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COMPLEX SYSTEMS BIOPHYSICS

The Realization of a Mechanical Signal during Gravitational Unloading: The Response of mTORC1 Targets to Eccentric Contractions

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Abstract—The aim of this study was to assess the response of key mTORC1 substrates to a bout of contractile stimuli under different times of functional unloading. Functional unloading of hind-limb muscles was carried out by the method of antiorthostatic suspension. Twenty-eight Wistar rats were divided into four groups: control, and hindlimb suspension for 1, 3, and 7 days. After hindlimb suspension, isolated soleus muscles of rats were subjected to a bout of ex vivo eccentric contractions. The contents of phosphorylated forms of p70s6k and 4E-BP1 were then determined using western blotting. It was found that an eccentric load resulted in a significant increase in p70s6k phosphorylation and reduced 4E-BP1 phosphorylation both in control and suspended rats, but in the case of suspension the response was dramatically reduced. Thus, it can be concluded that a bout of eccentric contractions of isolated rat soleus muscle during functional unloading causes a weaker activation of the Akt-mTORC1-p70s6k signaling pathway compared with the control. This may indicate that it is important to maintain muscle tone for a more efficient muscle perception of an external mechanical signal and subsequent activation of anabolic signaling pathways.

Keywords: gravitational unloading, soleus muscle, eccentric contractions, p-p70s6k, 4E-BP1

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INTRODUCTION

The development of effective preventive interventions that allow one to reduce the negative effects of a deep decrease in contractile activity of the extensor muscles causing their atony and atrophy during space flight, as well as in neurological and orthopedic diseases is an important problem of space physiology and rehabilitation medicine. However, so far there is no convincing data on the molecular mechanisms that carry out external mechanical signals in muscle fiber in response to functional unloading. Recent studies have shown the importance of studying mechanotransduction, that is, the conversion of an external signal into a biomechanical response in the form of intracellular signaling cascades [1]. Mechanical loading is one of the recognized mechanisms that control muscle mass. This has been confirmed by numerous in vivo studies, which showed that the chronic effect of mechanical loading on skeletal muscle leads to an increase in their mass, while chronic mechanical unloading results in muscle-mass reduction [2]. Changes in protein synthesis play the key role in the mechanical regulation of muscle mass. Stretch-sensitive ion channels, as well as focal adhesion kinase associated with complexes of focal adhesions and integrins are the hypothetical mechanosensors of the muscle cell [3]. At the same time, it is assumed that mechanosensory elements do not function independently, but are integrated with the cytoskeleton and also associated with the functioning of contractile elements of the muscle fiber [4]. The mTORC1/p70s6k signaling cascade is the key anabolic signaling pathway, which is activated in response to mechanical stimuli [5–7]. mTORC1 activity is usually assessed by phosphorylation of its main substrates, – p70 ribosomal kinase (p70s6k) and factor 4E-binding protein (4E-BP1). Considering that the aim of the study was to assess the response of key mTORC1 substrates to a bout of eccentric contractions (EC) in the rat soleus muscle under different times of functional unloading, we hypothesized that numerous changes in signaling pathways and cytoskeletal organization during functional unloading could lead to disturbance of mechanical signals in the postural muscle.

Abbreviations: p70s6k, p70 ribosomal protein S6 kinase; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein; EC, eccentric contractions.

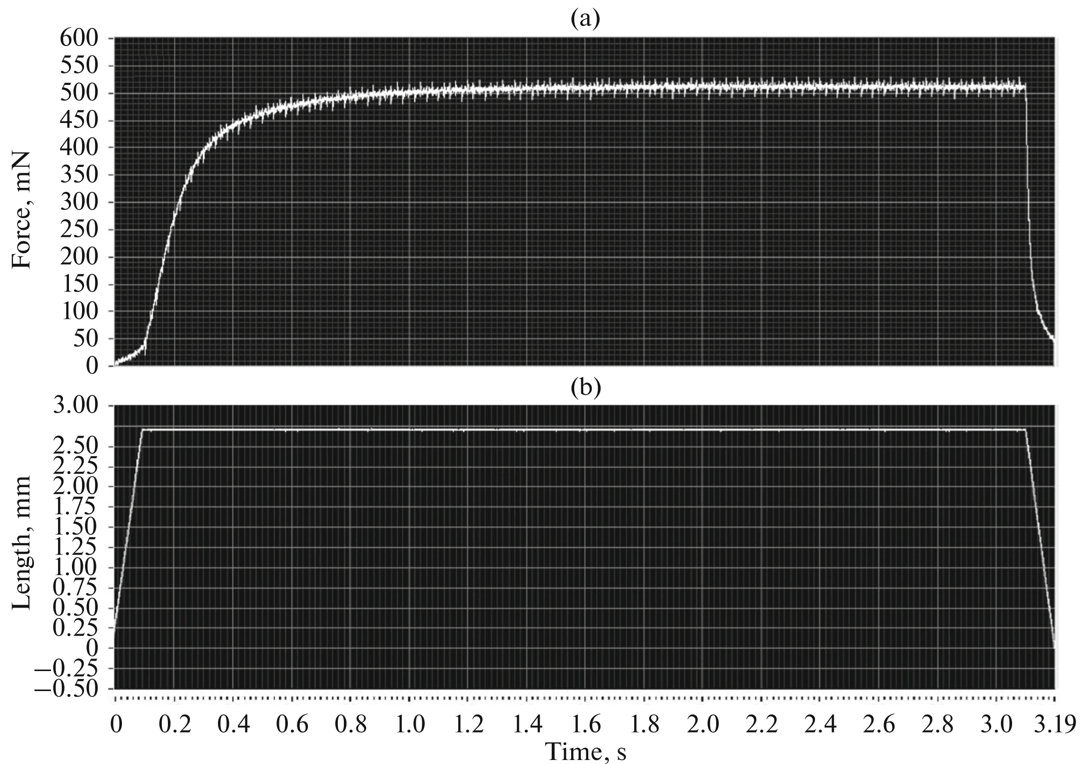

Fig. 1. Schematic representation of a tetanic eccentric contraction using a force sensor (a) and length sensor (b).

MATERIALS AND METHODS

Twenty-eight male Wistar rats with the weight of 220 ± 5 g, which were randomly divided into four groups (seven animals in each group): control, 1HS (suspension for 1 day), 3HS (suspension for 3 days), and 7HS (suspension for 7 days) were used in the study. The animals of the control group were maintained in the conditions of a vivarium and received regular food and water ad libitum. An antiorthostatic suspension of the hindlimbs in order to simulate gravitational unloading was carried out by the standard Il'in–Novikov method modified by Morey–Holton [8]. Rat soleus muscle served as the material of the study.

The program of experiments and all manipulations performed with animals were approved by the Biomedical Ethics Committee of the Institute of Biomedical Problems (Minutes no. 421 dated April 14, 2016).

Eccentric Contraction of Isolated Soleus Muscle

This procedure is based on the protocol given in [9]. Isolated rat soleus muscle was placed in Krebs–Henseleit solution (120 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 2 mM $MgSO_4$, and 5 mM HEPES) with constant perfusion of carbogen (95% O_2 + 5% CO_2) and maintaining of the temperature at 37°C. One end of the muscle was connected to a dynamometer/stress generator by silk threads through tendons and another to a fixed retainer. The muscle was then stretched to the optimum length (L_0). Eccentric contractions were performed using electric field stimulation (80 V, 50 Hz for 3 s). The muscle was stretched to 15% of L_0 during stimulation (the time of stretching and returning to the previous length was 100 ms) [10]. Each contraction was accompanied by a 10-s interval during which the muscle was at L_0. Measurements of muscle contraction and stretching parameters were performed using the DMC/DMA software (Aurora Scientific, United States). The indicators of the maximum tetanic contraction were normalized to the cross-sectional area of the muscle (the ratio of the muscle weight to the optimum length, multiplied by muscle density was 1.07 g/cm^3) [11]. A schematic representation of a tetanic eccentric contraction obtained by a force sensor and length sensor is shown in Fig. 1.

Determination of Phosphorylated p70s6k and 4E-BP1 by Western Blotting

The muscle soleus was sectioned to 20 μm slices using a cryostat, which then were placed into a cooled solution of RIRA Lysis Buffer System (with the addition of 0.5 M EDTA, 24 μL/mL; Na_3VO_4, 20 μL/mL; dithiothreitol, 4 μL/mL; phenylmethylsulfonyl fluoride, 20 μL/mL; aprotinin, 5 μL/mL; leupeptin,

5 μL/mL; pepstatin A, 5 μL/mL; and Phosrhatase Cocktail Inhibitor B, 40 μL/mL) at the buffer level of 130 μL per sample. Homogenization and centrifugation were then performed (for 15 min at +4°C and 12000 rpm). Thereafter, the supernatant was collected and the protein concentration was determined on a UV-2450 spectrophotometer (Shimadzu, Japan) by measuring the absorbance at 595 nm. The protein concentration in the samples was calculated using the calibration curve. Electrophoresis was performed according to the Laemmli method [12] on a 10% separating polyacrylamide gel in a Bio-Rad mini system (United States) for 1 hour at a current of 17 mA per gel. Transfer of proteins to nitrocellulose membrane (Bio-Rad, United States) was performed in a Trans-Blot mini system (Bio-Rad, United States) for 2 h at +4°C and a constant voltage of 100 V. The nitrocellulose membrane was then stained with Ponceau S to verify equal protein loading on all tracks. The membrane was then washed of the dye and blocked by a solution of 4% dry milk in a phosphate buffered saline containing 0.1% Tween 20 at room temperature for 1 h. Incubation (15 h, +4°C) with primary antibodies against p-p70s6k (Thr 389) at a dilution of 1 : 2000 (Santa-Cruz, United States), p-4E-BP-1 (Thr37/46) at a dilution of 1 : 1000 (Cell Signaling, United States) and monoclonal antibodies against glyceraldehyde 3-phosphate dehydrogenase (Abm, Canada) at a dilution of 1 : 10 000 was then performed. The membrane was then washed five times for 5 min in a phosphate buffered saline with 0.1% Tween 20. The membranes were then incubated with biotinylated goat anti-rabbit secondary antibodies for 1 h at a dilution of 1 : 1000 (Santa-Cruz, United States). Goat anti-mouse secondary antibodies at a dilution of 1 : 20000 were used for the detection of glyceraldehyde-3-phosphate dehydrogenase and puromycin (Bio-Rad, United States). Washing in PBS with 0.1% Tween 20 was carried out five times for 5 min. Protein bands were detected on the membrane using a Star TM Substrate Kit (Bio-Rad, United States). Analysis of protein bands was performed using a C-DiGit blot scanner (LI-COR Bioteshnology, United States) and corresponding Studio Digits program Image. The resulting signal from the protein bands was normalized to the reference protein (glyceraldehyde-3-phosphate dehydrogenase).

Statistical Processing

The data are presented as the mean ± standard error of the mean (M ± SEM). The significance of the differences between groups was determined by analysis of the variance (ANOVA method). The significance between intact muscle and the muscle subjected to eccentric contractions was determined using the paired *t*-test. The differences with a significance level of $p < 0.05$ were considered statistically significant.

Table 1. The weight of rats and the ratio of the soleus muscle weight to the rat body weight. The data are presented as the mean values ± standard error of the mean

Group	Weight of animals, g	Weight of the soleus muscle, mg/rat body weight, g
Control	220 ± 8	0.40 ± 0.011
1HS	214 ± 3	0.42 ± 0.013
3HS	222 ± 10	0.372 ± 0.015
7HS	210 ± 7	0.336 ± 0.018*

$p < 0.05$ compared with the control group.

RESULTS

The Weight of Animals and Soleus Muscle

The weight of rats from experimental groups (1HS, 3HS, and 7HS) did not significantly differ from the mass of the control animals (Table 1). The weight of the soleus muscle, normalized to the animal's body weight, was significantly reduced by 16% ($p < 0.05$) after suspension for 7 days relative to the control.

The Mechanical Characteristics of Muscle Contraction and the Cross-Sectional Area of the Soleus Muscle

Table 2 shows parameters such as the cross-sectional area of the muscle, the optimal length of the muscle (L_0), and the specific muscle tension normalized to the cross-sectional area. The cross-sectional area of the soleus muscle significantly changed ($p < 0.05$) only after 7-day gravitational unloading. The indicators of the contraction force (specific tension) normalized to the cross-sectional area did not differ in any of the experimental groups.

The Contents of Phosphorylated forms of p70s6k and 4E-BP1 in Rat Soleus Muscle

Phosphorylation of p70s6k, a substrate of mTORC1, was significantly increased in all groups with EC ($p < 0.05$) relative to the group with intact muscle (Fig. 2). However, there was a significant ($p < 0.05$) decrease in the content of phosphorylated p70S6K simultaneously with an increasing duration of suspension in groups with EC (1HS, 3HS, and 7HS + eccentric): on the first day, 15%; on the third day, 46%; on the seventh day, 60%. In the case of intact muscle a significant ($p < 0.05$) decrease in phosphorylation by 40% was observed only on the seventh day of suspension (Fig. 2).

The opposite change in phosphorylation was shown for another substrate of the mTORC1 system, 4E-BP1. A significant ($p < 0.05$) decrease in phosphorylation was observed in all groups with EC relative to the groups with intact muscle (Fig. 3). At the same time a significant ($p < 0.05$) decrease in the level of 4E-BP1 phosphorylation in all groups with EC and

Table 2. The mechanical and physical parameters of the soleus muscle. The data are presented as the mean values ± standard error of the mean

	Control	1HS	3HS	7HS
Optimal length of muscle – L_0, cm	1.99 ± 0.06	2.01 ± 0.04	1.89 ± 0.03	1.87 ± 0.04
cross-sectional area of muscle, cm^2	0.042 ± 0.002	0.041 ± 0.002	0.037 ± 0.002	0.033 ± 0.001*
Average contraction force, mN	777.4 ± 17.9	723.3 ± 23.6	742.5 ± 20.7	660.75 ± 24.4*
Normalized average contraction force, mN/cm^2	19368 ± 1405	18229 ± 1045	20195 ± 1044	20244 ± 1122

Significant difference from the control group ($p < 0.05$).

suspension was detected (1HS, 3HS, and 7HS + eccentric) relative to the control (control + eccentric): on the first day, 49%, on the third day, 51%; and on the seventh day, 47% (Fig. 3). For groups with intact muscle a significant ($p < 0.05$) decrease in 4E-BP1 phosphorylation by the same values as in the groups with EC was also shown.

DISCUSSION

The fact that a progressive decrease in the activation of the mTORC1 system (according to phosphorylated p70s6k) was first demonstrated in rat soleus muscle in response to EC ex vivo during functional unloading of hindlimbs was an important result of the present study. It can be assumed that a decrease in the anabolic response after EC could be associated with a significant reduction in contraction force of m. soleus as a result of gravitational unloading. However, the average contraction force normalized to the cross-sectional area did not differ significantly between the studied groups of animals, which suggests that the decrease in the mTORC1 response was not associated with the loss of specific tension of the soleus muscle. Therefore, it can be hypothesized that a decrease of the anabolic response during functional unloading could be caused by disturbance of both mechanosensory structures of the muscle fiber and signaling molecules involved in mechanotransduction and functioning upstream of mTORC1. In particular, the role of integrin-associated focal adhesion kinase in the perception of an external mechanical signal and subsequent triggering of an intracellular signaling cascade that leads to p70s6k hyperphosphorylation and activa-

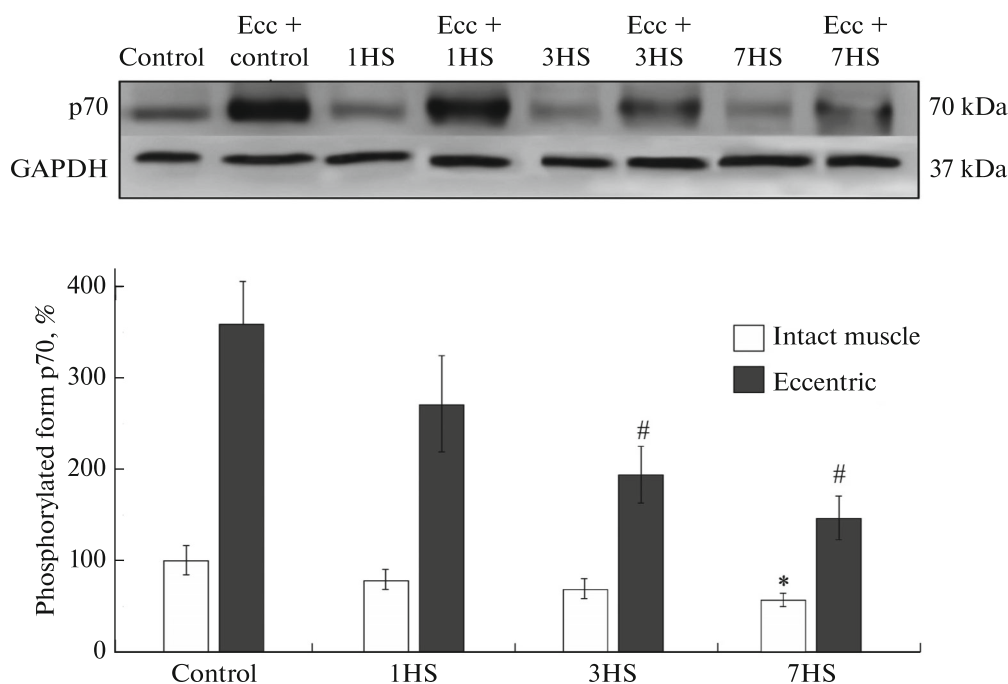

Fig. 2. The content of the phosphorylated form, p70s6k. The data are presented as the mean values ± standard error of the mean. * The significant difference of suspended groups from the control group ($p < 0.05$); #, the significant difference of the control group with eccentric from suspended groups with eccentric ($p < 0.05$)

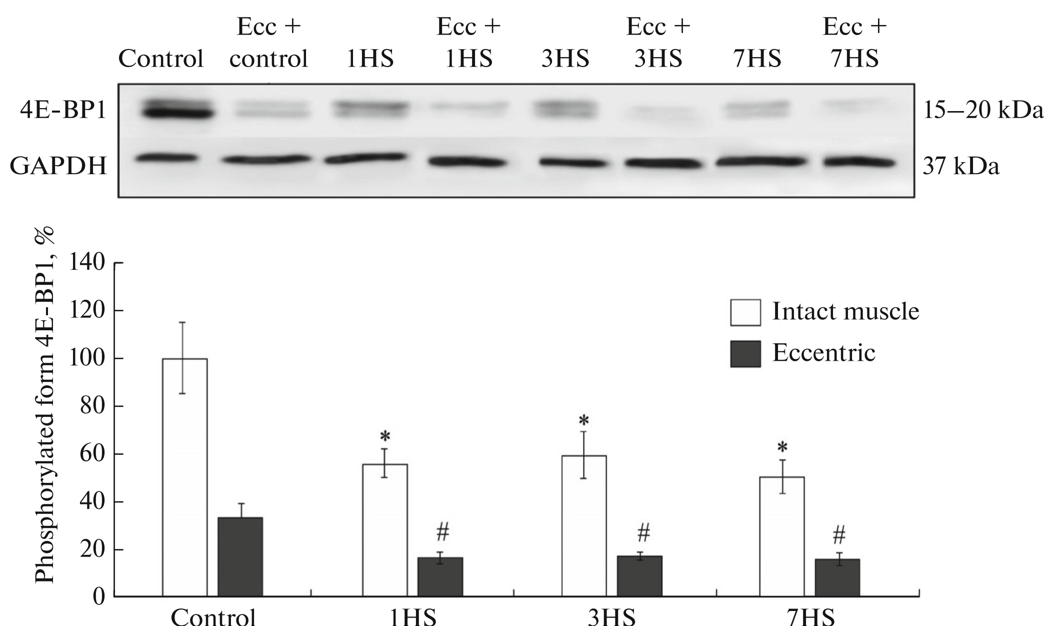

Fig. 3. The content of the phosphorylated form, 4E-BP1. The data are presented as the mean values ± standard error of the mean. * The significant difference of suspended groups from the control group ($p < 0.05$); #, the significant difference of the control group with eccentric from suspended groups with eccentric ($p < 0.05$)

tion of initiation of protein translation was shown previously [13–15]. It should be noted that a significant increase in the phosphorylated form of p70s6k with overexpression of focal adhesion kinase in mouse m. tibialis anterior was found, while the content of phospho-4E-BP1 remained unchanged [16]. Mitogen-activated protein kinases, p54JNK and ERK1/2, whose activation was observed in rat skeletal muscle in response to a series of isometric and eccentric contractions, may be another potential regulator of protein synthesis in response to mechanical signals [17, 18]. However, there is evidence that inhibition of ERK1/2 kinase did not result in the reduction of mTORC1 activation and protein synthesis in isolated mouse muscle in response to stretching [19]. At the same time it was shown that a key role in mechano-dependent mTORC1 activation and protein synthesis in skeletal muscle belongs to phosphatidic acid, which is capable of direct binding to the mTORC1 complex [9, 19, 20]. In addition, the reduction of the mTORC1 substrate response to eccentric contractions during functional unloading could be associated with impaired expression of stretch-sensitive calcium channels. Thus, it was shown that the use of an inhibitor of these channels, gadolinium salt, resulted in decreased concentration of Ca^{2+} and reduced mTORC1 activity in response to EC [21, 22]. Furthermore, an important role in the transmission of the mechanical signal from the muscle fiber surface to signaling molecules that control protein synthesis may belong to the cytoskeleton [23]. Data on a variety of regulatory functions of the intermediate filament protein desmin occur in the literature [24, 25]. Previously, a significant decrease in the content of desmin in rat soleus muscle after 3- and 7-day gravitational unloading was shown [26, 27]. It can be assumed that destruction of cytoskeletal proteins might contribute to the disturbance of mechanical signal transduction to mTORC1 in the rat soleus muscle under gravitational unloading.

Thus, a bout of eccentric contractions of isolated rat soleus muscle during functional unloading caused weaker activation of the Akt-TORC1-p70s6k signaling pathway compared with the control. This may indicate the need to maintain muscle tone for more efficient muscle perception of an external mechanical signal and subsequent activation of anabolic signaling pathways.

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