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Acute exposure to essential amino acids activates contraction mediated mTOR/p70 signaling in soleus muscle of elderly rats

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TITOLO
L'esposizione acuta agli amino acidi essenziali potenzia l'attivazione mediata da contrazione della via di segnale mTOR/p70 nel soleo intatto di ratto anziano

KEY WORDS
Amino acids, protein synthesis, ageing, nutritional supplements

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Summary
The aging process leads to sarcopenia that is a progressive skeletal muscle weakening due to reduction of mass and concomitant change in phenotype. Several studies indicate that amino acids supplementation and, in particular, branched chain amino acids (BCAA) and contraction may activate protein synthesis through the activation of mTOR/ $p70^{S6K}$ signaling pathway. In the present study we used an in vitro assay to analyze the effects of tetanic contractions and acute exposure to branched chain enriched amino acid mixture on mTOR/ $p70^{S6K}$ signaling activation in soleus muscle of elderly rats. *Methods*: Soleus muscles from Wistar male elderly rats (18 mo of age) were grouped as follows (n = 5 each): nc, not contracted; C, contracted (10 tetani of 0.5 sec/100Hz at 0.02Hz) and contracted and acutely incubated with BCAA enriched mixture at 1% (C+BCAA). Following treatment the activation level of mTOR and $p70^{S6K}$ was measured by Western blot. *Results:* Contracted muscles (C) showed no increase of mTOR activation relative to uncontracted muscles (nc) and a significant increase of the level of $p70^{S6K}$ activation (+80%). On the contrary following acute BCAA enriched mixture exposure contracted muscles displayed higher mTOR activation (Figure 1) in comparison with C and nc muscles (+40%) and a further increase in p70S6K in comparison with C (+180% vs nc and +55% vs C). *Conclusions:* Tetanic contractions and acute exposure to BCAA enriched mixture are able to synergically activate the mTOR/$p70^{S6K}$ signalling pathway in the soleus muscle of elderly rats.

Riassunto
Il processo di invecchiamento è associato ad una riduzione della massa muscolare con perdita di forza e cambiamenti del fenotipo. Diversi studi hanno dimostrato che miscele di aminoacidi essenziali e in particolare contenenti amino acidi ramificati (BCAA) nonché la contrazione muscolare possano stimolare direttamente le sintesi proteiche muscolari attraverso la regolazione della via di segnale mTOR/ $p70^{S6K}$. Nel presente studio abbiamo utilizzato un saggio in vitro per indagare quale sia l'effetto della contrazione e della esposizione acuta del muscolo soleo di ratto anziano ad una miscela contenente amino acidi essenziali sulla via di segnale mTOR/$p70^{S6K}$. *Metodi:*

I muscoli solei di ratti Wistar maschi anziani (18 mesi di età) sono stati suddivisi in tre gruppi sperimentali (n = 5 ciascuno): nc, non sottoposti a contrazione; C, sottoposti a contrazione (10 tetani di 0.5sec/100Hz, alla frequenza di 0.02Hz) e contratti ed esposti acutamente ad una miscela aminoacidica arricchita in BCAA all'1% (C+BCAA). Al termine del saggio il livello di espressione e di fosforilazione di mTOR e $p70^{S6K}$ è stato misurato mediante Western blot. *Risultati:* I muscoli contratti (C) hanno mostrato un livello di attivazione di mTOR paragonabile a quello dei muscoli non contratti (nc) e un aumento significativo del livello di attivazione di $p70^{S6K}$ (+80%). Al contrario l'esposizione acuta alla miscela aminoacidica ha determinato un aumento del livello di attivazione di mTOR rispetto ai muscoli nc e C (+40%) e un aumento significativo del livello di attivazione di $p70^{S6K}$ rispetto a nc (+180%) e C (+55%). *Conclusioni:* Sia la contrazione muscolare che l'esposizione acuta ad una miscela aminoacidica sono in grado di attivare la via di segnale mTOR/$p70^{S6K}$ in maniera sinergica nel muscolo soleo di ratto anziano.

Introduction

The aging process leads to sarcopenia (1) that is a progressive skeletal muscle weakening due to reduction of mass and concomitant change in phenotype including a loss of fast type II fibers (2). Several factors may contribute to sarcopenia of ageing including a reduced protein and caloric intake and an altered turnover of myofibrillar proteins (3). In particular a general (4) or selective (5) reduction in muscle protein synthesis (MPS) has been observed in the aged muscle thus indicating that the efficiency of the protein turnover and its responsiveness to specific stimuli including mechanical stress, electrical stimulation, insulin and amino acids may be gradually and partially lost.

Human studies have shown that in ageing the reduced MPS is to be attributed, at least in part, to an altered efficiency of the mTOR signaling pathway (6), which is now recognized as a fundamental controller of cell synthesis and survival through regulation of the translation process (3). In particular it has been shown that, although the basal levels of activation of mTORC 1 and downstream target kinases as S6K1 and 4EBP1 appears normal, the physiological increase of MPS induced by mechanical, electrical and nutritional stimuli is attenuated with age (7), particularly in fast twitch muscles (8). In agreement with these results, other studies have shown that an altered change in MPS after a single bouts of resistance exercise in elder unlike young subjects (9) being the activation response of the pathway significantly delayed and partially reduced. These data strongly suggest that the observed reduction of muscle growth induced by mechanical loading in the aged muscle may be in part due to a failure in activation of the signals involved in the control of translation.

Essential amino acids (EAA) are fundamental regulators of muscle protein synthesis (10) and supplementation with EAA may represent a potential countermeasure to muscle loss associated with aging (3, 11). Several in vitro and in vivo studies have shown that the EAA, in particular branched chain amino acids (BCAA) mixtures containing leucine and isoleucine, may directly stimulate muscle protein synthesis

and mitochondrial biogenesis (3) through the regulation of mTOR signals followed by translation initiation (12).

Numerous studies in vivo and in vitro have demonstrated that during muscle contraction the mechanical stress imposed by the load to the fibers membrane is able to activate a series of intrinsic responses within the muscle and this mechanotransduction is essential for the regulation of MPS. For instance bouts of resistance exercise can increase MPS for two or more days (13) and post-exercise protein intake may potentiate the rise of MPS up to exceed the amount of protein degraded, with the result of a clearly positive protein balance and a consequent protein accretion (14). Importantly mechanical load and nutrients as BCAA, although first acting at different levels, seem to have a common target on the mTOR biosynthetic pathway controlling protein synthesis. For example O'Neil and coworkers (15) have recently shown that eccentric exercise is able to activate S6K1 through changes in the level of phosphatidic acid (PA) followed to the activation of phospholipase D which plays an important role in the mediation of the MPS. In fact, blocking the synthesis of PA with L-butanol, an inhibitor of phospholipase D, completely abolishes the increased activation of S6K1 by eccentric exercise thus inhibiting MPS (16). Thus mechanical load and amino acids may act on the same metabolic pathway but is currently unknown whether this process may proceed through competitive or not competitive interactions with differentially located and potentially interacting sensors within the path and whether this interaction may also be seen in the aged muscle in which a reduced responsiveness to both stimuli has been described.

Here we used an in vitro assay to investigate whether acute exposure of skeletal muscle to BCAA enriched mixture would positively impact on contraction mediated mTOR/S6K1 activation in soleus muscle of elderly rats. Results from our study demonstrated that both mechanical load and amino acids are able to impact on the pathway in a not competitive fashion.

Materials and methods

Animal treatment

The study was carried out on 10 male Wistar rats of 18 mo of age which were treated according to the EU guidelines and with the approval of the Institutional Ethical Committee. Animals were maintained to 12/12 h light-dark cycle (7 a.m.-7 p.m.) and given unrestricted access to a standard diet (4.3 kcal% fat, 18.8 kcal% protein, 76.9 kcal% carbohydrate, Laboratorio Dottori Piccioni) and tap water. The day of sacrifice obtained with an ether overdose, intact soleus muscles were excised under a stereomicroscope at room temperature. The muscles from 5 rats were used as follows: the right soleus was contracted and incubated with amino acid mixture (C+BCAA) whereas the left muscle served as contracted unincubated (C) control. The muscles from the remaining 5 rats were used as follows: the right muscle was unincubated (C) but contracted whereas the left muscle served as uncontracted unincubated (nc) control. Once dissected each muscle was placed in an organ bath filled with 15 ml Krebs solution (NaCl 120 mm, KCl 2.4 mM, 2.5 mM $CaCl_2$, $MgSO_4$ 1.2 mm, Glucose 1 g/L, KH_2PO_4 1.2 mm, and $NaHCO_3$ 24.8 mm pH 7.4) bubbled with 95% O_2 and 5% CO_2 and maintained at the constant temperature of 22°C, containing or not 1% of the amino acid mixture (BCAA enriched mixtures composition: 31.25% leucine, 16.25% lysine, 15.52% valine, 15.52% isoleucine, 8.75% threonine, 3.75% cysteine, 3.75% histidine, 2.6% phenylalanine, 1.25% methionine, 0.75% tyrosine, 0.5% tryptophan). Once attached from one tendon to a force transducer (FT-03, Astro-med, West Warwick, RI USA) and from the other tendon to the hook of a movable shaft, electrical stimula-

tion was delivered through platinum field electrodes connected to a Grass stimulator (S48 stimulator, Astro-Med, Warwick, RI, USA). 10 tetanic isometric contractions were evoked (500 ms, 100 Hz, supramaximal amplitude) at L_o (the length at which the maximal isometric force is observed) at not fatiguing frequency (0.02 Hz). Immediately after the 10th tetanic contraction or the corresponding time for uncontracted soleus, the muscle was unhooked and its volume was calculated by means of an *ad hoc* built volumometer and for subsequent measurement of absolute tetanic force (mN) and specific tetanic force (mN/microL) of each tetanic contraction. Lastly after brief blotting the muscle sample was rapidly frozen in fluid nitrogen for successive analysis.

Activation of mTOR and p70S6K by Western Blot

The level of activation of mTOR and downstream target p70S6K in uncontracted/unincubated, contracted/unincubated and contracted/incubated soleus muscles was investigated by Western blotting.
The antibodies directed against p-mTOR (Ser2448) and p-p70S6K (Thr389) were obtained from Cell Signaling Technology (Danvers, MA, USA); anti-p70S6K were obtained from Upstate (Charlottesville, VA, USA), anti-mTOR was obtained from Sigma-Aldrich (Milano, Italy). Tissues were homogenized in lysis buffer (50 mM Tris.Cl pH 7.6, 1% Triton X100, 150 mM NaCl, 5 mM EDTA, 100 mM NaF, 2 mM NaPPi, 2 mM Na_3VO_4, 1 mM PMSF and 10 micrograms/mL each of leupetin, pepstatin, aprotinin) using a Dounce Tissue Grinder (Wheaton, Millville, NJ, USA). The homogenate was centrifuged at 16000 rpm, the supernatant was recovered and assayed for protein concentration by the Bradford Assay (Bio-Rad Laboratories, Milano, Italy). One hundred micrograms of protein extracts were run on a 7.5% SDS-PAGE for mTOR, p-mTOR or 15% SDS-PAGE for p70S6K and p-p70S6K and transferred on a PVDF membrane (Millipore, Milano, Italy). The membranes were stained with Ponceau Red (Sigma-Aldrich, Milano, Italy) to verify the protein transfer and were blocked at RT for 2 h with 10% non-fat dry-milk in TBST containing 0.1% Tween20. Thereafter, the blots were washed briefly and incubated with primary antibodies directed either against p70S6K and p-p70S6K1 (1:1000 O/N at 4°C), mTOR (1:3000 for 2 h at RT), p-mTor (1:1000 O/N at 4°C diluted with 5% non-fat milk or 5% BSA (only for the phoshospecific antibodies) in TBST 0.1% Tween20. The membranes were washed 3 times for 10 minutes with TBST. Then they were incubated for 1 hour, at room temperature, with anti-rabbit or anti-mouse (depending on the primary antibody) HRP-conjugated secondary antibody (Bio-Rad Laboratories, Milano, Italy) diluted 1/2000 in TBST containing 5% non-fat milk. The membranes were washed 3 times for 10 minutes, incubated in SuperSignal West Pico (Pierce Biotechnology, Rockford, IL, USA) chemiluminescent substrate and exposed to autoradiograph films (Fuji Photo Film Co., Dusseldorf, Germany). Optical densities of blot bands were finally determined by means of a computer-assisted densitometer. Results are expressed as phospho signal/total signal.

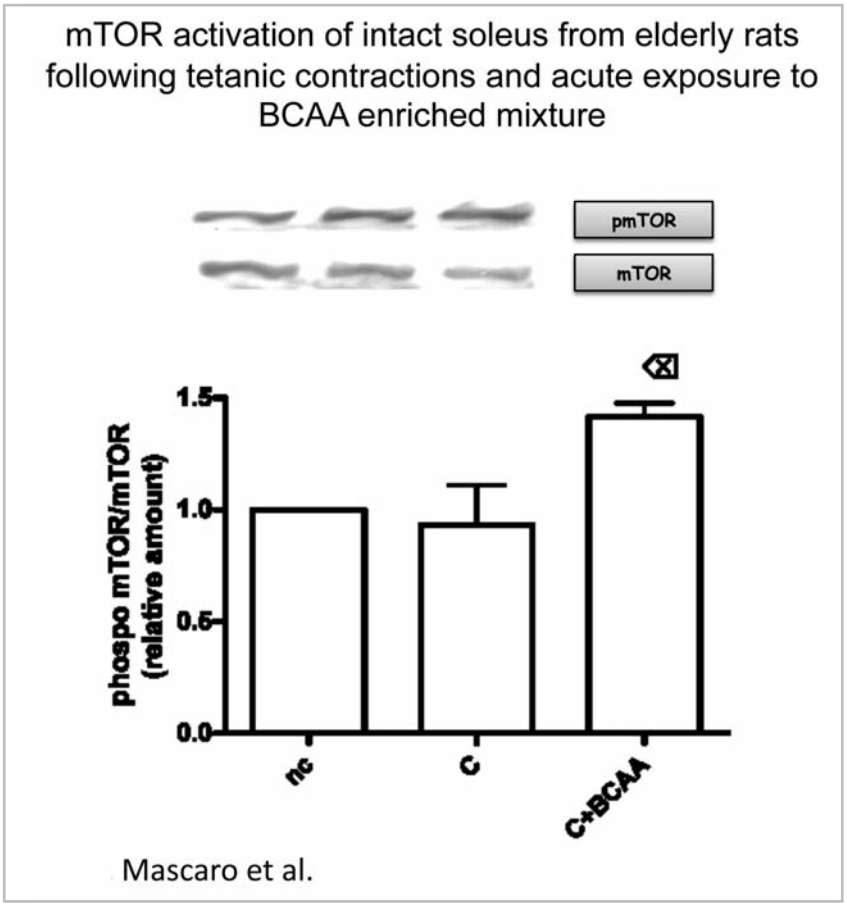
Statistical analysis

Data were expressed as means ± S.D. Statistical significance of the differences between means was assessed by one-way ANOVA followed by Student-Newman-Keuls test. A probability of less than 5% was considered significant ($p<0.05$).

Results

No significant change in absolute (C, 7.54±0.1; C+BCAA 7.6±0.15 mN) and specific tension (C, 0.05±0.002; 0.05±0.001 mN/microL) was observed between contracted (C) and contracted/incubated (C+BCAA) muscles.

Figure 1 - Extracts from uncontracted/unincubated (nc), contracted/unicubated (C), and contracted/incubated (C+BCAA) soleus from elderly rats were immunoblotted with anti-mTOR/phospho-[Ser2448]-mTOR antibodies as described in Methods. Upper panel: representative immunoblots; lower panel: amount of phospho-mTOR/total mTOR in the three groups of muscles. Values are means ± SD, Values in nc are taken as 1.0; circle means significantly different from the other groups, $p<0.05$, n=5 for each group.



Contracted muscles (C) showed no increase of mTOR activation relative to uncontracted muscles (nc) (Fig. 1). On the contrary an increased level of p70S6K activation (+80%) was observed in C *vs* nc muscles (Fig. 2).

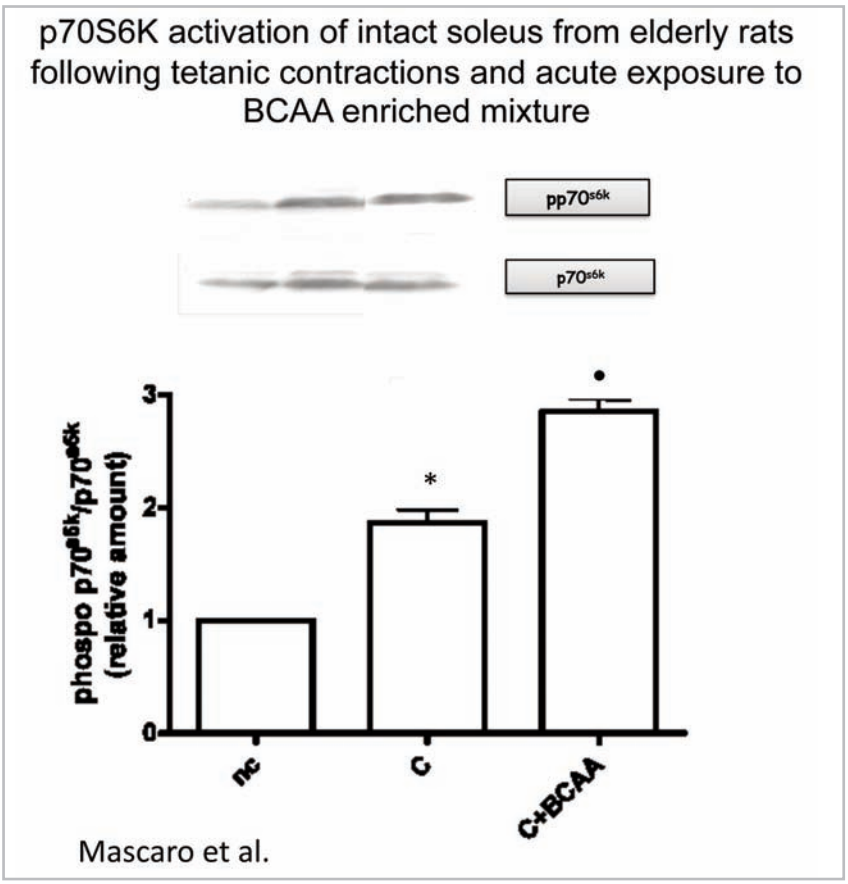
Following acute BCAA enriched mixture exposure contracted muscles displayed higher mTOR activation (Fig. 1) in comparison with contracted and uncontracted muscles (nc) (+40%) and a further increase in p70S6K (Fig. 2) activation in comparison with C (+180% vs nc and +55% *vs* C) muscles thus suggesting a synergistic effect on the signal.

Discussion

In our study the acute exposure to BCAA enriched mixture of intact soleus muscle from elderly rats subjected to mechanical load was followed by further activation of mTOR/p70S6K1 signaling pathway. This evidence demonstrated that both mechanical load and amino acids are able to independently activate the signal pathway thus presumably exerting an additive effect on translation and protein synthesis.

Until recently it was believed that the hypertrophic response following resistance exercise was primarily due to a transient increase of circulating hormones such as testosterone, GH and IGF-1 which certainly have anabolic effects during childhood and puberty or when co-administered at hyper-physiological doses. However their transient increase post-exercise is only partially responsible for the load-induced increase of MPS that precedes the known hypertrophic response occurring as a result of strength training. So far these hormones cannot be considered valid markers of muscle response to load (17).On the other side numerous studies focused on identifying the mechanisms that regulate MPS, have found that the contraction itself and diverse nutrients as amino acids (BCAA) play the role of fundamental regulators of the exercise-induced changes in protein synthesis. In particular muscle contraction appears crucial for a correct balance between protein synthesis and breakdown, since the lack of mechanical load produces significant

Figure 2 - Extracts from uncontracted/unincubated (nc), contracted/unicubated (C), and contracted/incubated (C+BCAA) soleus from elderly rats were immunoblotted with anti-p70S6K/phospho-[Thr389]-p70s6k antibodies as described in Methods. Upper panel: representative immunoblots; lower panel: amount of phospho-p70S6K /total p70S6K in the three groups of muscles. Values are means ± SD, Values in nc are taken as 1.0; asterisk means significantly different from nc and C+BCAA; circle means significantly different from nc and C, $p<0.05$, n=5 for each group.



metabolic changes resulting of a progressive loss of muscle mass (17). On the contrary, mechanical stimuli induce the activation of a series of intrinsic mechanisms which seem to act by increasing the activation level of p70S6K signal, similarly to what follows the interface of amino acids with mTOR. Thus muscle accretion may require the coordinative interaction of all these factors (hormonal, mechanical and nutritional) along the Akt/mTOR/p70S6K pathway.

We recently demonstrated that prolonged supplementation with a selected BCAA enriched mixture determines a greater activation of the mTOR/p70S6K signals followed by antisarcopenic effect, increased mitochondrial biogenesis, and reduced oxidative stress in skeletal muscle of aged mice (3). All these effects appeared potentiated by concomitant exercise training (3).

Here we investigated the level of activation of mTOR and p70SK1 in soleus muscle of elderly rats in resting state and following repeated contractions with or without incubation with BCAA enriched mixture.

The mechanical load imposed to the muscle consisted of repeated not fatiguing tetanic contractions. As expected no change in tetanic force was observed following acute exposure to BCAA in comparison with unincubated controls. In a second set of experiments based on the calculation of susceptibility to fatigue (fatigue index), obtained by imposing fatiguing tetanic contraction at increasing frequencies, the lack of functional improvement due to exposure to BCAA mixture was further confirmed as no change in fatigue index was observed between incubated and unincubated muscles (not shown).

In accordance with previous suggestions (16, 18) we found that imposed tetanic contractions were able to significantly activate p70S6K1 signal thus confirming the responsiveness of the pathway to mechanotransduction also in the elder. Importantly the contraction-mediated increase in p70S6K1 was not associated with concomitant apparent increased of mTOR activation. On the contrary acute exposure of contracting muscle to BCAA enriched mixture was associated with increased mTOR activation, in comparison with contracted and uncon-

tracted, muscles and to a further activation of p70S6K1 in comparison with contracted and unincubated muscles. These findings may suggest a different site or timing of attack for contraction and amino acids on the mTOR/p70S6K pathway and deserves future investigation.

These results highlighting a synergistic action of amino acids with muscle contraction to activate the mTOR pathway in the elderly mammal may put forward the rationale basis for a greater structural and functional effect of amino acids supplementation when associated with exercise. Indeed the synergistic effect of mechanical load and amino acids supplementation may contribute to overcome the age-related decreased responsiveness of the pathway to anabolic stimuli including load itself.

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