Absence of leucine in an essential amino acid supplement reduces activation of mTORC1 signaling following resistance exercise in young females

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Abstract

The purpose of the study was to investigate the specific effect of leucine on mTORC1 signaling and amino acid metabolism in connection with resistance exercise. Comparisons were made between ingestion of supplements with and without leucine. Eight young women performed leg press exercise on two occasions. In randomized order they received either an aqueous solution of essential amino acids with leucine (EAA) or without leucine (EAA-Leu), given as small boluses throughout the experiment. Muscle biopsies were taken after an overnight fast before exercise and 1h and 3h post-exercise and blood sampled repeatedly during the experiment. Plasma and muscle concentrations of leucine rose 60-140% ($P<0.05$) with EAA and fell 35-45% ($P<0.05$) with the EAA-Leu supplement. In the EAA-trial, plasma and muscle levels of tyrosine (not present in the supplement) and the sum of the EAA were 15-25% ($P<0.05$) lower during recovery. Phosphorylation of mTOR and p70S6k was elevated to a larger extent following 1h of recovery with leucine in the supplement (120 vs. 49% ($P<0.05$) and 59- vs. 8-fold ($P<0.05$) for EAA and EAA-Leu, respectively). The levels of MAFbx and MuRF-1 mRNA and of the corresponding proteins were not significantly altered after 3h recovery from exercise. In conclusion, the presence of leucine in the supplement enhances the stimulatory effect on mTORC1 signaling and reduces the level of tyrosine and the sum of the EAA in muscle and plasma, suggesting a stimulation of protein synthesis and/or inhibition of breakdown, leading to improvement in net protein balance.

Keywords: exercise, insulin, MAFbx, MuRF-1, muscle, p70S6k

Introduction

Regular strength training increases muscle mass and strength. The former is achieved when the protein synthesis exceeds degradation and can be observed already after 6 to 8 weeks training (Tesch 1988; Fry 2004). Even a single session of resistance exercise enhances the rate of protein synthesis significantly; however, also the rate of degradation increases, although to a lesser extent, resulting in an improved net balance (Biolo et al. 1995; Phillips et al. 1997). However, only when a protein or amino acid supplement is ingested in connection with the exercise, a positive net balance is achieved (Tipton et al. 1999). Interestingly, only the essential amino acids (EAA) are required for this effect, whereas the non-essential amino acids appear to have no impact on the net balance (Tipton et al. 1999; Børsheim et al. 2002).

Of the EAA, leucine has attracted most interest because of its ability to influence protein turnover, as demonstrated primarily in animal muscle. Direct stimulation of protein synthesis was first shown in preparations of rodent muscle (Buse and Reid 1975; Fulks et al. 1975; Tischler et al. 1982; Li and Jefferson 1978; Mitch and Clark 1984) and later in the intact animal following oral administration (Crozier et al. 2005). There is some evidence from isolated rodent muscle that leucine also reduces protein degradation (Buse and Reid 1975; Fulks et al. 1975; Tischler et al. 1982; Mitch and Clark 1984).

An indication that leucine has anabolic effects also on human muscle was first presented by Nair et al. (1992), who reported that infusion of leucine improves the net protein balance over the resting muscle. In more recent investigations on resting muscle, infusion or ingestion of leucine was found to stimulate protein synthesis and activate the regulatory enzyme 70-kD ribosomal protein S6 kinase (p70S6k) in the signaling pathway of the mechanistic target of rapamycin complex 1 (mTORC1, formerly known as the mammalian target of rapamycin) (Smith et al. 1998; Greiwe et al. 2001; Wilkinson et al.

2013). Activation of this pathway, including the downstream targets p70S6k and the eukaryotic initiation factor 4E binding protein 1 (4EBP-1), plays a key role in stimulating protein synthesis in skeletal muscle (Drummond et al. 2009; Dickinson et al. 2011). Although, these observations indicate that leucine stimulates protein synthesis in human muscle as well, this effect has not yet been confirmed following exercise.

When leucine-enriched supplements have been given to young subjects in connection with resistance exercise, little or no additional effect on protein synthesis or protein balance occurred (Katsanos et al. 2006; Koopman et al. 2005; Tipton et al. 2009; Glynn et al. 2010b). This lack of effect may, however, simply be due to the fact that the unenriched supplement itself already contains sufficient leucine to stimulate protein synthesis maximally. This suggestion is consistent with the recent finding that intake of whey protein with a small amount of leucine immediately after resistance exercise, enhanced the fractional rate of protein synthesis to the same extent as a larger amount (Churchward-Venne et al. 2012). However, whether total absence of leucine would attenuate the stimulatory effect remains unknown.

In the present investigation, the specific influence of leucine has been examined by excluding this amino acid from a supplement taken by young women in connection with resistance exercise, which we hypothesized would reduce the anabolic effect of the supplement. Following ingestion of a mixture of EAA with or without leucine (EAA-Leu), alterations in mTORC1-signalling, and the expression of genes encoding both positive and negative modulators of mTOR activity, as well as the ubiquitin ligases Muscle Atrophy F-box (MAFbx) and Muscle Ring Finger 1 (MuRF-1) were characterized. In addition, plasma levels of insulin and the concentrations of amino acids in both the plasma and muscle were determined.

Materials and methods

Subjects

Eight healthy female subjects participated in the study. They had a mean (± SE) age of 27 (± 2) years, height 167 (± 2) cm, weight 60 (± 3) kg and maximal oxygen uptake (VO_{2max}) of 2.67 (± 0.06) L min^{-1}. All were recreationally active, performing endurance and/or resistance exercise on a regular basis. They were informed both orally and in writing of the purpose of the study and the associated risks before agreeing to participate. The protocol was approved by the Regional Ethical Review Board in Stockholm and performed in accordance with the principles outlined in the Declaration of Helsinki.

Preparatory tests

In the first of the preparatory tests, the one-repetition maximum (1RM) on a leg press machine at a knee angle of 90-180° (243 Leg press 45°, Gymleco, Stockholm, Sweden) was determined (after warm-up) for each subject. The load was gradually increased until the subject was unable to perform no more than one single repetition. The subjects reached 1RM within 4-6 trials. The second and third preparatory tests were designed to familiarize the subjects with the intensity and frequency of repetition employed in the actual experimental set-up (described below). These tests were separated by an interval of approximately one week and the experiment was performed 8-13 days after the third test.

Maximal oxygen uptake was determined on a mechanically braked cycle ergometer (Monark 839E, Varberg, Sweden) some weeks after completion of the experiment. The work rate was gradually increased until exhaustion, in the manner described by Åstrand and Rodahl (1986), and oxygen uptake monitored continuously utilizing an on-line system (Amis 2001, Innovision A/S, Odense, Denmark).

Experimental protocol

During the two days prior to the experiment, the subjects were told to refrain from any intense physical activity and to eat a standardized diet containing ~15 energy (E) % protein, ~30 E% fat and ~55 E% carbohydrates. The caloric content was adjusted to the estimated basal metabolic rate and level of physical activity of each individual participant.

Following an overnight fast, the subjects arrived at the laboratory early in the morning and rested in a supine position for 30 min, after which a catheter was inserted into the antecubital vein of one arm and a resting blood sample was taken. Under local anaesthesia (2 % Carbocain, AstraZeneca, Södertälje, Sweden), a resting biopsy was taken from the vastus lateralis muscle of the right leg in four subjects and of the left leg in four subjects, using a Weil-Blakesley chonchotome, as described previously by Henriksson (1979). Thereafter, the subjects warmed-up by pedaling on a cycle ergometer (Monark 828E) at 60 W for 10 min and then performed a warm-up set of 10 repetitions at 40% 1RM. After warm-up, a second blood sample was taken and the subjects rested for three minutes before performing four sets of 10 repetitions at 80% 1RM with a 5-min interval between sets. The time required to complete each set was about 30-40 s, so this regime of resistance exercise was completed in approximately 20 min. Blood samples were drawn after the second set, immediately after completion of the protocol and following 15, 30, 60, 90, 120 and 180 min of recovery.

Biopsies from the vastus lateralis muscle (on the same leg as before the exercise) were taken after 1 and 3 hours of recovery. Thus, three biopsies were taken from the same leg, the first approximately 11-14 cm above the mid-patella and each subsequent one was taken from a new incision approximately 3-5 cm proximal to the previous biopsy. These samples were immediately frozen in liquid nitrogen and thereafter stored at -80°C for

subsequent analysis. During the second experiment, biopsies were taken at the same level, but from the opposite leg.

The subjects ingested 150 ml of a solution containing either EAA or EAA without leucine (EAA-Leu) before warming-up on the cycle ergometer and before warming-up on the leg press machine, after the third set of resistance exercise and following 15, 30, 60 and 90 min of recovery. The total amount of EAA supplied was 260 mg EAA kg^{-1} body weight (including 13.7% L-histidine, 9.4% L-isoleucine, 17.3% L-leucine, 18.0% L-lysine, 2.9% L-methionine, 14.4% L-phenylalanine, 13.7% L-threonine and 10.7% L-valine). This total intake of leucine (45 mg kg^{-1} body weight) was identical to that in a previous study on the effect of branched-chain amino acid (BCAA) intake on male subjects (Karlsson et al. 2004). In the other drink (EAA-Leu), L-leucine was replaced by L-glycine to provide the same amount of nitrogen in the two supplements. The current drinking protocol with repeated small boluses has been employed in previous studies (Karlsson et al. 2004; Apró and Blomstrand 2010; Borgenvik et al. 2012), in which anabolic effects of the supplement have been observed. The two experiments were performed in a randomized, double-blind, cross-over fashion. Both drinks were lemon-flavored, contained salts and artificial sweetener, and were indistinguishable in taste. On the first of the two experimental occasions, four subjects were supplied with EAA and the other four with EAA-Leu, each receiving the other solution on the second occasion. The participants were in various phases of their menstrual cycles and taking oral contraceptives or not, and the two experiments were always separated by four weeks. Figure 1 shows a schematic overview of the experimental protocol.

Analyses of plasma

Blood samples drawn from the venous catheter into heparinised tubes were transferred to Eppendorf tubes, centrifuged (10,000 g for 3 min) and the plasma stored at -80°C. For determination of amino acids, the plasma was first deproteinized by precipitation with ice-cold 5% trichloroacetic acid (1:5), maintained on ice for 20 min, centrifuged at 10,000 g for 3 min and the resulting supernatant stored at -80°C for later analysis. The concentrations of free amino acids in the supernatants were measured by reversed-phase high-performance liquid chromatography (HPLC, Waters Corporation, Milford, MA, USA) employing orthophthalaldehyde (OPA) as the derivatizing agent as described by Pfeifer et al. (1983). Plasma insulin was measured using a radioimmunoassay kit in accordance with the manufacturer's protocol (Millipore, Billerica, MA, USA). Glucose and lactate concentrations were analyzed as described by Bergmeyer (Bergmeyer 1974).

Analyses of muscle amino acids

The muscle biopsies were freeze-dried and blood and connective tissue subsequently dissected away under a light microscope (Carl Zeiss, Germany). Following transfer of 2-3 mg muscle tissue to Eppendorf tubes, the amino acids were extracted with ice-cold 5% trichloroacetic acid (40 µL per mg), and the tubes then maintained on ice for 30 min, centrifuged at 10,000 g for 3 min and the resulting supernatant removed and stored at -80°C for subsequent analysis of amino acids as described above.

Immunoblotting

Muscle samples (~ 3 mg) lyophilized and dissected free from blood and connective tissue were first homogenized in ice-cold buffer (80 µL mg^{-1} dry weight) containing 2 mmol L^{-1} HEPES, pH 7.4, 1 mmol L^{-1} EDTA, 5 mmol L^{-1} EGTA, 10 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} β-glycerophosphate, 1% TritonX-100, 1 mmol L^{-1} Na_3VO_4, 2 mmol L^{-1} dithiothreitol, 20

µg mL^{-1} leupeptin, 50 µg mL^{-1} aprotinin, 1% phosphatase inhibitor cocktail (Sigma P-2850) and 40 µg $µL^{-1}$ PMSF using a ground-glass homogenizer. The homogenization and Western blotting were performed according to the protocol described by Apró and Blomstrand (2010), with minor modifications. In brief, the homogenates were centrifuged at 10,000 g for 10 min at 4°C and the resulting supernatant stored at -80°C for later use. Protein was determined in aliquots of the supernatant diluted 1:10 in distilled water using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA). The samples were diluted in Laemmli sample buffer (Bio-Rad Laboratories, Richmond, CA, USA) and homogenizing buffer to obtain a final protein concentration of 1.5 µg $µL^{-1}$, heated at 95°C for 5 min and then kept at -20°C until analysis.

Samples containing 30 µg protein were separated by SDS-PAGE (300 V for 35 min) on precast Criterion cell gradient gels (4-20% acrylamide; Bio-Rad Laboratories) following which the gels were equilibrated in transfer buffer (25 mmol L^{-1} Tris base, 192 mmol L^{-1} glycine, and 10% methanol) for 30 min. All samples from each subject were run on the same gel. The proteins were then transferred to polyvinylidine fluoride membranes (Bio-Rad Laboratories) at a constant current of 300 mA for 3 h at 4°C and these membranes then stained with MemCode™ Reversible Protein Stain Kit (Pierce Biotechnology) to confirm successful transfer of proteins.

After blocking for 1 h at room temperature in Tris-buffered saline (TBS; 20 mmol L^{-1} Tris base, 137 mmol L^{-1} NaCl, pH 7.6) containing 5% non-fat dry milk, the membranes were incubated overnight with commercially available primary phosphospecific antibodies diluted in TBS supplemented with 0.1% Tween-20 containing 2.5% non-fat dry milk (TBS-TM). Next, the membranes were washed with TBS-TM, incubated for 1 h at room temperature with appropriate secondary antibodies, and washed again several times. The phosphorylated proteins were subsequently visualized by chemiluminescent detection with

a Molecular Imager ChemiDoc™ XRS system. All bands were quantified using the contour tool in the Quantity One® version 4.6.3 software (Bio-Rad Laboratories) and the levels of phosphorylated proteins expressed in arbitrary units relative to the level of α-tubulin.

Antibodies

Primary antibodies directed against phospho-mTOR (Ser^{2448}; diluted 1:500), phospho-$p70^{S6k}$ (Thr^{389}; 1:1000), phospho-Akt (Ser^{473}; 1:1,000), phospho-eEF2 (Thr^{56}; 1:2,000) and eIF3A (1:1,1000) (Cell Signaling Technology, Beverly, MA, USA), MAFbx (1:1,000) (Abcam, Cambridge, United Kingdom), MuRF-1 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-tubulin (1:5,000) (Sigma-Aldrich, St. Louis, MO, USA), and secondary rabbit and mouse (1:10,000) (Cell Signaling Technology) and goat antibodies (1:5,000) (Abcam) were purchased from the sources indicated.

Quantification of mRNA

Total RNA was extracted from 2-5 mg of freeze-dried muscle tissue homogenized in PureZOL RNA Isolation Reagent (Bio-Rad Laboratories) using a ground-glass homogenizer. The concentration and purity of the isolated RNA was assessed spectrophotometrically (Borgenvik et al. 2012). One microgram of RNA was then utilized to produce 20 µL complementary DNA (cDNA) with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The concentration of cDNA, annealing temperature, and conditions for the polymerase chain reaction (PCR) were optimized for each primer pair and maintained within the linear range for amplification. To allow direct comparison of relative protein levels, all samples from each participant were run in triplicate in parallel on the same 96-well plate.

Real-time quantitative PCR amplification (RT-qPCR) was performed on a Bio-Rad iCycler (Bio-Rad Laboratories) in a 25-µL volume containing 12.5 µL 2 x SYBR Green Supermix (Bio-Rad Laboratories), 0.5 µL of both the forward and reverse primers (10 µmol L^{-1}) and 11.5 µL template cDNA (for further details see Borgenvik et al. 2012). Use of the housekeeping GAPDH mRNA as an internal control has been validated previously under the same experimental conditions. The expression of each target gene was evaluated with the $2^{-\Delta Ct}$ method, where $\Delta C_t = (C_{t\ \text{Gene of interest}} - C_{t\ \text{GAPDH}})$. Analyses were performed on the biopsy samples taken before and three hours after exercise.

Statistical analyses

All data are expressed as means ± SE and were checked for normal distribution before performing parametric statistical analyses. A two-way repeated measures ANOVA was applied to evaluate changes over time and differences between EAA and EAA-Leu (time, supplement) with respect to the concentrations of amino acids and the levels of protein phosphorylation and mRNA in muscle, as well as plasma levels of glucose, lactate, insulin and amino acids. In addition, the area under the concentration versus time curve for plasma insulin was compared using Students´ t-test for paired observations. Unless otherwise indicated, the values presented in the text are means ± SE and a P-value <0.05 was considered to be statistically significant.

Results

Resistance exercise

The mean value for the maximal strength of the participants, 1RM, was 188 (± 14) kg and the average workload during the exercise protocol was 150 (± 11) kg, which corresponds

to 80 (± 0.05) % of 1RM. All 8 subjects completed the entire exercise protocol (four sets of 10 repetitions) on both occasions.

Plasma levels of glucose, lactate and insulin

The glucose concentration decreased slightly ($P<0.05$) in both conditions, from 5.2 ± 0.3 to 4.7 ± 0.4 mmol L^{-1} (EAA) and from 5.2 ± 0.2 to 5.0 ± 0.2 mmol L^{-1} (EAA-Leu) at the end of the experiment, and the lactate increased ($P<0.05$ for both conditions) from 1.1 ± 0.3 to 3.8 ± 0.7 mmol L^{-1} (EAA) and from 1.3 ± 0.3 to 4.2 ± 0.5 mmol L^{-1} (EAA-Leu) at the end of exercise and had returned to basal 1 h after exercise.

Both supplements elevated the plasma level of insulin, but the effect of EAA was more pronounced, yielding an area under the concentration versus time curve for the entire experimental period that was 24% larger ($P<0.05$). Taking this curve into consideration only up to one hour of recovery (when the insulin level had returned to the basal value in both cases), the impact of EAA was 33% greater (Fig. 2).

Plasma concentrations of amino acids

Table 1 documents the plasma concentrations of amino acids before, during and after exercise as well as in the recovery period in subjects receiving the two different supplements. With the EAA supplement, the concentration of leucine increased continuously during exercise and 30 min recovery, at which time the level was 2.4-fold higher than the basal value. This level remained relatively constant during the subsequent 1.5 h of recovery and, despite attenuation during the third hour, was still 54% higher than pre-exercise at the end of the recovery period. In the case of supplementation with EAA-Leu, the leucine level fell by 25% during the experimental period (Fig. 3a).

The plasma levels of the other two BCAA, isoleucine and valine, rose during exercise with both supplements, but 30 min (isoleucine) or 90 min (valine) into recovery the levels were lower when EAA were ingested, resulting in final values 30 and 57% lower, respectively, than with EAA-Leu. A similar trend was observed for the sum of EAA (excluding leucine) and in the case of tyrosine (not included in the supplements), i.e., the plasma level was enhanced to a similar extent during exercise in both cases, but then leveled off and decreased during recovery to a final value with EAA that was 30% lower than with EAA-Leu ($P<0.05$) (Fig. 3c, e). As expected, the concentration of glycine was markedly elevated by ingestion of the EAA-Leu supplement since this amino acid was included in the supplement instead of leucine.

Amino acid concentrations in muscle

Table 2 documents alterations in the concentration of amino acids in muscle during and after exercise with both supplements. With EAA the level of leucine was elevated 64% 1 h after exercise and remained elevated during 3 h of recovery, whereas this level fell 40-45% in association with ingestion of EAA-Leu (Fig. 3b). The levels of isoleucine and valine rose during the first hour of recovery in both cases, but to a lesser extent with EAA than EAA-Leu (35 vs. 79% for valine and 56 vs. 167% for isoleucine, respectively). Moreover, these concentrations continued to increase during 1-3 h of recovery with the EAA-Leu supplement, but were attenuated during this same time period with EAA, resulting in final values of valine and isoleucine that were 30% and 60% lower, respectively, in the latter case. A similar pattern was observed with respect to the levels of tyrosine and the sum of EAA (excluding leucine) (Fig. 3d, f). The concentration of glycine was higher with the EAA-Leu supplement, as expected since glycine was added to the supplement to replace leucine.

Protein phosphorylation in muscle

After one and 3 h of recovery, phosphorylation of Akt at Ser473 was not different to before exercise in any of the conditions (Fig. 4a). After resistance exercise, phosphorylation of mTOR was enhanced with both supplements, but more so with EAA than EAA-Leu (2.2-fold vs. 46%, respectively, after 1 h of recovery; $P<0.05$) (Fig. 4b). Similar patterns were observed regarding the phosphorylation of p70S6k which was elevated 1 and 3 h following exercise with both supplements, but to a larger extent with EAA (59-fold increase with EAA vs. 8-fold increase with EAA-Leu after 1 h of recovery; $P<0.05$) (Fig. 4c). Phosphorylation of the elongation factor eEF2 was reduced 30-50% ($P<0.05$) following exercise in both cases (Fig. 4d). The two-way ANOVA revealed a main effect of time, as well as an interaction between time and supplement for phosphorylation of mTOR and p70S6k, and a main effect of time with respect to eEF2.

mRNA levels in muscle

The level of REDD1 mRNA was reduced 60% after 3 h of recovery from resistance exercise with both supplements, whereas the level of REDD2 and hVsp34 mRNA did not change significantly under any condition. The expression of Rheb mRNA was elevated after 3 h of recovery with both supplements, as was the level of cMyc mRNA (Fig. 5). For both these genes a main effect of time was achieved in the ANOVA analysis.

With respect to the two ubiquitin ligases MAFbx and MuRF-1, the levels of encoding mRNA and corresponding protein were not significantly altered after 3 h of recovery from exercise (Fig. 6a-d), although the level of MAFbx mRNA tended to be reduced with both supplements ($P=0.12$). Expression of MuRF-1 mRNA, was lowered 40% only when EAA was ingested (reduced in 6 of the 8 subjects), although the interaction

between supplement and time did not reach significance in the ANOVA. The level of initiating factor eIF3A protein, a substrate for proteolysis by MAFbx, was the same after 3 h of recovery as before the exercise with both supplements (Fig. 6e).

Discussion

The present investigation reveals that lack of leucine in an EAA supplement taken in combination with resistance exercise attenuates the anabolic effect. With leucine present, signaling through mTORC1 was enhanced and, furthermore, a smaller or no increase in plasma and muscle concentrations of tyrosine and the sum of EAA was observed during the recovery period suggesting an improvement in net protein balance. Together, these findings indicate that leucine plays a particularly important role in stimulating anabolic processes in human muscle following exercise, as previously shown in experimental animals in resting conditions (Anthony et al. 2000; Escobar et al. 2005, 2006).

In agreement with our hypothesis, inclusion of leucine in the EAA supplement enhanced stimulation of mTORC1 signaling, as seen from the more extensive increases in phosphorylation of mTOR and p70S6k during the recovery period. However, after three hours of recovery, this difference between the supplements had disappeared; phosphorylation of mTOR and p70S6k remained elevated but to a similar extent, probably as a lasting effect of the exercise. Nutrition appears to have a more transient effect on anabolic processes as judged from data on resting muscle (Atherton et al. 2010a). Phosphorylation of the elongation factor eEF2 was attenuated by exercise, in agreement with earlier reports (Dreyer et al. 2006; Mascher et al. 2008; Apro and Blomstrand 2010). However, dephosphorylation of eEF2 was similar with and without leucine in the supplement, in agreement with the observation that in isolated C2C12 muscle cells, leucine

activates mTOR and the downstream proteins p70S6k and 4EBP-1, but is without effect on elongation factors (Atherton et al. 2010b).

As expected, both plasma and muscle concentrations of leucine were markedly elevated by ingestion of the EAA mixture. This increase can have a direct stimulatory effect on mTORC1-signalling in muscle, as previously seen in incubated cells. Addition of leucine to the incubation medium promotes p70S6k phosphorylation/activation to a greater extent than any other amino acid, clearly emphasizing the unique influence of leucine (Patti et al. 1998; Shigemitsu et al. 1999; Kimball and Jefferson 2002; Atherton et al. 2010b). One mechanism that may underlie this stimulatory effect of leucine has recently been shown to involve leucyl-tRNA synthetase, which, upon sensing the higher intracellular level of leucine, initiates molecular events leading to the activation of mTORC1 (Han et al. 2012).

In addition to such a direct stimulatory effect on anabolic signaling, leucine can act indirectly by stimulating insulin secretion (van Loon et al. 2000). With the EAA supplement, plasma insulin levels peaked at approximately 40 mU L^{-1} versus 30 mU L^{-1} with EAA-Leu, a difference which is relatively minor and unlikely to substantially enhance activation of mTORC1 and protein synthesis (Greenhaff et al. 2008), although an effect of the higher insulin cannot be excluded. Despite the elevated levels of insulin caused by both supplements, no change in Akt-Ser473 phosphorylation was detected. This might be due to the fact that the first biopsy following exercise was taken after one hour of recovery, when the insulin level had returned to the basal value and, perhaps also any augmentation of Akt-Ser473 phosphorylation, which appears to mirror the insulin response (Atherton et al. 2010a).

During ingestion of the EAA-Leu supplement the levels of EAA rose continuously throughout the two and three hours recovery for plasma and muscle,

respectively. In contrast, intake of EAA elevated the plasma and muscle concentrations of these amino acids initially, but their concentrations reached a plateau after 30-60 min and fell during the third hour of recovery, and the concentrations of EAA in both plasma and muscle were significantly lower when leucine was present in the supplement (Fig. 3). The same pattern was observed for tyrosine, which, like phenylalanine, is not metabolized in skeletal muscle. The reduction observed in both plasma and muscle could indicate that leucine stimulates the utilization of amino acids for synthesis of new proteins and/or that leucine reduces the rate of protein breakdown (Nair et al. 1992). Similar observations have been reported previously during ingestion of BCAA in connection with exercise or infusion of BCAA or leucine alone at rest, strongly suggesting that the net balance of muscle protein is improved by leucine (Eriksson et al. 1981; Alvestrand et al. 1990; Borgenvik et al. 2012). The present results support the conclusion that oral intake of leucine exerts a particularly potent stimulatory effect on anabolic processes in human muscle, in agreement with a recent study on resting muscle (Wilkinson et al. 2013).

In addition to acute stimulation of mTOR through phosphorylation, the activity of this protein can be modulated by altering the level of proteins that regulate its activity. However, in contrast to the more pronounced effect of EAA versus EAA-Leu on mTORC1- signaling, these two supplements influenced the gene expression of positive and negative modulators of mTOR activity to similar extent. Expression of cMyc and Rheb (positive regulators) was enhanced and that of REDD1 (a negative regulator) reduced after 3 h of recovery, changes that may promote an increase in mTOR activity, both with and without leucine in the supplement. Similar changes occur following resistance exercise without nutrition (Apró et al. 2013), suggesting that this effect is induced by exercise rather than the amino acid supplements.

In contrast to its stimulatory effect on protein synthesis, minor effects of EAA on the rate of protein breakdown following an acute session of resistance exercise have been reported (Tipton et al. 1999; Rasmussen et al. 2000; Borsheim et al. 2002). Consistent with this, intake of EAA and carbohydrates in combination with resistance exercise led to an increase in mRNA expression of MuRF-1 and no effect on MAFbx, the same changes as previously have been reported after exercise alone (Mascher et al. 2008; Glynn et al. 2010a). In the present study, both supplements prevented the expected elevation in the level of MuRF mRNA and, furthermore, a tendency towards a lower level of MAFbx mRNA after 3 h of recovery from resistance exercise was observed. A pronounced, although non-significant reduction in the level of MuRF-1 mRNA was observed during intake of EAA, however, this was not accompanied by a reduction in the level of the corresponding protein (Fig. 6). Thus, leucine *per se* appears to exert little influence, since our data indicate that supplementation with EAA, either with or without leucine, attenuates the induction of catabolic processes by resistance exercise. However, the absence of a placebo situation in the present investigation, disallows a definite conclusion.

Insulin is considered to be an important regulator of protein breakdown, for example demonstrated in the study by Biolo et al. (1999), where infusion of insulin following resistance exercise reduced the post exercise increase in protein breakdown. It is therefore possible that leucine, indirectly through insulin, could have reduced protein degradation and consequently contributed to improve the net protein balance in the EAA trial. Speaking against this view is the fact that large differences in insulin secretion generated similar effects on protein breakdown and on catabolic gene expression (Glynn et al. 2010a). Hence, it is unlikely that the differences of approximately 30% in insulin concentration observed here between the EAA and EAA-Leu trial would have resulted in detectable differences in proteolysis that would have been attributed to insulin.

The discrepancies between our present findings and those of earlier reports with regard to MuRF-1 and MAFbx mRNA may, at least to some extent, be due to differences in experimental design. In the present study, the amino acid supplements were provided during the exercise itself, as well as early in the recovery period, which may be more effective than intake 30-60 min after completion of the exercise (Rasmussen et al. 2000; Borsheim et al. 2002; Glynn et al. 2010a). Enhancing levels of amino acids in muscle already during exercise may reduce the requirement for protein degradation to supply free amino acids for the synthesis of new proteins (Phillips et al. 1997).

There are few reports on the effects of resistance exercise on women. However, two recent articles showed an increase in the fractional rate of protein synthesis and in mTORC1-signaling following resistance exercise in female subjects, both with (West et al. 2012) and without nutritional supplement (Dreyer et al. 2010). Our present results are in line with the former study and show that resistance exercise in combination with nutrition stimulates the mTORC1-pathway in females. In addition, we demonstrate that without leucine in the supplement, the stimulation is attenuated.

The lack of direct measurement of the rate of protein synthesis in muscle in the present investigation limits to some extent the conclusions that can be drawn. Although there are a number of reports documenting parallel changes in mTORC1-signalling and the fractional rate of protein synthesis (Cuthbertson et al. 2005; Kumar et al. 2009; Atherton et al. 2010a), this is not a consistent finding (Greenhaff et al. 2008). However, the several-fold greater phosphorylation of mTOR and p70S6k during intake of EAA in comparison to EAA-Leu strongly supports a role for leucine in the regulation of muscle protein synthesis, even though quantification of this role on the basis of alterations in phosphorylation may not be possible.

In conclusion, our present findings demonstrate the beneficial effects of including leucine in an EAA supplement ingested by women in combination with resistance exercise. Leucine enhances the stimulatory effect of such a supplement on signaling through the mTORC1 pathway and prevents the progressive increase in or even reduces the levels of tyrosine and the sum of EAA in both the plasma and muscle tissue. These observations indicate that leucine stimulates the incorporation of amino acids into new proteins, thereby improving the net balance. Furthermore, the results support the conclusion that oral intake of leucine in combination with resistance exercise exerts a particularly potent stimulatory effect on anabolic processes in human muscle.

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Figure legends

Figure 1. Schematic overview of the experimental protocol. WU refers to warm-up and RE refers to resistance exercise (4 x 10 repetitions at 80% 1RM). The subjects were in randomized order supplied a drink containing either EAA or EAA-Leu during the experiment.

Figure 2. Plasma levels of insulin in subjects receiving either the EAA or EAA-Leu supplement during and after resistance exercise. The filled boxes represent the EAA supplement and the gray triangles the EAA-Leu supplement. The area under the curve (AUC) was calculated from time point 0 min to 220 min. The black bar represents the EAA supplement and the grey bar the EAA-Leu supplement. The values given are means ± SE for 8 subjects in each case. $^{*}P < 0.05$ in comparison to resting level, $^{\#}P < 0.05$ in comparison to EAA-Leu.

Figure 3. Plasma and muscle levels of leucine (a and b), essential amino acids (EAA; c and d) and tyrosine (e and f) in subjects receiving either the EAA or EAA-Leu supplement during and after resistance exercise. The filled boxes represent the EAA supplement and the gray triangles the EAA-Leu supplement. Regarding the plasma and muscle levels, EAA include the sum of histidine, isoleucine, lysine, methionine, phenylalanine, threonine and valine. The values presented are means ± SE for 8 subjects in each case. $^{*}P < 0.05$ in comparison resting level (Pre-Ex), $^{\#}P < 0.05$ in comparison to EAA-Leu.

Figure 4. Phosphorylation of Akt at Ser^{473} (a), mTOR at Ser^{2448} (b), p70S6k at Thr^{389} (c) and eEF2 at Thr^{56} (d) before resistance exercise (Pre-Ex) and following 1 and 3 h of recovery (1h Post and 3h Post) in the muscle of subjects receiving either the EAA (black

bars) or the EAA-Leu (white bars) supplement. Representative immunoblots from one subject are shown above each graph. Since the order of supplements was randomized, some of the bands have been rearranged here for clarity. The values presented are in arbitrary units relative to the level of α-tubulin and represent the mean ± SE for 8 subjects in each case. $^{*}P < 0.05$ in comparison to Pre-Ex, $^{\#}P < 0.05$ in comparison to EAA-Leu.

Figure 5. Levels of mRNA encoding REDD1 (a), REDD2 (b), Rheb (c) and cMyc (d) before resistance exercise (Pre-Ex) and following 3 h of recovery (3h Post) in the muscle of subjects receiving either the EAA (black bars) or the EAA-Leu (gray bars) supplement. The levels of mRNA were calculated by the $2^{-\Delta Ct}$ procedure and normalized to that of GAPDH. The values shown are in arbitrary units, and represent the mean ± SE for 8 subjects in each case. $^{*}P < 0.05$ in comparison to Pre-Ex.

Figure 6. Levels of mRNA encoding MAFbx (a) and MuRF-1 (b) and of MAFbx (c), MuRF-1 (d) and eIF3a (f) total protein before resistance exercise (Pre-Ex) and following 3 h of recovery (3h Post) in the muscle of subjects receiving either the EAA (black bars) or the EAA-Leu (gray and white bars) supplement. The levels of mRNA were calculated by the $2^{-\Delta Ct}$ procedure and normalized to that of GAPDH. The levels of protein are expressed in arbitrary units relative to the content of α-tubulin. Representative immunoblots from one subject are shown above each protein graph. Since the order of supplement was randomized, some bands have been rearranged here for clarity. Values given are in arbitrary units, and represent the mean ± SE for 8 subjects in each case.

Table 1. Plasma concentrations of amino acids before, during and after resistance exercise, as well as repeatedly during recovery, in subjects receiving a supplement containing essential amino acids with (EAA) or without leucine (EAA-Leu). The values (μmol · L^{-1}) given are means ± SE for 8 subjects in each case. *$P < 0.05$ in comparison to the value at rest, #$P < 0.05$ in comparison to EAA-Leu, n.s. not significant.

Amino Acid	Supplement	Rest	Exercise			Recovery						Main effects		Int. effects
			Before	During	After	15	30	60	90	120	180	Time	Suppl	Time x Suppl
Histidine	EAA-Leu	84 ± 4	89 ± 4	120 ± 5*	150 ± 10*	155 ± 10*	155 ± 15*	165 ± 20*	150 ± 15*	165 ± 15*	105 ± 10*	P<0.05	n.s	n.s
	EAA	87 ± 5	92 ± 5	115 ± 5*	145 ± 5*	155 ± 10*	155 ± 15*	165 ± 5*	145 ± 10*	160 ± 10*	100 ± 5*			
Glycine	EAA-Leu	215 ± 25	225 ± 20	310 ± 30*	370 ± 40*	375 ± 40*	390 ± 40*	410 ± 50*	365 ± 55*	405 ± 40*	275 ± 40*	P<0.05	P<0.05	P<0.05
	EAA	200 ± 30	200 ± 30	195 ± 30#	190 ± 25#	175 ± 30#	175 ± 25#	170 ± 25#	160 ± 20#	160 ± 20#	160 ± 20#			
Threonine	EAA-Leu	140 ± 15	155 ± 20	175 ± 10*	215 ± 25*	210 ± 10*	255 ± 20*	265 ± 25*	245 ± 15*	305 ± 30*	245 ± 25*	P<0.05	n.s	P<0.05
	EAA	110 ± 15	125 ± 15	145 ± 15*#	160 ± 15*#	180 ± 20*#	190 ± 20*#	195 ± 20*#	205 ± 20*#	220 ± 25*#	170 ± 20*#			
Tyrosine	EAA-Leu	49 ± 2	51 ± 2	59 ± 3*	60 ± 3*	61 ± 4*	66 ± 4*	68 ± 5*	67 ± 5*	77 ± 5*	68 ± 5*	P<0.05	n.s	P<0.05
	EAA	50 ± 2	51 ± 2	56 ± 2*#	59 ± 3*#	60 ± 3*	62 ± 4*#	60 ± 4*#	58 ± 4*#	57 ± 4*#	48 ± 3*#			
Methionine	EAA-Leu	25 ± 1	29 ± 3*	36 ± 3*	37 ± 2*	37 ± 2*	42 ± 3*	40 ± 2*	40 ± 1*	42 ± 1*	33 ± 1*	P<0.05	n.s	P<0.05
	EAA	24 ± 1	26 ± 1	32 ± 1*#	35 ± 1*#	34 ± 1*	37 ± 2*#	33 ± 2*#	33 ± 2*#	32 ± 1*#	22 ± 1*#			
Valine	EAA-Leu	190 ± 5	210 ± 10	290 ± 15*	305 ± 15*	315 ± 10*	340 ± 10*	370 ± 20*	395 ± 15*	465 ± 20*	410 ± 10*	P<0.05	n.s	P<0.05
	EAA	205 ± 15	230 ± 10	285 ± 10*	315 ± 10*	330 ± 15*	355 ± 15*	350 ± 15*	365 ± 10*	380 ± 15*#	290 ± 5*#			
Phenylalanine	EAA-Leu	50 ± 2	60 ± 4	82 ± 6*	84 ± 4*	92 ± 5*	105 ± 8*	105 ± 10*	110 ± 5*	115 ± 5*	85 ± 4*	P<0.05	n.s	n.s
	EAA	54 ± 2	62 ± 2	79 ± 3*	89 ± 3*	95 ± 3*	110 ± 5*	110 ± 5*	115 ± 5*	115 ± 5*	81 ± 2*			
Isoleucine	EAA-Leu	52 ± 2	75 ± 7*	120 ± 10*	130 ± 5*	130 ± 5*	160 ± 10*	165 ± 10*	185 ± 10*	220 ± 10*	180 ± 5*	P<0.05	P<0.05	P<0.05
	EAA	55 ± 2	73 ± 3*	110 ± 5*	120 ± 5*	125 ± 5*	135 ± 5*#	120 ± 5*#	130 ± 5*#	130 ± 5*#	77 ± 2*#			
Leucine	EAA-Leu	100 ± 5	105 ± 5	110 ± 5	95 ± 5	87 ± 3	77 ± 3*	66 ± 4*	67 ± 3*	67 ± 5*	76 ± 6*	P<0.05	P<0.05	P<0.05
	EAA	110 ± 5	140 ± 5*#	200 ± 10*#	230 ± 10*#	235 ± 10*#	260 ± 15*#	240 ± 15*#	260 ± 5*#	265 ± 5*#	170 ± 5*#			
Lysine	EAA-Leu	175 ± 5	195 ± 10	255 ±10*	285 ± 15*	285 ± 15*	305 ± 20*	320 ± 25*	290 ± 10*	3320 ± 15*	215 ± 10*	P<0.05	n.s	n.s
	EAA	170 ± 10	195 ± 10	245 ± 15*	275 ± 15*	275 ± 20*	305 ± 25*	290 ± 20*	300 ± 20*	300 ± 20*	200 ± 15*			
∑ EAA§	EAA-Leu	710 ± 10	820 ± 40	1070 ± 50*	1210 ± 50*	1220 ± 40*	1360 ± 60*	1430 ± 90*	1410 ± 50*	1630 ± 70*	1270± 50*	P<0.05	P<0.05	P<0.05
	EAA	700 ± 40	800 ± 30	1010 ± 40*	1140 ± 40*	1190 ± 40*	1290 ± 70*	1260 ± 50*#	1290 ± 50*#	1340 ± 50*#	940 ± 40*#			

§∑ EAA includes the sum of histidine, isoleucine, lysine, methionine, phenylalanine, threonine and valine

Table 2. Concentrations of muscle amino acids before and 1 h and 3 h after resistance exercise in subjects receiving the EAA or the EAA-Leu supplement. The values (μmol · kg dry muscle^{-1}) shown are means ± SE for 8 subjects in each case. *$P < 0.05$ in comparison to the value at rest, $^{\#}P < 0.05$ in comparison to EAA-Leu, n.s. not significant.

Amino Acid	Condition	Rest	Recovery, minutes		Main effects		Int. effects
			60	180	Time	Suppl	Time x Suppl
Histidine	EAA-Leu	1030 ± 60	1110 ± 60	1230 ± 100	n.s	n.s	n.s
	EAA	1030 ± 50	1140 ± 80	1050 ± 90			
Glycine	EAA-Leu	2910 ± 330	3380 ± 370*	3730 ± 290*	P<0.05	P<0.05	P<0.05
	EAA	2710 ± 190	2740 ± 230$^{\#}$	2540 ± 300$^{\#}$			
Threonine	EAA-Leu	1780 ± 230	2520 ± 100*	2950 ± 300*	P<0.05	n.s	n.s
	EAA	1480 ± 120	2210 ± 180*	2310 ± 180*			
Tyrosine	EAA-Leu	210 ± 10	265 ± 15*	270 ± 20*	P<0.05	P<0.05	P<0.05
	EAA	215 ± 10	240 ± 20	180 ± 15*$^{\#}$			
Methionine	EAA-Leu	110 ± 10	160 ± 10*	145 ± 20*	P<0.05	n.s	P<0.05
	EAA	120 ± 10	150 ± 10	90 ± 15$^{\#}$			
Valine	EAA-Leu	685 ± 15	1220 ± 60*	1420 ± 60*	P<0.05	P<0.05	P<0.05
	EAA	780 ± 50	1050 ± 70*$^{\#}$	990 ± 70*$^{\#}$			
Phenylalanine	EAA-Leu	180 ± 5	365 ± 25*	310 ± 10*	P<0.05	n.s	n.s
	EAA	195 ± 10	370 ± 20*	275 ± 5*			
Isoleucine	EAA-Leu	215 ± 10	575 ± 30*	670 ± 40*	P<0.05	P<0.05	P<0.05
	EAA	245 ± 10	380 ± 25*$^{\#}$	275 ± 20$^{\#}$			
Leucine	EAA-Leu	415 ± 10	235 ± 15*	255 ± 20*	P<0.05	P<0.05	P<0.05
	EAA	470 ± 20	770 ± 45*$^{\#}$	620 ± 35*$^{\#}$			
Lysine	EAA-Leu	2150 ± 400	2410 ± 190	2870 ± 440*	P<0.05	n.s	n.s
	EAA	1920 ± 160	2250 ± 260	2000 ± 290*			
∑ EAA§	EAA-Leu	6150 ± 670	8360 ± 230*	9580 ± 870*	P<0.05	n.s	P<0.05
	EAA	5780 ± 240	7550 ± 390*	7000 ± 410*$^{\#}$			

§∑ EAA includes the sum of histidine, isoleucine, lysine, methionine, phenylalanine, threonine and valine

Figure 1

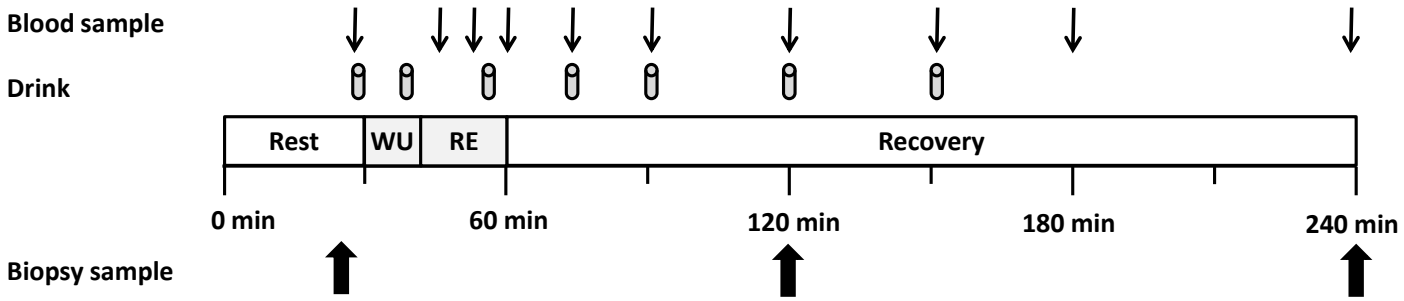

Figure 2

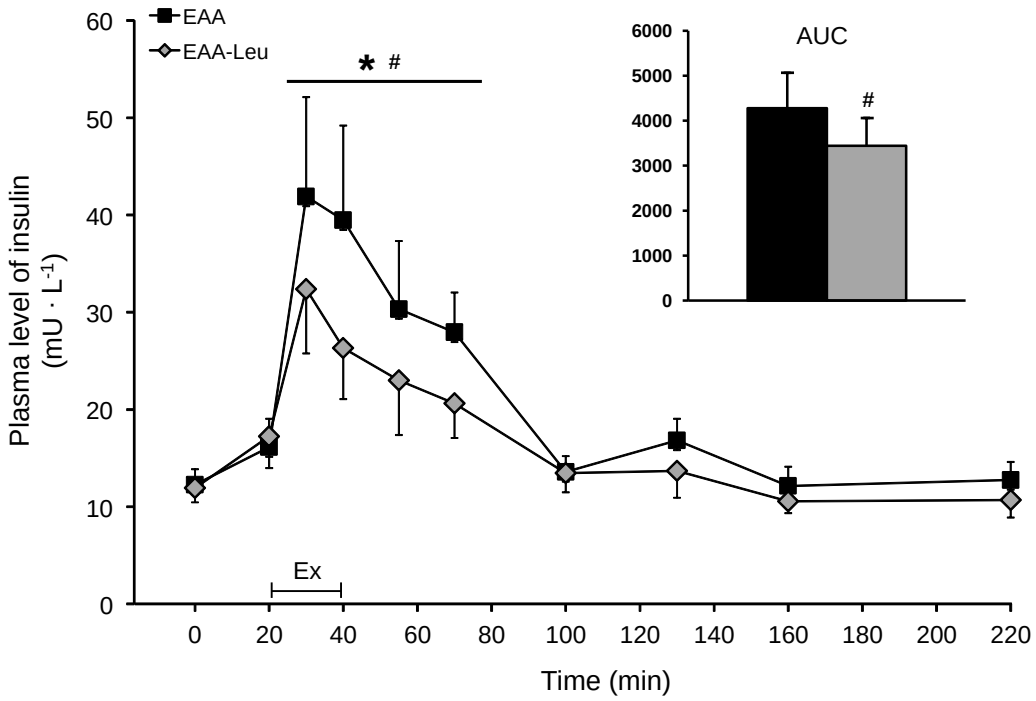

Figure 3

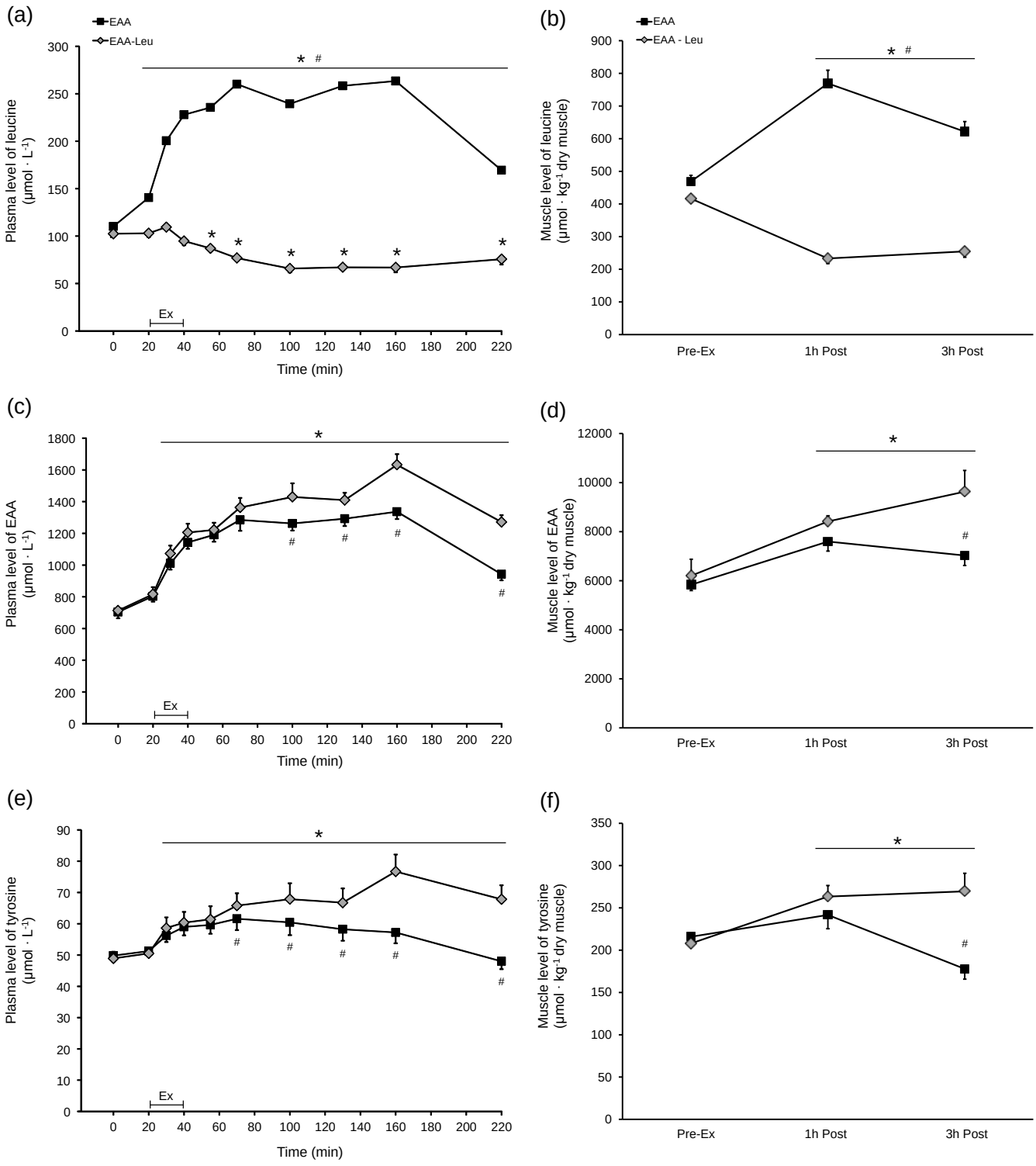

Figure 4

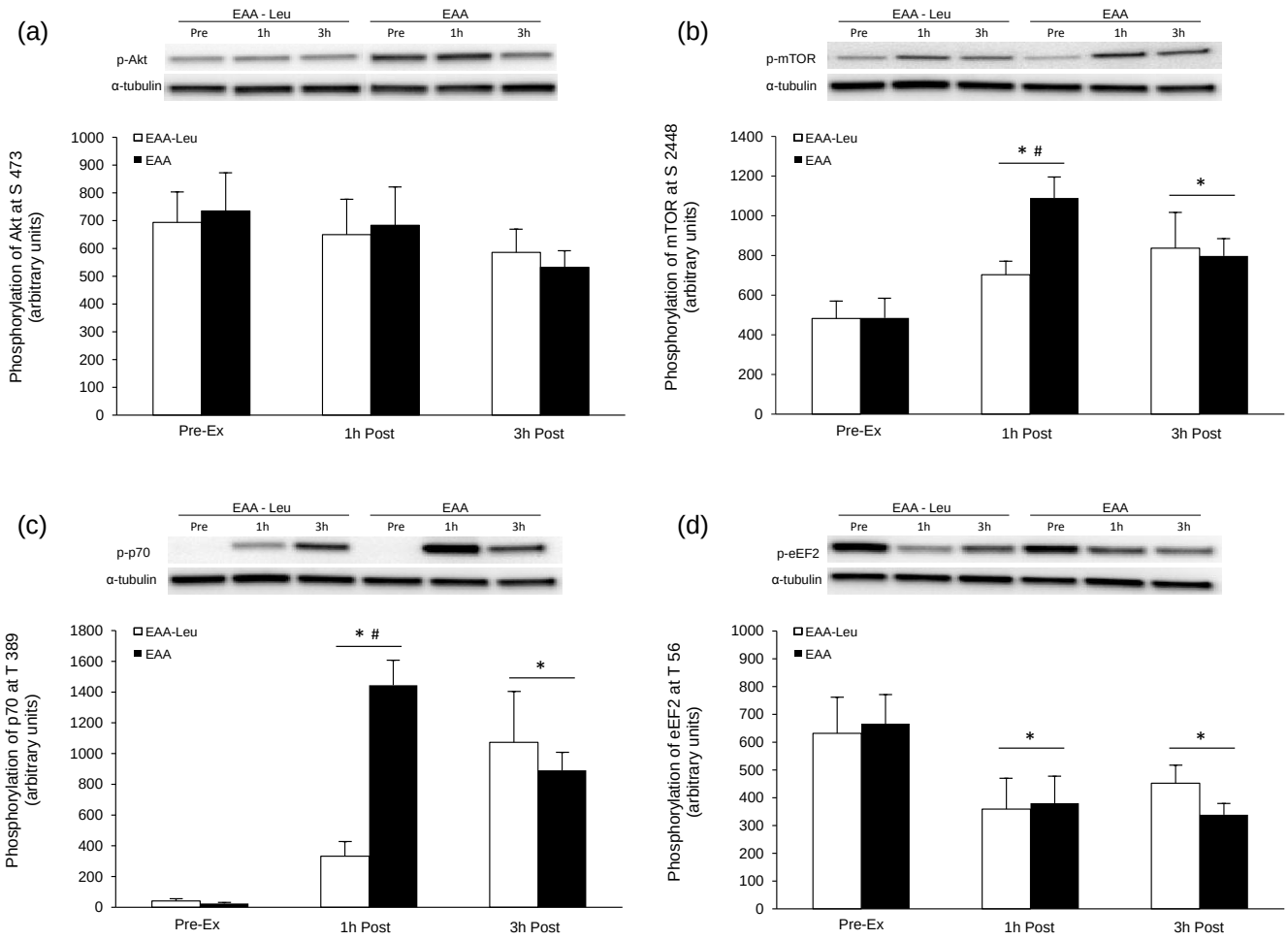

Figure 5

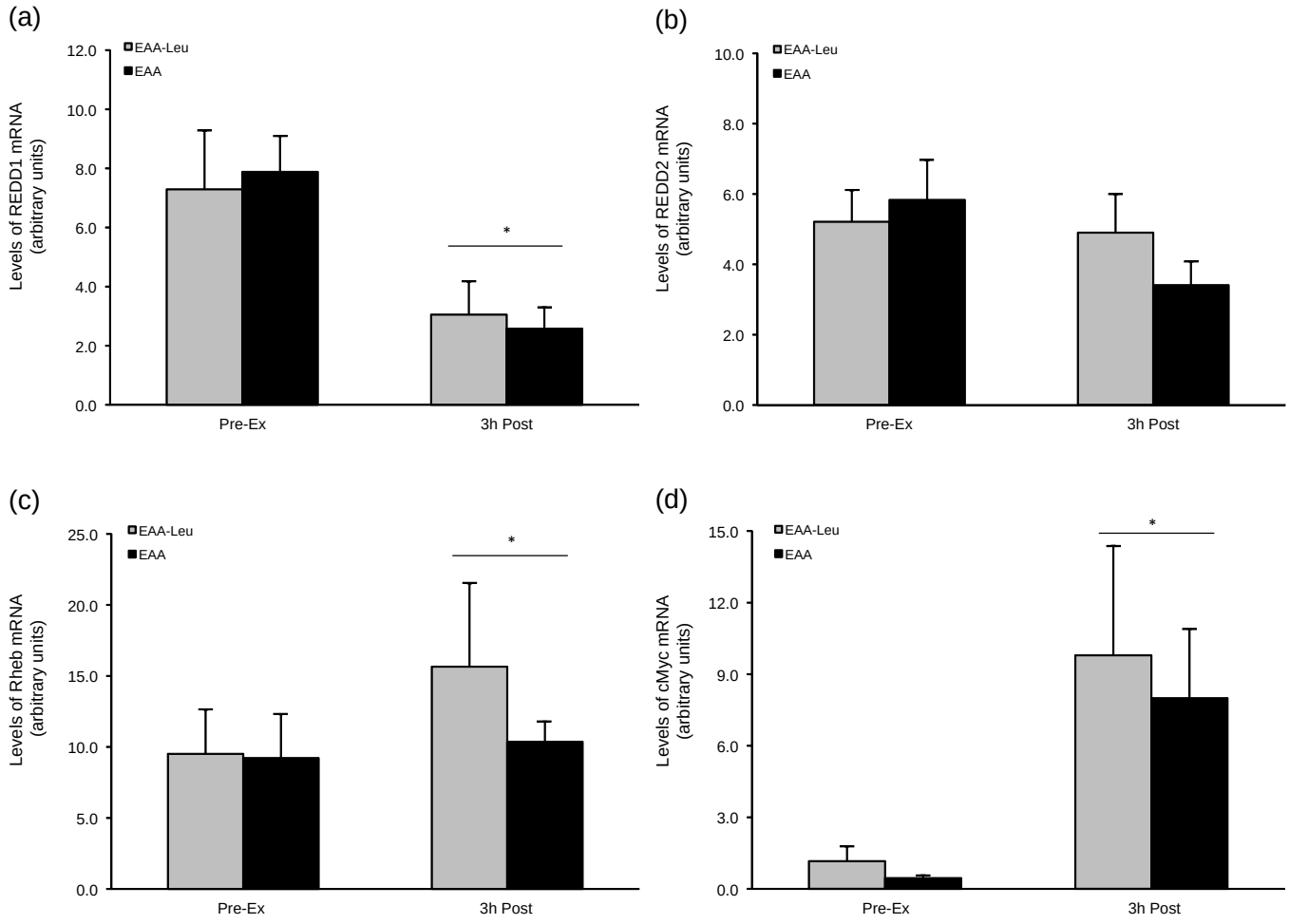

Figure 6