


Branched-chain amino acids administration suppresses endurance exercise-related activation of ubiquitin proteasome signaling in trained human skeletal muscle

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Abstract We tested whether post exercise ingestion of branched-chain amino acids (BCAA < 10 g) is sufficient to activate signaling associated with muscle protein synthesis and suppress exercise-induced activation of mechanisms associated with proteolysis in endurance-trained human skeletal muscle. Nine endurance-trained athletes performed a cycling bout with and without BCAA ingestion (0.1 g/kg). Post exercise ACC^{Ser79/222} phosphorylation (endogenous marker of AMPK activity) was increased (~3-fold, $P < 0.05$) in both sessions. No changes were observed in *IGF1* mRNA isoform expression or phosphorylation of the key anabolic markers — p70S6K1^{Thr389} and eEF2^{Thr56} — between the sessions. BCAA administration suppressed exercise-induced expression of mTORC1 inhibitor *DDIT4* mRNA, eliminated activation of the ubiquitin proteasome system, detected in the control session as decreased FOXO1^{Ser256} phosphorylation (0.83-fold change, $P < 0.05$) and increased *TRIM63* (*MURF1*) expression (2.4-fold, $P < 0.05$). Therefore, in endurance-trained human skeletal muscle, post exercise BCAA ingestion partially suppresses exercise-induced expression of *PGC-1 α* mRNA, activation of ubiquitin proteasome signaling, and suppresses *DDIT4* mRNA expression.

Keywords Endurance exercise · Branched-chain amino acids · Proteolysis · Mitochondrial biogenesis

Introduction

Endurance training leads to increased skeletal muscle oxidative capacity, mitochondrial volume density, and aerobic performance. During acute aerobic exercise, various metabolites accumulate and the glycogen store is decreased in skeletal muscle. These changes activate various signaling cascades, including the AMP-activated protein kinase (AMPK)–peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α ; also known as PPARGCA) pathway that plays an important role in regulating mitochondrial biogenesis. Activated PGC-1 α translocates to the nucleus and regulates genes encoding mitochondrial transcription factors and other mitochondrial proteins as well as its own gene expression [1].

Aerobic training has been shown to induce muscle hypertrophy in both young and old untrained men [2]. However, a recent meta-analysis revealed that aerobic training-induced muscle hypertrophy and strength increments are significantly and negatively related to both frequency of endurance training (in the range from 1 to 5 days per week) and average duration of endurance sessions (in the range from 20 to 60 min per day) [3]. A further increase in frequency, duration and intensity of aerobic training typical for well-trained endurance athletes may not induce muscle hypertrophy or even decrease muscle mass. Indeed, high-intensity endurance training is reported to cause a decrease of the cross-sectional area of muscle fiber and muscle strength [4–6], which may have a negative effect on performance in elite endurance athletes. These changes appear to be related to a negative protein balance

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in endurance-trained muscle fibers [7]. The latter effect may be explained by an antagonistic relationship between the signaling pathways regulating mitochondrial biogenesis and protein synthesis [8] as well as endurance exercise-induced activation of the ubiquitin proteasome system [9–12].

Essential amino acids, in particular leucine, are broadly utilized to activate the mammalian target of rapamycin complex 1 (mTORC1), the key regulator of protein synthesis [13]. Earlier studies have demonstrated that leucine administration decreases the activities of markers of the ubiquitin proteasome system and prevents loss of soleus muscle mass during hindlimb immobilization in rats [14, 15].

Post-exercise protein-carbohydrate feeding leads to increased skeletal muscle anabolic signaling and fractional synthetic rate, reduced rate of protein breakdown after endurance exercise [16–19], and a marked increase in the insulin level in blood from ~ 5 to 30 mU/l. The ablation of hypoinsulinemia per se may suppress the protein breakdown rate and modulate anabolic signaling [20–22]. However, activation of the insulin signaling pathway could deactivate PGC-1 α protein [23, 24] and theoretically suppress exercise-induced mitochondrial biogenesis.

Here, we tested the hypothesis that post exercise oral ingestion of branched-chain amino acids (BCAA < 10 g) that does not induce a marked insulin response is sufficient to activate signaling associated with muscle protein synthesis and suppress exercise-induced activation of signaling associated with proteolysis in endurance-trained human skeletal muscle. We administered a BCAA dose of 0.1 g/kg body mass (including 0.05 g/kg leucine) to achieve near-maximal stimulation of muscle protein synthesis [16, 25] and, on the other hand, avoid a marked rise in the insulin level in blood immediately after exercise. Feeding status substantially affects anabolic and proteolytic signaling and protein synthesis in skeletal muscle. The main objective of this investigation was to modulate post exercise anabolic and proteolytic signaling through administration of additional BCAA under normal feeding conditions typical for endurance athletes.

Methods

Participants

Nine amateur endurance-trained athletes [18–30 years old, median (interquartile range) maximal oxygen consumption rate ($\dot{V} O_{2\max}$) 57 (53–61) ml/kg/min, body mass 68 (66–74) kg] participated in this study. All participants were informed about the study design and provided their written

consent for participation. The study was approved by the Human Ethics Committee of the Institute of Biomedical Problems (Moscow, Russia) and complied with the guidelines of the Declaration of Helsinki.

Preliminary experiments

All participants performed an incremental ramp test on an electromagnetic bicycle ergometer (Ergoselect 200; Ergoline) until exhaustion. Initial load, load increment and revolution rate were 0 W, 15 W/min, and 60–70 rpm, respectively. The pulmonary oxygen consumption rate ($\dot{V} O_2$) was measured using MetaMax 3B (Cortex). Capillary blood samples (20 μ l) were obtained from a fingertip every 2 min, the blood lactate concentration evaluated using a Biosen C-line analyzer (EKF Diagnostics), and workload corresponding to the anaerobic threshold [AT] calculated at a lactate level of 4 mM. The highest $\dot{V} O_2$ value for 30 s was taken as the $\dot{V} O_{2\max}$.

Main study

Each participant performed two test sessions in a random order: endurance exercise (E) and endurance exercise followed by BCAA ingestion (EA) with 1 week in between (Fig. 1). The exercise bout (70 min) consisted of a warm-up (5 min cycling at 50% AT) and intermittent exercise [(3 min cycling at 60% AT plus 2 min cycling at 95% AT) \times 13]. In a previous study, muscle protein synthesis was shown to be upregulated 90–180 min after oral amino acid administration [26]. To stimulate muscle anabolic signaling over an extended period after exercise during the EA session, participants ingested 0.1 g/kg body mass of BCAA in the form of capsules (leucine, isoleucine, and valine [2:1:1], ~ 234 kJ; AST Sport Science) at two time-points: immediately after and 5 h after the end of the exercise session.

Participants arrived at the laboratory at 09:00 and consumed a standardized breakfast (3624 kJ; 24 g protein, 157 g carbohydrate, 15 g fat). Cycling began 1 h and 45 min after breakfast. A standardized lunch (4849 kJ; 37 g protein, 126 g carbohydrate, 67 g fat) was provided 2 h after the termination of cycling. After completing the experimental session, subjects went home and ate their usual dinner. Each subject returned to the laboratory the next morning after overnight fasting for the biopsy. Biopsy samples from m. vastus lateralis were obtained under local anesthesia (2 ml 2% lidocaine) via a micro biopsy technique [27] prior to and 40 min, 5 h, and 22 h after the termination of cycling. Muscle samples were quickly blotted with gauze to remove superficial blood, frozen in liquid nitrogen, and stored at -80 °C until further analysis.

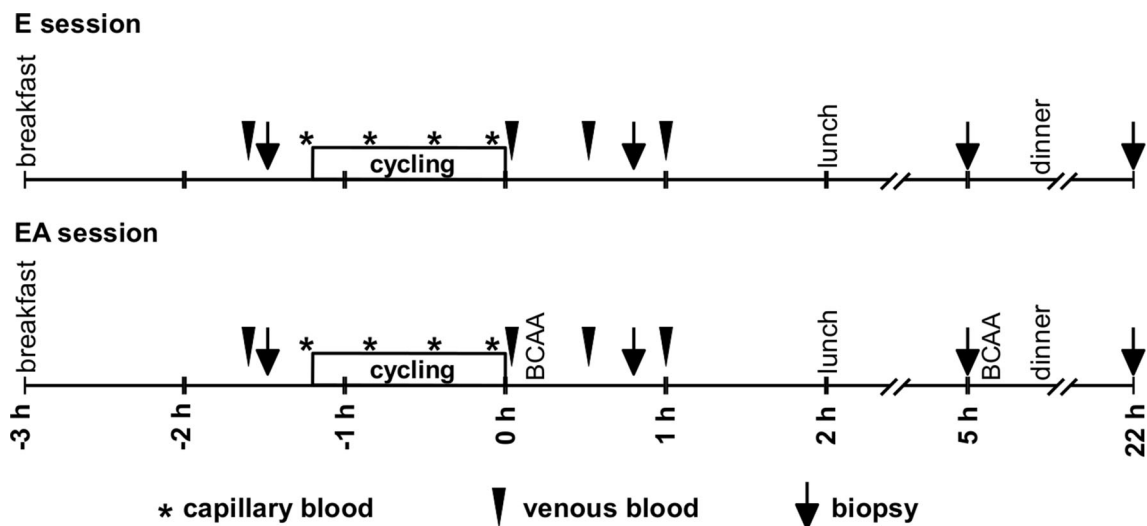


Fig. 1 Overview of study design. Each participant performed two test sessions in a random order: endurance exercise (E) and endurance exercise followed by BCAA ingestion (EA) with 1 week in between

The first biopsy was performed 15 cm proximal to the condylus lateralis ossis femoris and subsequent biopsies taken 2-cm proximal to the previous one. Biopsy samples during another session were acquired from a different leg. The leg for the first biopsy was selected in a random order.

Venous blood samples were acquired from the v. intermedia cubiti via a catheter prior to, immediately after, and 30 and 60 min after the termination of cycling. Cortisol and insulin levels were evaluated via enzyme-linked immunosorbent assay (ELISA) using the ELISA-Cortisol Kit (ImmunoTek) and Insulin ELISA Kit (Merckodia). The lactate level was measured in capillary blood; samples were taken prior to, and at 20, 40 and 65 min during cycling.

RNA extraction

Frozen samples (~20 mg) were sectioned into 20- μ m slices using an ultratome (Leica Microsystems) and RNA extracted using the RNeasy Mini Kit (Qiagen). RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). After DNase treatment (Fermentas), cDNA was obtained from 1 μ g of total RNA using the MMLV Reverse Transcriptase kit (Evrogen).

Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed using the Rotor-Gene Q cycler (Qiagen). The annealing temperature was optimized for each primer pair. The thermal profile included an initial heat denaturing step at 95 $^{\circ}$ C for 5 min, followed by 45 cycles of denaturation at 95 $^{\circ}$ C for

15 s, annealing (56–60 $^{\circ}$ C) for 30 s and extension at 72 $^{\circ}$ C for 30 s. Amplified genes were quantified via fluorescence using the EvaGreen Master Mix (Syntol). The specificity of amplification was monitored via melting curve analysis and agarose gel (1%) electrophoresis. Each sample was run in triplicate and a non-template control included in each run. Target gene mRNA expression levels were calculated using the efficiency-corrected Δ Ct method with the formula:

$$\sqrt[2]{(1 + E_{\text{ref1}})^{C_{\text{tref1}}} \times (1 + E_{\text{ref2}})^{C_{\text{tref2}}}} / (1 + E_{\text{tar}})^{C_{\text{tar}}}$$

. PCR efficiency (E) was calculated using standard curves corresponding to target and reference genes (*RPLP0*, *GAPDH*). Each standard curve included six points (diluted PCR product), with triplicate data obtained for each point. The primer sequences are shown in Table 1.

Western blot

Frozen samples (~10 mg) were homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitors (50 mmol/l β -glycerophosphatase, 50 mmol/l NaF, 1 mmol/l Na_3VO_4 , 20 μ g/ml aprotinin, 50 μ g/ml leupeptin, 20 μ g/ml pepstatin and 1 mmol/l PMSF). Samples were centrifuged for 10 min at 10,000 \times g, at 4 $^{\circ}$ C. The protein content was analyzed with the bicinchoninic acid assay. Samples were mixed with Laemmli buffer (20 μ g protein per lane) and loaded onto a 10% T polyacrylamide gel, and electrophoresis performed in the Mini-PROTEAN Tetra Cell system (Bio-Rad) at 20 mA per gel. Proteins were transferred onto nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad) in Towbin buffer for 30 min at 25 V. Membranes were stained with Ponceau S to verify consistent loading of protein,

Table 1 Primers used in this study

Transcript	Strand	Sequence, 5'–3'	Product size, bp
<i>Total PGC-1α</i>	Forward	CAGCCTCTTTGCCAGATCTT	101
	Reverse	TCACTGCACCACTTGAGTCCAC	
<i>NT-PGC-1α</i>	Forward	TCACACCAAACCCACAGAGA	172
	Reverse	CTGGAAGATATGGCACAT	
<i>TFAM</i>	Forward	AGATTCCAAGAAGCTAAGGGTGATT	85
	Reverse	TTTCAGAGTCAGACAGATTTTTCCA	
<i>TFB2 M</i>	Forward	CAAGGAAGGCGTCTAAGGC	118
	Reverse	AGCAGTAGGTGTGGAGGTC	
<i>CS</i>	Forward	GAGAAGGCAGCGGTATTG	196
	Reverse	AGGTAAGGGTCGGAAAGG	
<i>VEGFA</i>	Forward	TACCTCCACCATGCCAAG	148
	Reverse	GGTACTCCTGGAAGATGTC	
<i>TRIM63 (MURF1)</i>	Forward	CTCAGTGTCCATGTCTGGAGGCCGTT	147
	Reverse	GGCCGACTGGAGCACTCCTGTTTGTA	
<i>FBXO32 (Atrogin-1)</i>	Forward	GTCCAAAGAGTCGGCAAGTC	147
	Reverse	AGGCAGGTCAGTGAAGGTG	
<i>Myostatin</i>	Forward	CATGATCTTGCTGTAACCTCC	195
	Reverse	CGATAATCCAATCCCATCC	
<i>CTSL</i>	Forward	AAGTGAAGGCTGCAATGGT	483
	Reverse	CTCACACAGTGGGGTAGCTG	
<i>BNIP3</i>	Forward	TCTGGACGGAGTAGCTCCAA	296
	Reverse	TTCATGACGCTCGTGTTCCT	
<i>IGF1-Ea</i>	Forward	ATGCTCTTCAGTTCGTGTGTG	258
	Reverse	GCACTCCCTCTACTTGCGTTC	
<i>IGF1-Ec (MGF)</i>	Forward	ACCAACAAGAACACGAAGTC	281
	Reverse	CAAGGTGCAAATCACTCCTA	
<i>DDIT4 (REDD1)</i>	Forward	GGTTTGACCGCTCCACGAG	98
	Reverse	ATCCAGGTAAGCCGTGTCTTC	
<i>RPLP0</i>	Forward	CACTGAGATCAGGGACATGTTG	77
	Reverse	CTTCACATGGGGCAATGG	
<i>GAPDH</i>	Forward	CAAGGTCATCCATGACAACCTTTG	496

followed by washing and incubation in 5% non-fat dry milk for 1 h. Next, membranes were incubated overnight at 4 °C with anti-p70S6K1 (sc-230), anti-phospho-FOXO1^{Ser256} (sc-101681), anti-p38 (sc-728), anti-phospho-AMPK α 1/2^{Thr172} (sc-33524, all from Santa Cruz Biotechnology), anti-FOXO1 (ab52857), anti-phospho-p70S6K1^{Thr389} (ab2571), anti-phospho-ACC^{Ser79/222} (ab68191), anti-phospho-eEF2^{Thr56} (ab115165), anti-eEF2 (ab33523), anti-phospho-p38^{Thr180+Tyr182} (ab4822), anti-AMPK α 1/2 (ab80039) and anti-GAPDH (ab9485, all from Abcam).

The next day, membranes were incubated with HRP-linked anti-rabbit secondary antibody (Cell Signaling) for 1 h and washed with PBS–Tween 20 after each step (3 times for 5 min each). Following incubation of membranes with ECL substrate (Bio-Rad), luminescent signals were captured using the ChemiDoc Imaging System (Bio-Rad). Densitometry was performed using Image Lab 5.0 (Bio-

Rad). All data are expressed as the ratio of phosphorylated protein to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical analysis

Data are expressed as median and interquartile range, since sample volumes were small ($n = 9$) with non-normal data distribution. To compare post exercise values with the initial levels, nonparametric ANOVA (Friedman's test) with Dunn's multiple comparison test was used. We investigated the differences between control (E) and experimental (EA) sessions (separately for every time-point) using the Wilcoxon matched-pairs test. The level of significance was 0.05. Statistical analysis was performed using GraphPad Prism 6 software.

Results

Physiological indices

Before cycling (immediately after baseline biopsy), the blood lactate levels were 2.6 (2.3–2.7) mmol/l in the E session and 2.4 (2.0–2.5) mmol/l in the EA session. The mean blood lactate levels during the cycling were 4.2 (3.5–5.4) and 3.5 (3.0–4.7) mmol/l in the E and EA sessions respectively, and did not differ between the sessions. The plasma insulin level was decreased immediately after cycling (0.14-fold change; $P < 0.001$) for all sessions, and maintained below 6 mU/l for the first hour after termination of cycling activity (Table 2). Notably, ingestion of BCAA immediately after cycling in the EA session did not lead to an increase in the insulin level (Table 2). The plasma cortisol level was not affected either by cycling or by the combination of cycling with BCAA (Table 2).

Regulation of mitochondrial biogenesis

AMP-activated protein kinase (AMPK α 1/2) and p38 mitogen-activated protein kinase (p38) contents as well as p38^{Thr180+Tyr182} phosphorylation level were not altered during both sessions (data not shown). However, the AMPK α 1/2^{Thr172} phosphorylation level was increased by a 1.6-fold change ($P < 0.05$; Fig. 2a, 5) at 40 min after termination of cycling in the EA experiment. The phosphorylation level of acetyl coenzyme A carboxylase (ACC^{Ser79/222}), an AMPK substrate used as an endogenous marker of its activity, was increased 40 min after cycling in both the E and EA sessions (by 3.5- and 3.1-fold changes, respectively, $P < 0.05$ for both; Fig. 2b, 5). Expression of total PGC-1 α mRNA at 5 h after cycling exercise was increased to a higher extent in the E session (4.4-fold change, $P < 0.05$), compared to the EA session (Fig. 2c). Levels of N-truncated PGC-1 α gene isoforms were increased in both the E and EA sessions (3.9- and 5.6-fold, respectively, $P < 0.05$ for both), with no significant differences between the sessions (Fig. 2d). In contrast, expression patterns of PGC-1 α -related genes were distinct between the E and EA sessions. Expression of the mitochondrial transcription factor A (TFAM) gene was

increased only after the E session by ~1.3-fold change at 22-h recovery ($P < 0.05$, Fig. 2e). Expression of the vascular endothelial growth factor A (VEGFA) gene increased in the E session (3.5- and 2.5-fold change at the 40-min and 5-h recovery, $P < 0.05$ for both, Fig. 2f), while mitochondrial transcription factor B2 (TFB2M) and citrate synthase (CS) gene levels remained unchanged in both sessions (data not shown).

Regulation of proteolysis

The forkhead box O1 (FOXO1) content did not change during both sessions (data not shown). FOXO1^{Ser256} phosphorylation was decreased (0.83-fold change, $P < 0.05$, Fig. 3a, 5) up to 22 h of recovery in the E session. BCAA administration completely eliminated this cycling-induced decrease in FOXO1^{Ser256} phosphorylation and affected expression of the FOXO1 target genes, E3 ubiquitin ligases MURF1 and Atrogin-1. Expression of TRIM63 (tripartite motif containing 63, also known as MURF1) was increased (2.4-fold change, $P < 0.05$, Fig. 3b) in the E session at 40 min after termination of cycling. MURF1 mRNA levels between the E and EA sessions were significantly different ($P < 0.05$). Moreover, in contrast to the E session, BCAA ingestion induced a decrease (0.59-fold change, $P < 0.05$, Fig. 3c) in the expression of F-box protein 32 (FBXO32, also known as Atrogin-1) at 5 h after termination of cycling.

Expression levels of the autophagic-lysosomal system-related genes, cathepsin L (CTSL) and BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), did not differ between the sessions (data not shown). The myostatin (MSTN) gene displayed significant decrease (more than 0.5-fold change; $P < 0.05$, Fig. 3d) in both exercise sessions, with no significant differences between the sessions.

Regulation of synthesis

The mRNA levels of insulin-like growth factor 1, isoform Ea (IGF1-Ea) and isoform Ec (IGF1-Ec, also known as mechano growth factor, MGF), potential regulators of

Table 2 Blood insulin and cortisol levels before, immediately after, 30 and 60 min after the termination of intermittent cycling during the E and EA sessions

	Session	Before	After	30 min	60 min
Insulin, mU/l	E	26.7 (17.3–32.4)	3.6* (2.0–6.5)	5.2* (2.6–7.1)	3.8* (2.8–6.7)
	EA	26.8 (15.9–43.6)	3.80* (1.9–5.6)	5.7 (3.9–17.8)	5.1* (3.7–8.1)
Cortisol, ng/ml	E	155 (122–268)	255 (201–351)	229 (155–287)	155 (118–245)
	EA	127 (97–217)	312 (206–350)	213 (125–240)	134 (97–198)

Each value represents the median and interquartile range

* Difference from the initial level ($P < 0.05$)

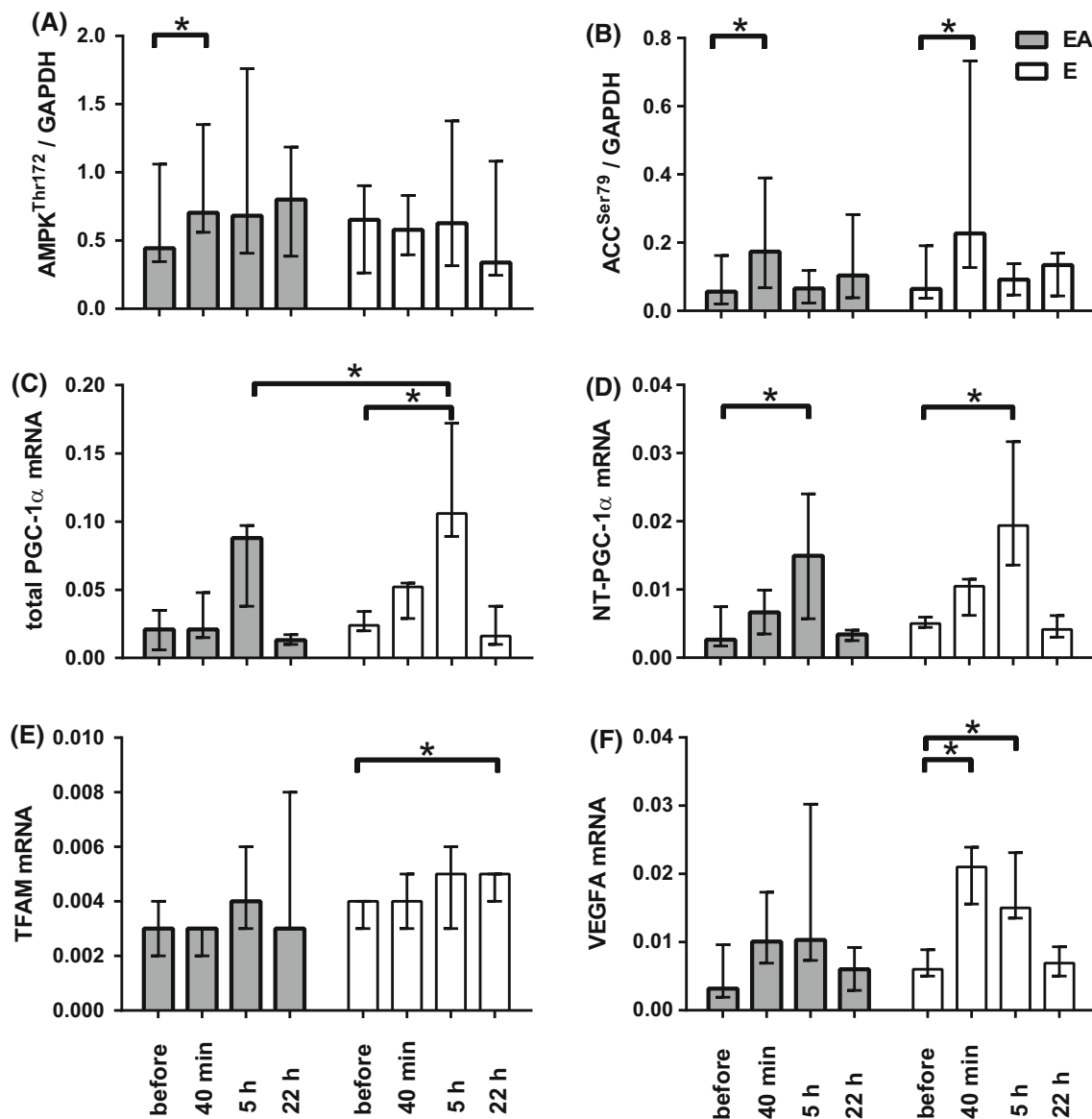


Fig. 2 Proteins and genes related to the regulation of mitochondrial biogenesis in skeletal muscle before, 40 min, and 5 and 22 h after the termination of the endurance exercise (E) and endurance exercise followed by BCAA ingestion (EA). Phosphorylation levels of AMPK^{Thr172} (a) and ACC^{Ser79} (b) and mRNA abundance of total

PGC-1 α (c), NT-PGC-1 α (d), TFAM (e), and VEGFA (f). Each target protein was normalized to GAPDH, each target mRNA was normalized to two reference genes (*GAPDH* and *RPLP0*), each value represents the median and interquartile range, *significant difference ($P < 0.05$)

myogenesis, were not different between the sessions (Fig. 4c, d). Additionally, p70 ribosomal protein S6 kinase 1 (p70S6K1) and eukaryotic elongation factor 2 (eEF2) contents remained unchanged in both sessions (data not shown). No changes were observed in the phosphorylation levels of the key anabolic markers, p70S6K1^{Thr389} or eEF2^{Thr56}, between the sessions (Fig. 4a, b, 5). However, expression of DNA damage inducible transcript 4 (*DDIT4*, also known as *REDD1*), a negative regulator of mTORC1, at 40 min after termination of cycling in the EA session was lower ($P < 0.05$) than that in the E session (Fig. 4e).

Discussion

In the current study, we conducted intermittent cycling exercise tests, since this type of exercise induces a greater increase in *PGC-1 α* and *TFAM* mRNA levels than continuous cycling [28]. Molecular response to acute aerobic exercise is more specific in endurance-trained skeletal muscle than untrained muscle [29–32]. Therefore, endurance-trained athletes were enrolled and only a few activated signaling proteins and genes involved in the regulation of mitochondrial biogenesis detected after the cycling bout. As expected, intermittent cycling (E session)

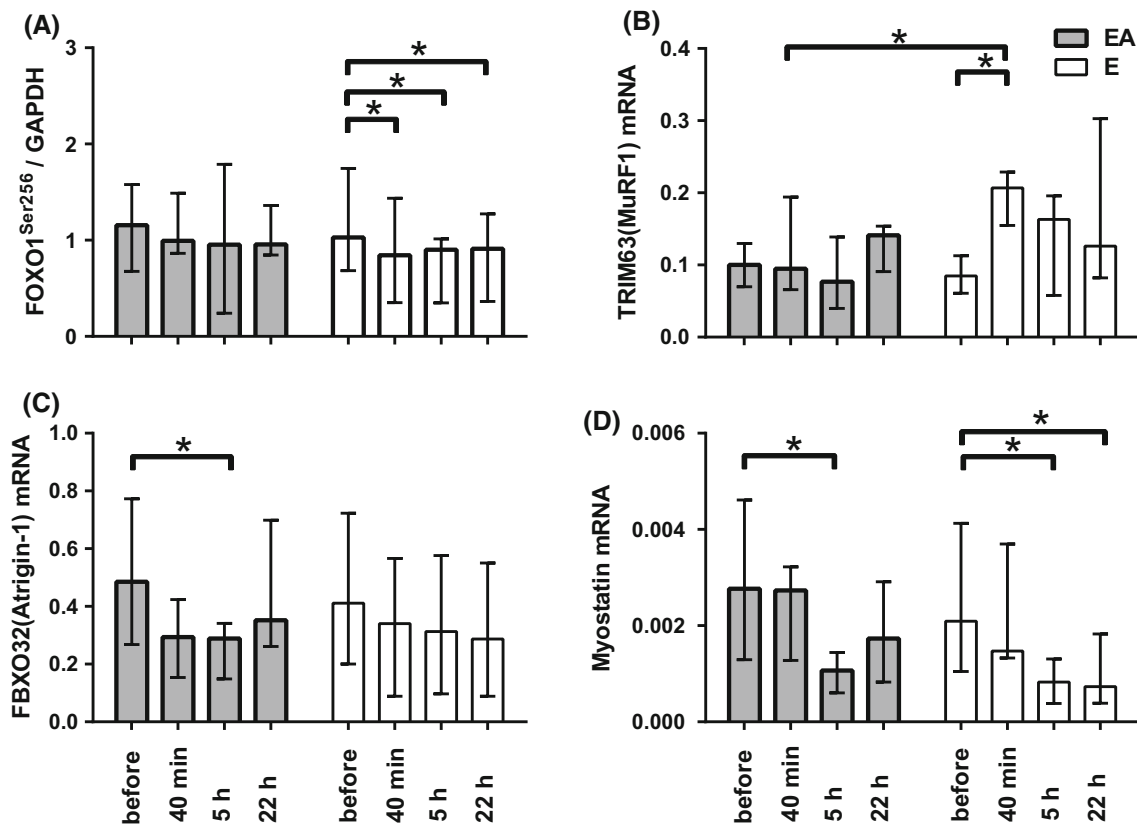


Fig. 3 Proteins and genes related to regulation of proteolysis in skeletal muscle before, 40 min, and 5 and 22 h after the termination of endurance exercise (E) and endurance exercise followed by BCAA ingestion (EA). Phosphorylation level of FOXO1^{Ser256} (a) and mRNA abundance of TRIM63 [MURF1] (b), FBXO32 [Atrogin-1] (c), and

Myostatin (d). Each target protein was normalized to GAPDH, each target mRNA was normalized to two reference genes (GAPDH and RPLP0), each value represents the median and interquartile range, *significant difference (P < 0.05)

activated AMPK (evaluated based on ACC^{Ser79/222} phosphorylation) and increased the expression of total PGC-1 α , N-truncated PGC-1 α mRNA, and the PGC-1 α -related genes TFAM and VEGFA. BCAA ingested after the cycling (EA session) had no effect on the exercise-induced activation of AMPK and expression of N-truncated PGC-1 α mRNA, but suppressed TFAM, VEGFA, and total PGC-1 α mRNA. However, the potential influence of these effects on mitochondrial biogenesis at the protein level remains to be established (Fig. 6).

We observed no changes in the levels of markers of the autophagic-lysosomal system (BNIP3 and CTSL) after exercise in both sessions. On the other hand, ubiquitin proteasome signaling was upregulated in response to E sessions, but not EA sessions. FOXO1^{Ser256} phosphorylation was decreased and the mRNA level of the E3 ubiquitin protein ligase, MURF1, increased after the exercise in E session. Our data are in accordance with findings from previous studies conducted on exercise-trained men [9–12]. These events may be evoked by a fall in the blood insulin level and consequent deactivation of Akt (V-Akt murine thymoma

viral oncogene homolog). Indeed, suppression of insulin signaling has been shown to be associated with increased expression of E3 ubiquitin ligases in mouse skeletal muscle [33] and rat heart [34] while increase in blood insulin from ~5 to 30 mU/l induces a decrease in protein breakdown in humans [20, 22]. In our study, exercise-induced activation of ubiquitin proteasome signaling (determined based on decreased FOXO1^{Ser256} phosphorylation and increased MURF1 mRNA expression) was ablated following BCAA ingestion. This effect of BCAA on ubiquitin proteasome signaling is consistent with findings from studies on immobilized rat skeletal muscle [14, 15]. Expression of MURF1 is reported to be regulated via insulin [33, 34] as well as cortisol [35] and AMPK-related signaling [36, 37]. In our study, post exercise AMPK activation (evaluated based on the phosphorylation level of ACC^{Ser79/222}), as well as cortisol and insulin levels did not differ between sessions. Despite the lack of differences in the insulin levels at 30 min after exercise between sessions, several individuals in the EA group displayed insulin levels higher than 10 mU/l. We cannot exclude the possibility that this

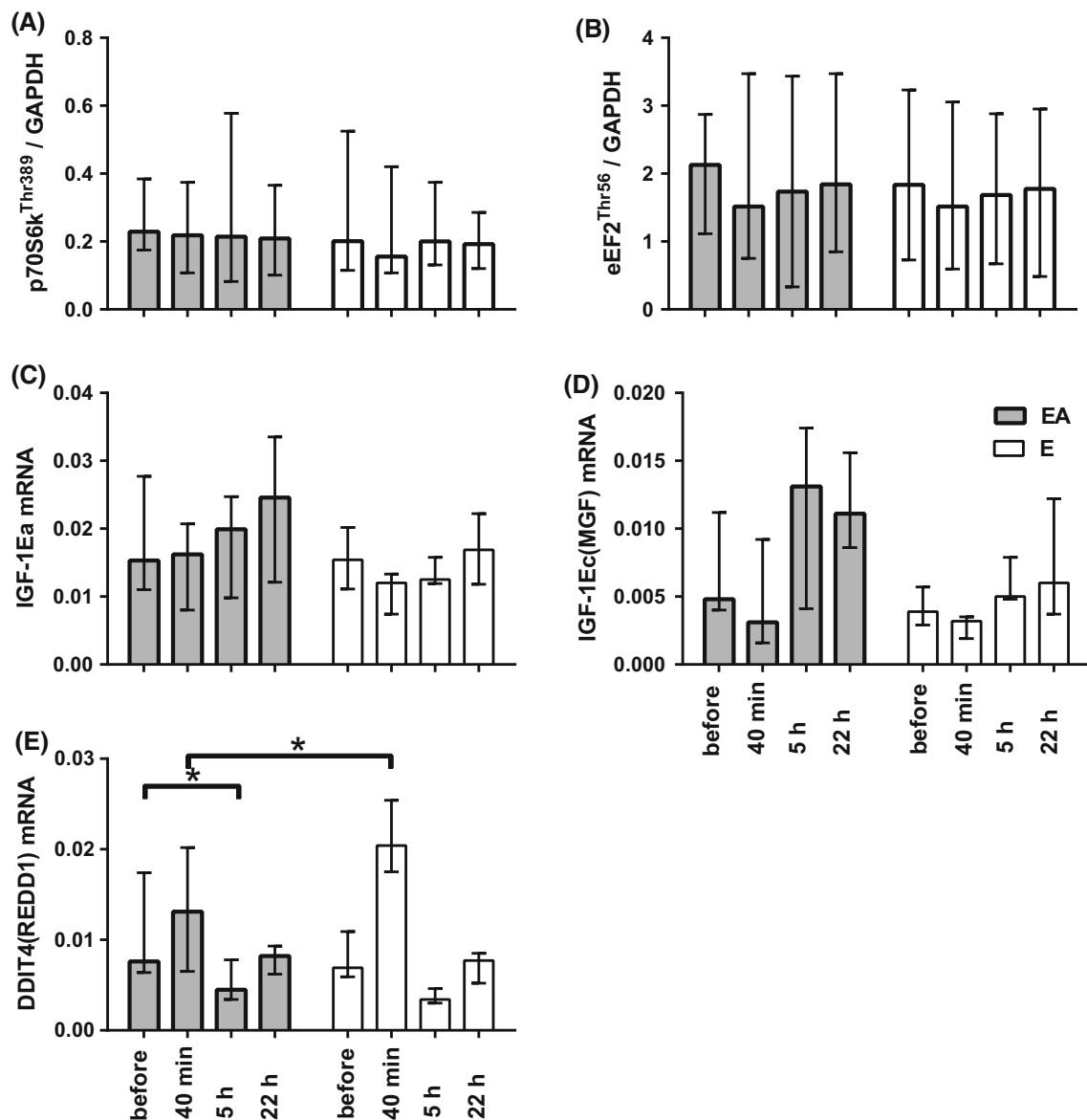


Fig. 4 Proteins and genes related to regulation of protein synthesis in skeletal muscle before, 40 min, and 5 and 22 h after the termination of endurance exercise (E) and endurance exercise followed by BCAA ingestion (EA). Phosphorylation levels of p70S6K1^{Thr389} (a) and eEF2^{Thr56} (b), mRNA abundance of *IGF1-Ea* (c), *IGF1-Ec* [*MGF* (d), and *DDIT4* [*REDD1*] (e). Each target protein was normalized to GAPDH, each target mRNA was normalized to two reference genes (*GAPDH* and *RPLP0*), each value represents the median and interquartile range, *significant difference ($P < 0.05$)

(d), and *DDIT4* [*REDD1*] (e). Each target protein was normalized to GAPDH, each target mRNA was normalized to two reference genes (*GAPDH* and *RPLP0*), each value represents the median and interquartile range, *significant difference ($P < 0.05$)

transient increase in insulin partially serves to suppress ubiquitin proteasome signaling. On the other hand, BCAA-dependent suppression of ubiquitin proteasome signaling may be related to other signaling mechanisms, for instance the activation of FOXO1-related kinase(s) and/or the inhibition of phosphatase(s). The inhibitory effect of BCAA on ubiquitin proteasome signaling supports the potential effectiveness of this approach in preventing muscle mass decrease induced by intense training in endurance athletes. Direct measurements of muscle protein breakdown are necessary to confirm this suggestion.

Amino acid administration modulates *IGF1* gene expression [38, 39], anabolic signaling and the protein synthesis rate [26]. Contrary to our hypothesis, we observed no changes in *IGF1-Ea* and *IGF1-Ec* (*MGF*) mRNA isoform expression or phosphorylation levels of key anabolic markers (p70S6K1^{Thr389} and eEF2^{Thr56}) during the EA session. Unexpectedly, BCAA administration had no effect on regulation of anabolic signaling. It might be related to metabolic perturbations in working muscle and in blood induced by intermittent endurance exercise in trained muscle. Amino acid administration under conditions of a low background blood insulin level (~ 5 mU/l) is

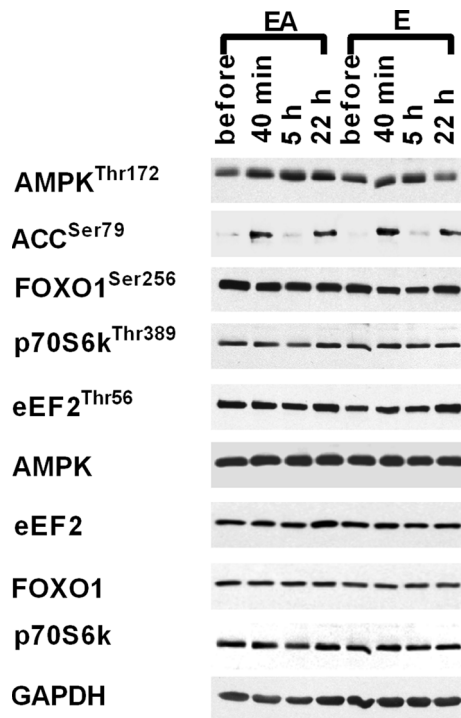


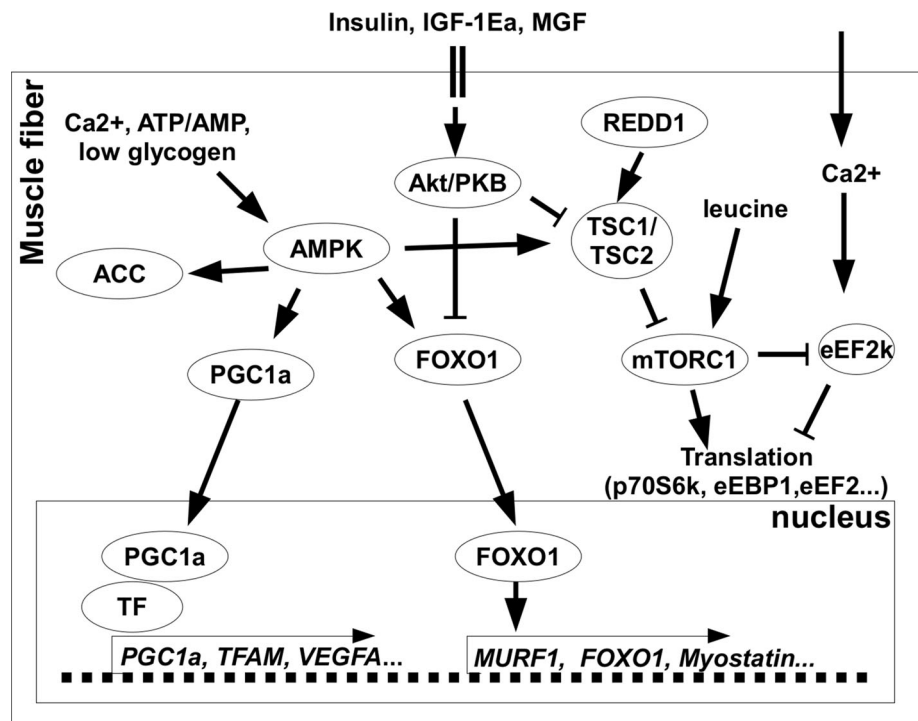
Fig. 5 Representative immunoblots of phosphorylated proteins (AMPK^{Thr172}, ACC^{Ser79}, FOXO1^{Ser256}, eEF2^{Thr56}, p70S6k^{Thr389}) and total proteins (AMPK, FOXO1, p70S6k, eEF2, GAPDH) before, 40 min, and 5 and 22 h after the termination of intermittent cycling during E and EA sessions

reported to induce an increase in muscle protein synthesis rate without affecting the phosphorylation levels of p70S6K1^{Thr389} and eEF2^{Thr56} [21]. Therefore, we cannot exclude the possibility that BCAA administration affects muscle protein synthesis during the EA session.

DDIT4 (REDD1) may mediate attenuation of mTORC1 signaling via activation of hamartin-tuberin complex (TSC1/2), a negative regulator of mTORC1. *DDIT4* mRNA and protein levels have been shown to increase in rat skeletal muscle immediately after an endurance exercise bout [40]. Murakami et al. (2011) reported that pre-exercise feeding of BCAA does not attenuate exercise-induced expression of both *DDIT4* mRNA and protein in rat skeletal muscle [41]. In our experiments, *DDIT4* gene expression was lower at 40 min after exercise in the EA session, compared with the E session. The discrepancies in findings between these studies may be attributable to the time of BCAA ingestion, i.e., before exercise in the study by Murakami and co-workers and just after exercise in our experiments.

Effects of BCAA in our study were observed mainly at 40 min and 5 h after the termination of exercise and first BCAA ingestion. The effect of BCAA ingestion on signaling and protein synthesis in human muscle lasts for a few hours [26, 42]. We think this finding may explain the lack of the effect of BCAA at 22 h of recovery in our work.

Fig. 6 The proposed molecular mechanism for the interaction between mitochondrial biogenesis, protein synthesis and degradation in skeletal muscle of endurance trained individuals after high intensity endurance exercise combined with BCAA ingestion



In conclusion, in endurance-trained human skeletal muscle, post exercise BCAA ingestion partially suppresses endurance exercise-induced expression of *PGC-1 α* mRNA, activation of ubiquitin proteasome signaling (FOXO-1 phosphorylation, *MURF1* mRNA expression), and suppresses *DDIT4* mRNA expression, an inhibitor of mTORC1. Therefore, BCAA administration may present an effective tool for preventing muscle mass decrease induced by intense training in endurance athletes. Further evaluation of muscle protein synthesis and breakdown after acute exercise combined with BCAA and studies investigating the effects of long-term endurance training along with BCAA administration are necessary to assess and optimize the efficiency of this approach.

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Author contributions The study was designed by DVP, EAL and OLV. Data were collected and analyzed by EAL, TFV, EML and DVP. Data interpretation and manuscript preparation were undertaken by EAL, OLV, and DVP. All authors approved the final version of the paper.

Compliance with ethical standards

Conflict of interest Evgeny A. Lysenko declares that he has no conflict of interest. Tatiana F. Vepkhvadze declares that she has no conflict of interest. Egor M. Lednev declares that he has no conflict of interest. Olga L. Vinogradova declares that she has no conflict of interest. Daniil V. Popov declares that he has no conflict of interest. This study was funded by the Russian Science Foundation (Grant no. 14-15-00768, control experiment) and the Russian Foundation for Basic Research (Grant no. 14-04-01807a, BCAA experiment).

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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