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Research Article

BRANCHED CHAIN AMINO ACIDS (BCAA) SUPPLEMENTATION INCREASES THE SKELETAL MUSCLE PROTEIN SYNTHESIS UNDER CALORIE RESTRICTED CONDITION AFTER ENDURANCE EXERCISE

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A B S T R A C T

Branched-chain amino acids (BCAA) are essential amino acids that serve as essential substrates and important regulators in the synthesis of body proteins. Our primary objective was to investigate the effect of calorie restriction with endurance exercise and supplementation of branched-chain amino acids (BCAA; leucine, isoleucine &Valine) on fractional protein synthesis rate. Further mTOR signaling pathway (4E-BP1 Thr37/46 and S6K1 Thr389) was studied to understand the molecular mechanism, involved in the regulation of translation initiation in skeletal muscle. Thirty six rats were randomly divided into three groups- -*Ad libitum* with exercise (Ad+ Ex) as controls, Calorie restricted with exercise (CR+ Ex) and Calorie restricted with exercise and supplemented with branched chain amino acid (CR +Ex +BCAA). Fractional rates of protein synthesis and indexes of translation initiation were measured after endurance exercise (2 h post exercise). Muscle protein fractional synthesis rate was increased ($P < 0.05$, $P < 0.01$) in CR + Ex +BCAA supplemented group in comparison to control and CR group. Administration of BCAA during CR with exercise increased ($P < 0.05$) phosphorylation of PKB, mTOR, eukaryotic initiation factor (eIF) 4E-binding protein-1, 70 kDa ribosomal protein S6 kinase (S6K1) and ribosomal protein S6. BCAA also increased ($P < 0.05$) the phosphorylation of ribosomal protein S6 kinase in skeletal muscles. The results suggest that the BCAA supplementation under calorie restricted condition with endurance exercise acts as nutrient signal to stimulate protein synthesis in skeletal muscles of rats by activation of mTOR signaling pathway and its components including 4E-BP1 and S6K1.

INTRODUCTION

Physically demanding occupations often require periods of sustained work without the provision of adequate calories and dietary proteins, which create the energy deficit or condition of calorie restriction (CR). The resultant negative energy and nitrogen balance often leads to a reduced physical (Johnson *et al*., 1994; Nindl *et al*., 2002) and cognitive (Gomez-Merino *et al*., 2004) performance of an individual. Reduced Physical performances are often accompanied by a significant loss of muscle mass (Friedl *et al*., 2000; Nindl *et al*., 2003). For instance, an earlier finding revealed that during a chronic energy restriction for 62 days there was a 20 % decrease in body mass with 6 % from fat-free mass that leads to decrease in maximal lifting strength (Nindl *et al*., 2007). Further, significant losses in body mass, fat free mass and lower-body physical performance have occurred as early as within 3 days (Nindl *et al*., 2003).

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Under such conditions, loss of body mass could prove to be detrimental to physical performance, thereby aggrevating the risk of injuries for the individual or group. Information on supplementation of Branched Chain Amino acids (BCAA's) under CR condition on protein metabolism and physical performance could signify the importance of adequate caloric intake. Further, the elucidation so involved molecular mechanism could be used to design interventions to minimize protein losses, thus potentially reducing susceptibility to injury. To the best of our knowledge there is no reported study on the effects of prolonged negative energy balance with BCAA supplementation on protein kinetics, physical performance and protein synthesis signaling cascade in rats.

However, increased amino acid availability and exercise both directly increase protein synthesis in skeletal muscle. BCAA-isoleucine, valine, and in particular leucine, have anabolic effects on protein metabolism by increasing the rate of protein synthesis and decreasing the rate of protein degradation in resting human skeletal muscle (Louard *et al*., 1990; Nair *et al*., 1992). During recovery from exercise, BCAA have an anabolic effect on human skeletal muscle (Blomstrand and

Saltin, 2001). Furthermore, administration of BCAA increases the phosphorylation of proteins involved in the regulation of protein synthesis, including $p70^{S6k}$, in human skeletal muscle (Liu *et al*., 2001). Branched-chain amino acid (BCAA) supplementation enhances adaptability to exercise training of mice with a muscle-specific defect in the control of BCAA catabolism (Xu *et al*., 2018). Branched-chain amino acids supplementation attenuates the accumulation of blood lactate dehydrogenase during distance running (Koba *et al*., 2007).

Amino acids are the main anabolic factors for muscle protein synthesis by activating the initiation translation of proteins through a specific intracellular pathway: the rapamycin sensitive mTOR-signaling pathway (Kimball *et al*., 1994; Proud, 2002). This pathway involves two key regulatory proteins, 70 kDa ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein-1(4E-BP1). The phosphorylation of 4E-BP1 probably induced by mTOR activation, results in dissociation of the 4E-BP1·eIF4E complex. Modification of the complex allows the formation of an active component, which induces one of the first steps of the translation initiation. In addition, the activation of pathway is associated with an increase in the activity of the S6K1 and subsequently the phosphorylation of ribosomal protein S6. Further rpS6 phosphorylation plays an important role in regulating the synthesis of proteins involved in the production of the translational apparatus (ribosomal proteins, translation initiation, and elongation factors).

Therefore, the present study was designed to investigate the effects of administration of BCAA under calorie restricted condition with endurance exercise on skeletal muscle protein synthesis and mammalian target of rapamycin (mTOR) dependent upstream and downstream components (PKB,4E-BP1 and S6K1), which are key proteins involved in the regulation of translation initiation.

MATERIALS AND METHODS

Animals and diet

All procedures and protocols used in the present study were approved by the Animal Care and Use Committee of the Institute and followed the guidelines documented in the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Male albino rats (Sprague Dawley), weighing 180-200 g, were housed in cages (46 × 24 × 20 cm) with two animals per cage in a temperature (22±1ºC), humidity and light-controlled room (lights on at 6:30 hrs, lights off at 18:30 hrs). Rats were provided with the standard rodent chow diet (Lipton India, Kolkata) and water *ad libitum*. As per the study design, male albino Sprague Dawly rats (n=36) were randomly divided into three groups (12 animals/group)- 1) *Ad libitum* food intake with exercise (Ad+ Ex) as controls, 2) Calorie restricted and exercised (CR+ Ex) and 3) Calorie restricted exercised and supplemented with branched chain amino acid (CR +Ex +BCAA).

Branched chain amino acid Supplementation protocol

A solution of mixture of BCAA (45% leucine, 30% valine, and 25% isoleucine) was prepared in tap water. A dosage of 100 mg/Kg body weight was supplemented orally (intragastric route using rodent feeding needle) to calorie restricted rats daily for 30 days. The corresponding Control group received a matched volume of tap water only. The supplementation dose of BCAA and composition of BCAA was selected on the basis of a reported study (Blomstand & Saltin 2001).

Exercise protocol

Initially, rats were acclimatized to the two lane rodent treadmill (Columbus Instruments, USA) by walking at a speed of 10m/min for 3 days. After acclimatization rats were run daily at 15 m/min for 45 mins for another six days as a part of familiarization program with the treadmill. The training program consisted of running on the treadmill at a speed of either 20 m/ min upto 25m/min and at 0º inclination for 30 days. Running at this speed is considered to be a light to moderate intensity exercise which is the evaluator of aerobic power (Lee *et al*., 2001). Rats were considered as exhausted when after receiving a mild electric shock (1.4mA current; 3Hz frequency) from an electric grid placed at the rear of the running lane in the treadmill, refused to continue running on the lane.

Sample collection

Blood samples were taken at rest (before supplementation) and immediately after resistance exercise. Blood was collected in the heparinised tubes and plasma was separated and stored in aliquots at -80°C until analysis. Gastrocnemius (mixed fibers) and quadriceps (red fibers) were removed and immediately stored at -80°C for posterior analyses.

Protein turnover rate

Fractional synthetic rate of proteins and degradation rate [Protein turnover] were measured according to the reported method (Vary *et al*., 1998). Muscles were incubated at 37 °C in a standard Krebs-Henseleit medium : 120 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM $CaCl_2$, (pH 7.4) containing 5 mM glucose, 5mM HEPES, 0.1% BSA, 0.17mM Leucine. After 30 min of pre-incubation, muscles were transferred to a fresh medium (2 ml) and incubated for a further 2 h, with a change of medium every 60 min. During the final 60min of the incubation period, 2ml of KH buffer was supplemented with 0.5mM Leucine (BARC, India). At the end of the incubation, muscles were removed from the incubation buffer, trimmed of connective tissue, immersed into 2ml of ice cold 10% (wt/vol) TCA and weighed. The rate of protein synthesis was estimated by the incorporation of radioactive phenylalanine into muscle protein. Muscles were homogenized in 2ml of 10% TCA using polytron homogenizer. The homogenate was centrifuged at 10,000g for 10 min at 4^oC. The supernatant was decanted and the pellet was washed 3 times with additional 10% TCA to remove any acid soluble radioactivity. The resulting pellet was dissolved in 1N NaoH and incubated at 37^o C for 30 min. Aliquots were assayed for protein using the Folin's Lowry method, using BSA as a standard (Lowry *et al*., 1951). Another aliquot was assayed for radioactivity by liquid scintillation counter using corrections for quenching [Disintegrations/min]. Rates of protein synthesis expressed as µmole of leucine incorporated per hour per milligram of protein, were calculated by dividing the amount of radioactivity incorporated into muscle protein over a 1-h period by the specific radioactivity of the leucine in the incubation medium.

Blood glucose, plasma insulin, IGF-1 and amino acids

Blood glucose was estimated by glucose oxidase method (Randox Ltd.) and determined immediately after sample collection. Plasma concentrations of insulin and IGF-1 were determined using EIA/ELISA kit (Krishgen Biosystems, India). Individual plasma amino acid concentrations were measured with an HPLC method (PICO-TAG reverse-phase column; Waters, Milford, MA) as previously described (Heinrikson *et al*., 1984).

Tissue processing

A portion of frozen epitrochlearis was weighed, transferred to prechilled polypropylene tubes, and homogenized in ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM sodium pyrophosphate, 2 mM Na_3VO_4, 10 mM NaF, 2 mM EDTA, 2 nM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 1% Nonidet P-40, and 10% glycerol). The homogenate was centrifuged at 10,000 *g* at 4°C for 10 min, and the supernatant was stored at -80°C until analysis. Protein was determined in aliquots of the supernatant using a Bradford protein assay (Bio-Rad Laboratories).

Protein western blot analysis

For phosphorylated forms of PKB and mTOR, aliquots of the supernatant (50 µg protein) were electrophoresed on an 8.5 and 7.5% polyacrylamide gel, respectively. For 4E-BP1, S6K1 phosphorylation and rpS6 aliquots of supernatant containing 50 µg protein were respectively electrophoresed on a 15 or 8.5% polyacrylamide gel. After gel electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered saline (TBS; 10 mM Tris, pH 7.6, and 100 mM NaCl) containing 5% BSA for overnight at 4°C. After blocking, membranes were incubated with rabbit polyclonal anti phospho- PKB antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal anti phospho-mTOR (Ser 2448), rabbit polyclonal anti 4E-BP1 antibody, rabbit polyclonal p70 S6 kinase and rabbit polyclonal anti rpS6 (Cell Signaling Technology, Inc., Beverly, MA) overnight at room temperature followed by incubation with anti rabbit coupled to horseradish peroxidase for 1 hr at room temperature. The blots were developed using an enhanced chemiluminescense kit (ECL, Amresco) and quantified by densitometric scanning with a Gel Doc 1000, in combination with Molecular Analyst software (version 1.5; Bio-Rad Laboratories). All data were expressed as the change in phosphorylation in arbitrary units.

Statistical analysis

Results were expressed as mean ±SEM. To determine the effect of treatment on fractional protein synthesis rates and the abundance of translation initiation factors, analysis of variance (ANOVA) was performed with commercially available software (SPSS version 15.0). Differences of $P<0.05$ were considered significant.

RESULTS

Body weight

Body weight of the control group during the experiment was unaltered, while the CR and CR+BCAA groups showed mean weight losses of 13.0 ±5.6% and 16.2 ±8.9%, respectively. There were no significant differences in weight loss between the CR and CR+BCAA groups. Although CR, as expected led to a significant decrease in body weight ($p<0.05$ *vs.* AL) the absolute muscle weights remained unaltered with the diet and exercise (Table 1).

Table 1 Body mass and muscle mass in C, CR and CR+BCAA rats

Variables	C	CR	CR+ BCAA
Body mass (g)			
Initial	212±12	210±8	205±10
Final	220 ± 4	165±10**	180±12
Muscle (g)	1.64 ± 1.4	1.32 ± 1.9	1.51 ± 2.4

Values are mean± SEM. C, control rats; CR, calorie restricted with exercise; CR +BCAA, calorie restricted and branched chain amino acid, n=12 in each group. *P<0.05, **P<0.05 vs. control.

Run to exhaustion time

Run to exhaustion time from day 1 to day 30 was measured in case of all the groups. A significant ($p<0.001$) increase in runtime was found from day 3 to day 29 of the regime in CR group with BCAA supplementation in comparison to control group. There was a 53.6% improvement in endurance performance of CR +BCAA group in comparison to control group (Fig 1).

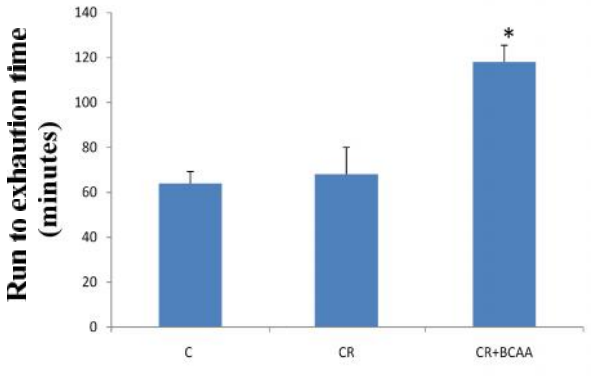


Figure 1 Exercise tolerance test quantified as run time to exhaustion on treadmill (minutes). Values are expressed as mean± SEM (n=12). *P<0.01 *vs.* Control group.

Muscle protein turnover rate

Skeletal muscle protein fractional synthesis rate was increased ($p<0.05$, $p<0.01$) significantly in CR + Exer +BCAA supplemented group in comparison to control and CR group. Muscle protein fractional synthetic rate (FSR) decreased immediately following endurance exercise in CR rats (Fig. 2).

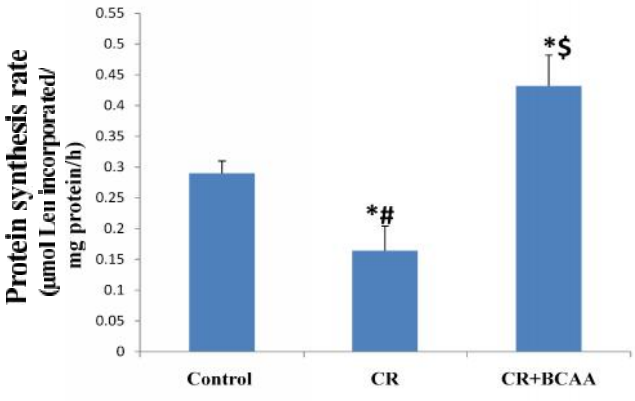


Figure 2 Muscles from all experimental groups were incubated with L-[^{14}C] Leucine for 2h and protein synthesis was then assessed based on the incorporation of radiolabelled amino acid. Results are expressed as means ± SEM (n=12 rats in each group). *P<0.05 vs. control, P<0.05 vs.CR+BCAA, $P<0.01 vs. CR group.

Plasma glucose, Insulin and amino acids

Plasma glucose levels were decreased ($p<0.05$) significantly in CR rats after endurance exercise in comparison to control. Plasma glucose levels were not affected by the BCAA administration after endurance exercise (Fig.3). No remarkable change was observed in case of Plasma insulin concentration in CR, CR+ BCAA rats in comparison control rats (Fig. 4).

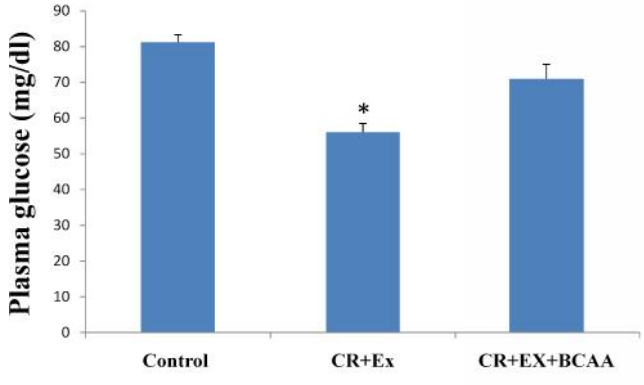


Figure 3 Plasma glucose levels after treadmill exercise in control, CR &CR+BCAA. Results are expressed as means ± SEM (n=12 rats in each group). *P<0.05 vs. control, P<0.05 vs.CR+BCAA, $P<0.01 vs. CR group.

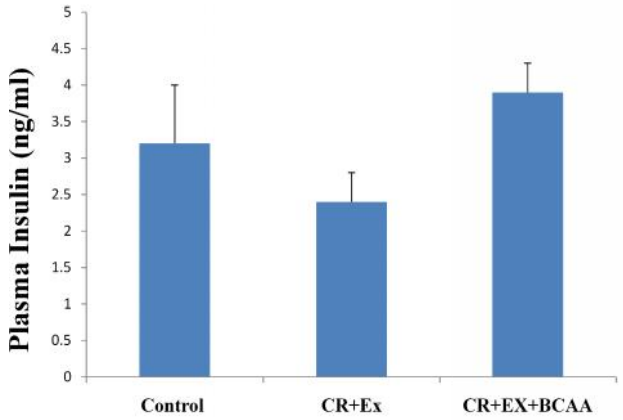


Figure 4 Plasma insulin levels after treadmill exercise in control, CR &CR+BCAA. Results are expressed as means ± SEM (n=12 rats in each group). *P<0.05 vs. control, P<0.05 vs.CR+BCAA, $P<0.01 vs. CR group.

The plasma amino acid data for Ad libitum, CR and CR+BCAA after exercise are presented in Table 2. Ingestion of BCAA led to an increase in the plasma concentration of isoleucine, leucine, and valine amino acids, after the endurance exercise. The BCAA levels were significantly elevated in CR+BCAA after exercise. Although no significant difference was observed in NEAA and EAA levels in CR+BCAA group in comparison to control, after exercise. Whereas, slight decrease was observed in BCAA levels in CR group after exercise but it was not significant.

Table 2 The plasma amino acid data of C, CR, CR+BCAA rats

	C	CR	CR+BCAA
NEAA	1436±51	1288±55	1312±56
EAA	716±32	626±28	704±30
BCAA	364±12	262±8	456±58*#

Values are represented as Mean ±SEM (n=12 in each group). Units are µM. NEAA, nonessential amino acids: EAA, essential amino acids: BCAA, branched chain amino acids. *P<0.05, **P<0.05 vs. control, # P<0.01 vs. CR.

Western blot analysis

Upstream regulators of mTOR signaling

The Phosphorylation of PKB at ser 473 ($p<0.01$, $p<0.05$) was found to be increased after endurance exercise in CR+BCAA group in comparison with control and CR rats (Fig. 5 A).

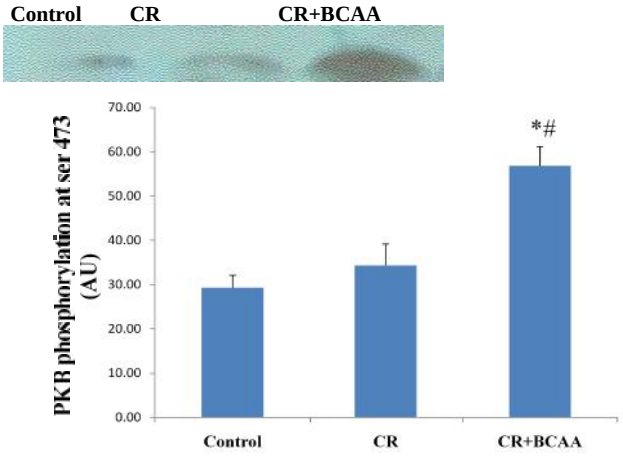
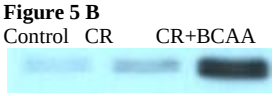


Figure 5 A Phosphorylation of PKB: upstream regulators of mTOR after treadmill exercise. Data are expressed as mean ± SEM (n=12). ∗ (P < 0.01*) vs* . control, # P < 0.01) vs.CR.

mTOR and downstream regulators of mTOR signalling (4E-BP1 and S6K1)

An appreciable increase in phosphorylation of mTOR at Ser2448 was significantly noticed ($p<0.01$) in CR+BCAA after endurance-exercise in comparison to control and CR groups (Fig. 5B). Furthermore, the phosphorylation of 4E-BP1 at Thr37/46 and phosphorylation of S6K1 at Thr389 was significantly enhanced in CR+BCAA compared to control and CR groups after endurance-exercise ($P<0.05$, $P<0.01$), (Fig. 5C&D).

Figure 5 B



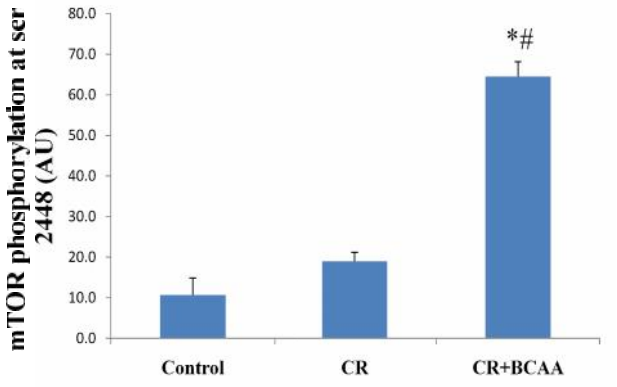


Figure 5 C

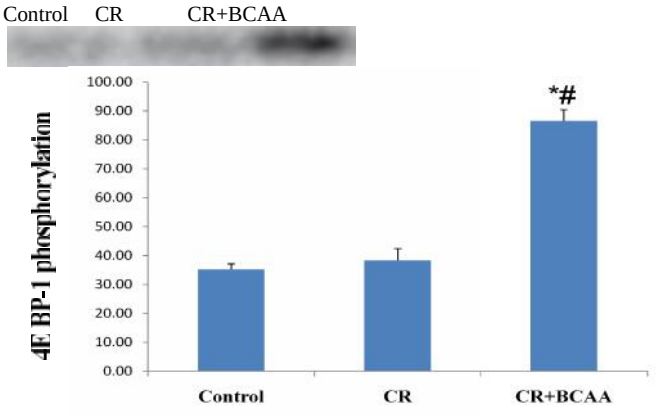


Figure 5 D

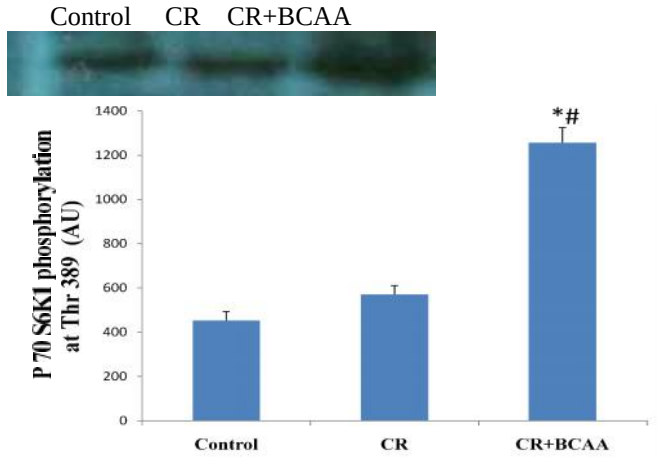


Figure 5 B, C & D. Phosphorylation of mTOR and downstream indicators of mTOR signalling (4E-BP1 and S6K1) after treadmill exercise. Data are expressed as means ± SEM (n=12). ∗ (P < 0.01*) vs* . control, # P < 0.01) vs.CR.

As observed for S6K1, phosphorylation of rpS6 was also higher (P<0.01) by BCAA treatment in muscles compared with control rats (Fig. 5E).

Figure 5 E

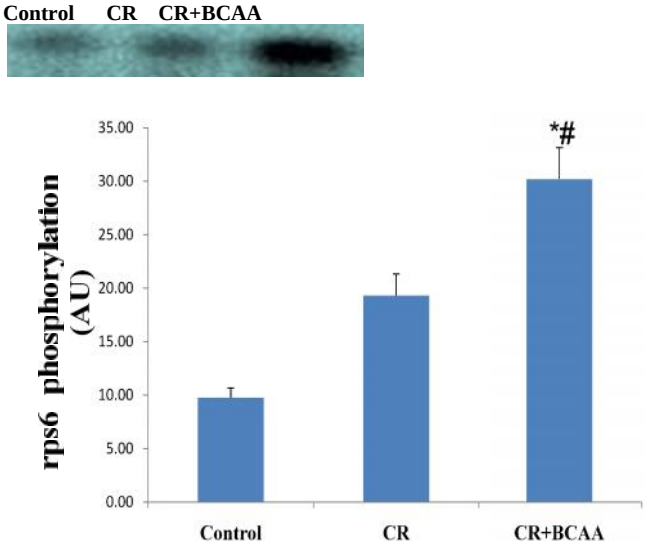


Figure 5 E Phosphorylation of the ribosomal protein S6 in skeletal muscle after treadmill exercise. Data are expressed as means ± SEM (n=12). ∗ (P < 0.01*) vs* . control, # P < 0.01) vs.CR.

DISCUSSION

The major findings of the present study was that branched-chain amino acid supplementation under calorie restricted condition increases the endurance performance of rats along with increase in the skeletal muscle fractional protein synthesis rate (post exercise). The latter found to be associated with increased levels of phosphorylated forms of protein kinase B (PKB), mTOR, 4E-BP1, S6K1 and rpS6.

Branched-chain amino acid (BCAA) supplements could theoretically benefit physical performance in several ways. They could supply TCA cycle intermediates, decrease the use of other energy sources (sparing glycogen), inhibit muscle protein breakdown, or limit the transport of tryptophan into the brain. However, the exact mechanism for the action of branched chain amino acids on the improvement of performance is unknown. Furthermore, our findings are in agreement with reported studies (Mourier *et al*., 1997) on wrestlers with moderate energy restriction and BCAA supplementation for 19 days maintains a high level of performance and BCAA supplementation in trained individuals performing resistance training while on a hypocaloric diet can maintain lean mass and preserve skeletal muscle performance while losing fat mass (Wesley *et al*., 2016).

In the present study, oral administration of BCAA with moderate CR increases skeletal muscle protein synthesis rate and it was associated with an enhanced phosphorylation of mTOR, 4E-BP1, S6K1. Amino acids are the most potent anabolic factors that are known to increase synergistically muscle protein synthesis in human adults (Volpi *et al*., 2000; Wolfe, 2002). The protein synthetic response, which occurs in disparate tissues but is most profound in skeletal muscle (Davis *et al*., 1996), is independently induced by the postprandial rise in insulin and amino acids (O'Connor *et al*., 2003). In a recent study (Bohe *et al*., 2001) in which a mixture of amino acids was infused to human subjects at rest, skeletal muscle protein synthesis was not altered during the first 30 min of amino acid infusion, but then rose rapidly between 30 and 60 min of infusion, and remained elevated for another hour of amino acid infusion. The amino acid-induced stimulation of skeletal muscle protein synthesis is modulated by the enhanced activation state of translation initiation factors that lead to increased eIF4G-eIF4E complex assembly (O'Connor *et al*., 2003).One of the previously reported study (Escobar *et al*., 2005) demonstrated that infusion of leucine alone, to increase its concentration in plasma to mimic postprandial levels, resulted in the stimulation of protein synthesis in skeletal muscle of neonatal pigs. Furthermore, the leucine-induced stimulation of protein synthesis was associated with enhanced phosphorylation of 4E-BP1, S6K1, and rpS6 (Escobar *et al*., 2005). It is generally considered that S6K1 and 4EBP-1 are phosphorylated simultaneously when the mammalian target of rapamycin (mTOR) kinase cascade, part of the insulin-signaling network, including PKB is activated (Taha and Klip 1999). However, in several circumstances, such as in human muscle (Hillier *et al*., 2000), it has been possible to dissociate the activation of S6K1 and 4E-BP1. Amino acids themselves activate the phosphorylation of both 4EBP-1 and S6K1 (O'Connor *et al*., 2003; Liu *et al*., 2002), and this effect requires mTOR activity (Proud, 2002). However, whether or not amino acids directly promote

activation of mTOR is unknown (Vary *et al*., 1999), but this activation seems not to involve the phosphorylation of PKB (Hillier *et al*., 2000; Wang *et al*., 1998).

Calorie restriction with exercise in combination with BCAA ingestion markedly enhanced site-specific phosphorylation of 4E BP-1 at Thr 37/46 and p70S6k at Thr389 in rat skeletal muscle, after exercise. Infusion of leucine for 2 h is associated with an increased p70S6k phosphorylation in skeletal muscle from human subjects at rest (Greiwe, *et al*., 2001). Phosphorylation of this residue is critical for the activity of p70S6k (Dufner *et al*., 1999). Moreover, phosphorylation of the ribosomal protein S6 was increased in skeletal muscle indicating that p70S6k was activated.

Another interesting finding was the change in plasma branched chain amino acids concentrations after exercise in BCAA supplemented group. These results directly suggest that branched chain amino acid concentrations are regulating mTOR signalling after endurance exercise, since mTOR phosphorylation was increased with increased BCAA concentrations. Other potential mechanisms, such as enhanced signaling through PKB and/or other regulators of mTOR signalling, are most likely to be responsible for the increase in muscle protein synthesis post-exercise (Anthony *et al*., 2002).

In conclusion, the data of the present study suggests that the moderate calorie restriction and BCAA supplementation helps in the maintenance of physical performance and also increases skeletal muscle protein synthesis after exercise, it is associated with an increase in PKB, mTOR and S6K1 phosphorylation a upstream and downstream components of the mTOR signalling pathway and a key regulator of translation initiation.

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