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Effect of Short-term Dry Immersion on Proteolytic Signaling in the Human Soleus Muscle

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Abstract—We analyzed the signaling processes initiating proteolytic events in the human soleus muscle during short-term exposure under the non-weight-bearing conditions. Dry immersion (DI) was used to induce weight deprivation in the m. soleus for 3 days. Western blotting was used to determine the level of insulin receptor substrate 1 (IRS-1), total and phosphorylated neuronal NO synthase (nNOS), and adenosine monophosphate-activated protein kinase (AMPK), which control the anabolic and catabolic signaling pathways, and the level of cytoskeletal protein desmin and Ca^{2+}-activated protease calpain. By day 3 of DI, calpain-dependent proteolysis manifests itself by reductions in both the total content and level of nNOS phosphorilation. The rate of AMPK phosphorylation was significantly decreased.

Keywords: dry immersion, m. soleus, IRS-1, AMPK, nNOS, desmin, calpain

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Space flights (SFs) and simulated microgravity have significant effect on the structural and functional properties of skeletal muscles in mammals [1, 2]. Changes in skeletal muscles in weightlessness can be reproduced in on-earth microgravity model of dry immersion (DI) [1–3]. In contrast to the bed-rest model, the DI model includes almost no weight-bearing reactions [4]. These models are different in the speed of changes in transverse stiffness of muscle fibers and muscle power, as well as in the process of motor unit recruiting in voluntary movements [2]. The DI model is most suitable for investigating changes in skeletal muscles under non-weight-bearing conditions.

It has been found earlier that seven days of DI lead to a number of changes in the postural muscles at the cellular level [5–7]. The findings of these studies included a decrease in the diameter and maximal power of isometric contraction of permeabilized fibers of m. soleus, a decrease in the calcium sensitivity, and transverse stiffness of fibers (primarily of type I), and a decrease in the relative concentration of titin, nebulin, desmin and α-actinin. Thus, seven days under non-weight-bearing conditions resulted in changes in a number of structural and functional parameters of the human soleus muscle. These changes occurred during a significant reduction of the electric activity of this muscle [8, 9]. Earlier studies showed that slow type motor units are not already involved in contraction after three days of DI [10]. The absence of electric activity in m. soleus in rats was observed almost immediately in the first hours of non-weight-bearing conditions [11]. Such inhibition of slow type motor units during the first hours or days of non-weight-bearing conditions leads to the activation of catabolic signaling pathways and proteplytic processes, which results in the destruction of cytoskeletal and contractile proteins.

In particular, a few days of antiorthostatic bed-rest test lead to an increase in the level of calcium ions in the myoplasm [12–14] and activation of calpains resulting in the destruction of several cytoskeletal proteins [15, 16] in m. soleus in rats. The destruction of desmin, a typical substrate of μ-calpain, is observed in m. soleus of rats as early as after three days of antiorthostatic bed rest [17]. At the same time, nitrogen oxide is one of the most active endogenous inhibitors of μ-calpain [18]. Nitrogen oxide is produced in muscle fibers by both neuronal and endothelial NO synthases. Interestingly, the activity of S-nitrosyled molecules of μ-calpain is significantly lower than that of calpain molecules unaffected by nitrogen oxide [18]. Experimental studies in rats, studies in rats during real SFs and studies in humans during seven days of DI showed that these conditions led to the reduction of the level of neuronal NO synthase (nNOS) and its translocation from sarcolemma to cytosol [6, 20, 22, 23]. Moreover, the total reduction of the level of nitrogen oxide in m. soleus was observed in an experiment with antiorthostatic bed rest test in rats [24]. A decrease in the level of nNOS in microgravity and its translocation to cytoplasm could be explained by the

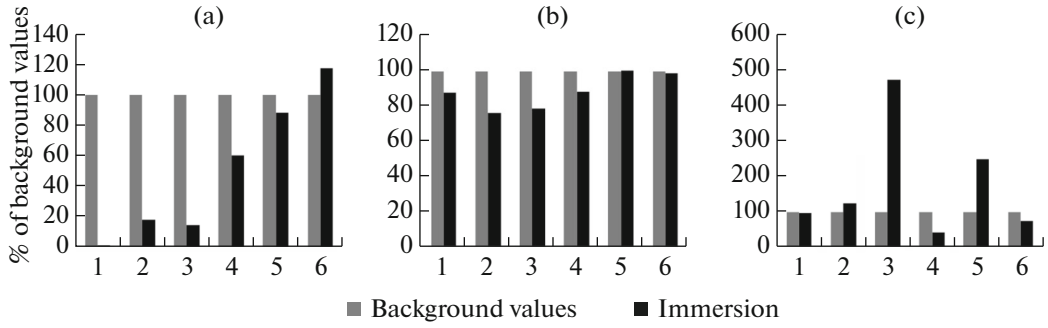

Fig. 1. Individual concentrations of (a) calpain, (b) desmin and (c) IRS-1 in m. soleus ($n = 6$) before and after immersion.

effect of μ-calpain [25, 26]. However, it is still unclear why the proteolytic effect of calpain on nNOS at early stages of weightlessness is stronger than the inhibiting effect of nitrogen oxide on calpain activity. Calpain-dependent destruction of nNOS may be preceded by a decrease in its activity (NO production). Obviously, the reduction of nNOS activity (as well as the activity of endothelial NOS) can be caused not only by its destruction, but also by its dephosphorylation [27]. Phosphorylation of nNOS is known to be mainly provided by the components of two signaling pathways: insulin/insulin-like growth factor 1 (IGF-1)-dependent proteinkinases [28] and adenosine monophosphate-activated protein kinase (AMPK) [27]. Since the activation of insulin/IGF-1-dependent pathways in weightlessness is primarily caused by the destruction of insulin receptor substrate 1 (IRS-1) [29], the state of this protein kinase in human m. soleus at early stages of weightlessness is an interesting object for studies. The phosphorylation rate of AMPK under these conditions is also of great interest.

The purpose of this study was to analyze signaling processes leading to the initiation of proteolytic events in m. soleus in humans after short-term exposure under the non-weight-bearing conditions.

MATERIALS AND METHODS

Dry Immersion Model

For creating non-weight-bearing conditions, we used the on-earth model of hypogravity, dry immersion (DI) model. When placed in immersion medium, the body is evenly affected by the buoyant force of water, and weight-bearing reactions are eliminated (Fig. 1). The DI model was used for three days. The method of microbiopsy [30] was used for collecting samples of soleus muscle from six volunteers (men with a mean age of 22 years and mean weight of 67.7 kg) before and after immersion. The studies were performed under standard conditions: the subjects were placed in horizontal position in basins 200 × 100 × 100 cm in size filled with water; the water temperature was kept at a level of 33 ± 0.5°C. The regimen of subjects was determined by time intervals chosen for examinations, preventive measures, meals, and sanitary activities. The schedule was as close to the real schedule of SFs as possible, including 8 h of sleep, three meals per day, medical control procedures, and experimental examinations. The subjects were removed from the bath for the sanitary measures for 15–20 min once a day with the use of a special elevator. All the subjects gave written consent for participating in the study. The study protocol was approved by the Bioethics Commission of the Institute of Biomedical Problems, which is the physiological section of the Russian Bioethics Committee (UNESCO Commission of the Russian Federation). All the procedures corresponded with the Helsinki Declaration and international and Russian laws.

Gel-Electrophoresis and Immunoblotting for the Measurement of the IRS-1, Desmin, Calpain, Phospho-NOS, Total NOS, and phospho-AMPK Levels

Slices of m. soleus tissue (20 μm) were made with the use of a cryostate and then placed into cool RIPA lysis buffer (the buffer also contained 0.5 M EDTA 24 (μL/mL), Na_3VO_4 (20 μL/mL), DTT (4 μL/mL), PMSF (20 μL/mL), aportinin (5 μL/mL), leupeptin (5 μL/mL), pepsatin A (5 μL/mL), and phosphatase inhibitor cocktail B (40 μL/mL)); 130 μL of buffer were used per sample. Then, the samples were homogenized and centrifuged for 15 min at a temperature of +4°C at 12000 rpm. We collected the supernatant and determined protein concentration using a UV-2450 spectrophotometer (Shimadzu, Japan) and measured absorption at a wave length of 595 nm. Protein concentration was determined basing on a calibration curve. Electrophoresis was performed according to the method of Laemmli using 10% polyacrylamide gel in a Bio-Rad mini-system (United States) during 1 h at 17 mA per gel. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad, United State) using a mini Trans-Blot system (Bio-Rad, United State) during 2 h at a temperature of +4°C and constant 100 V. The membrane with transferred proteins was blocked in 4% solution of dry milk in PBST (PBS + 0.1% Tween 20) at room temperature for 1 h. Then,

Table 1. Concentrations of desmin, calpain and IRS-1 in human m. soleus before and after immersion

Groups	Parameters, %		
	desmin	calpain	insulin receptor substrate 1
Control	100 (73.35–118.42)	100 (65–121)	100 (67.34–317.12)
Immersion	83.96* (59.72–115.23)	33 (9.2–70)	157.05 (128.92–298.67)

Here and in Table 2, the values are given in the form of median and interquartile ranges in % of the control values (before immersion); * $p < 0.05$.

the membranes were incubated (for 15 h) with primary polyclonal antibodies against IRS-1 (Santa Cruz, United States) diluted to 1 : 500, primary polyclonal antibodies to desmin (Santa Cruz, United States) diluted to 1 : 1000 in 4% milk, phospho-AMPK 1/2 (Thr172) (1 : 10000; Santa Cruz), phospho-nNOS (Ser 1417) (1 : 100000; Milipore Chemicals, United States), total nNOS (1 : 10000; BD Transduction Laboratories, United States), monoclonal antibodies GAPDH (Abcam, United State) diluted to 1 : 3000, and primary polyclonal antibodies against calpain (Cell Signaling, United State) diluted to 1 : 20000. The membranes were washed in PBST three times for 5 min. Then, the membranes were incubated with biotinylated secondary antibodies GAR (Santa Cruz, United States) diluted to 1 : 1000 for 1 h. Secondary antibodies GAM diluted to 1 : 20000 were used for GADPH. Then, PBST was washed three times for 5 min. Protein bands were detected on a film using a Star TM Substrate Kit (Bio-Rad, United States). Protein bands were analyzed using a GS-800 densitometer (Quantity One software, Bio-Rad, United States). The optical density of protein bands was normalized to reference GAPDH protein.

RESULTS AND DISCUSSION

Three days of DI resulted in a decrease in the level of cytoskeletal protein desmin in m. soleus by 10% as compared with initial values (Table 1). In 4 out of 6 subjects, the level of desmin was decreased by 12 to 24% (Fig. 1b).

The total concentration of calpains, calcium-dependent proteases, in m. soleus was decreased by 67% as compared with initial values (Table 1). In five out of six subjects, the level of calpain was decreased by 12 to 98% (Fig. 1a).

We also noted a decrease in the level of total nNOS in m. soleus by 26% as compared with control values; however, such decrease did not reach statistical significance (Table 2). At the same time, we observed a significant decrease in the level of phosphorylated nNOS by 43% as compared with control values (Table 2).

Since a decrease in the concentration of phosphorylated nNOS can result from the reduction of protein kinase activity of IGF-1/IRS-1/Akt cascade, the level of IRS-1 is an important factor. In general, the concentration of IRS-1 in m. soleus after three days of DI did nor differ from control values; however, an increase in this concentration was observed in two subjects (Fig. 1c).

The phosphorylation rate of AMPK is another important factor that provides the reduction of phosphorylation rate of nNOS. After three days of DI, the concentration of phosphorylated AMPK was significantly decreased by 36% compared with control values (Table 2). In five out of seven subjects, the level of phosphorylated AMPK was decreased by 26 to 84%; in one subject this value was increased by 29% (Fig. 2c).

A short-term experiment with non-weight-bearing conditions in humans and animals result in increased proteolytic activity of calpains, which can be estimated in vivo basing on the state of substrates, some cytoskeletal proteins, such as desmin [16]. It was found earlier that seven days of DI result in a decrease in the concentration of desmin in the human m. soleus by approximately 30% [7]. A small but significant decrease (by 10%) in the level of desmin was already observed after three days of DI. Thus, we suppose that the proteolysis of cytoskeletal proteins known as μ-calpain substrates starts o the early stages of non-weight-bearing experiments. A decrease in the level of nNOS in m. soleus took place in this period, which indicates activation of calpain-dependent processes. A significant decrease in the concentration of μ-calpain

Table 2. Concentrations of phosphorylated AMPK and total and phosphorylated NOS in human m. soleus before and after immersion

Groups	Parameters, %		
	phospho-AMPK	phospho-nNOS	total nNOS
Control	100 (93.18–106.20)	100 (64.32–124.62)	100 (75.66–136.46)
Immersion	57.31* (34.90–100.81)	46.21* (32.03–64.82)	76.94 (47.10–119.31)

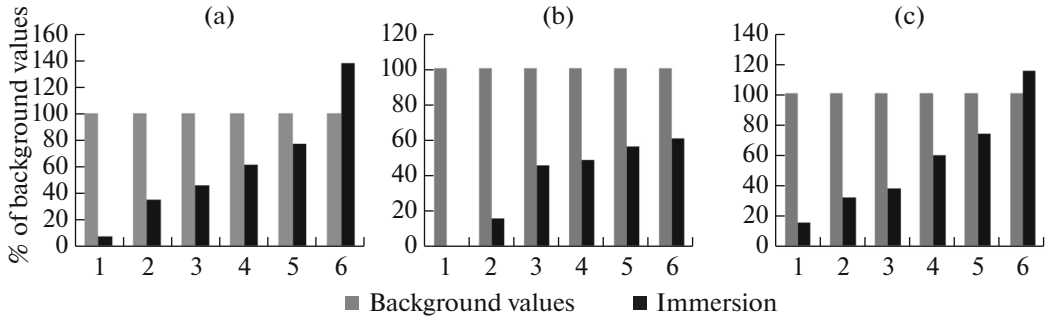

Fig. 2. Individual concentrations of (a) total NOS, (b) phosphorylated NOS, and (c) phosphorylated AMPK.

may result from the autolysis of calpain molecules, which is obviously caused by their activation [31]. The concentration of nNOS has often been reduced in non-weight-bearing experiments in leg muscles of humans and animals [6, 20–23]. However, these findings were mainly associated with long-term experiments. A decrease in the level of nNOS in human m. soleus after a short-term non-weight-bearing experiment was first observed in this study. We also observed the activation of calpain-dependent proteolysis in human m. soleus at early stages of non-weight-bearing conditions.

Since nitrogen oxide is one of the inhibitors of μ-calpain, it is unclear why nNOS cannot reduced the activity of calpain at early stages of the process. We suppose that the activity of nNOS is decreased as a result of changes in its phosphorylation rate.

Indeed, the level of phosphorylated nNOS in m. soleus was significantly reduced (by more than 40%). The inactivation of AMPK [27], proteinkinase D [32] and IGF-1/insulin/Akt/mTOR signaling pathway [28] is known to produce the same effect (a decrease in the phosphorylation rate of nNOS and production rate of nitrogen oxide). We did not observe any significant changes in the concentration of IRS-1 in m. soleus of subjects before and after DI. In one of the studies [29], a non-weight-bearing experiment (Morey–Holton test) led to the destruction of IRS-1 with the help of ubiqitin ligase Cbl-b. A decrease in the concentration of IRS-1 in m. soleus in rats was also observed after 14 days of head-down tilt bed-rest test [33]. However, no studies were performed to determine the level of this key component of IRS-1/mTOR cascade in human muscles at early stages of weightlessness. The absence of changes in the level of IRS-1 after three days of non-weight-bearing experiment indicates that an increase in the expression rate of E3-ubiqitin ligases associated with IRS-1 destruction is not typical of early stages of weightlessness. According to these data, a decrease in the prosphorylation rate of nNOS, most probably, is not caused by changes in the activity of canonical IRS-1/AKT/mTOR cascade.

At the same time, we observed a significant decreased in the concentration of phosphorylated (at the Thr 172 site) form of AMPK in m. soleus of subjects. Changes in the phosphorylation rate of AMPK at this site in m. soleus in rats were previously recorded after two weeks of head-down tilt bed-rest test in two studies [33, 34]. The phosphorylation rate was decreased in study [33] and increased in study [34]. No literature data are available on changes in the phosphorylation rate of AMPK after short-term weightlessness in either humans or animals. As we know the phosphorylation rate of AMPK is regulated by both systemic factors (such as interleukin-6 [35]) and cellular factors, first of all the ratio of phosphorylated and non-phosphorylated macroergic nucleotides (ATP/ADP/AMP) [36]. A decreased phosphorylation rate of AMPK can lead to a decrease in the phosphorylation rate of both neuronal and endothelial NOS and production rate of nitrogen oxide [27].

CONCLUSIONS

(1) Under the non-weight-bearing conditions of the DI model for three days, the proteolytic processes in the human body are activated, while nNOS, one of the most important negative regulators of proteolytic processes, is inactivated.

(2) The phosphorylation rate of AMPK is decreased, which may be the trigger of primary atrophic changes.

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