interest The authors have no conflicts of interest to declare.

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