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Protective Effect of L-Arginine Administration on Proteins of Unloaded *m. soleus*

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Abstract—Cytoskeletal and contractile proteins degenerate during functional unloading of muscle. The ratio of myosin heavy chain (MHC) expression changes simultaneously. We have supposed that NO can be a signal molecule related to the regulation of protein metabolism upon muscle unloading. To test this hypothesis, Wistar rats underwent functional unloading for 14 days without and with peroral administration of L-arginine (500 mg/kg) as NO precursor. Significant decreases in *m. soleus* mass, NO, nNOS, dystrophin, Hsp90, p-S6K, and type I MHC mRNA contents were found in the group of animals with unloading without preparation compared to those in control and in the group with unloading and administration of L-arginine; at the same time, increased contents of atrogin-1/MAFbx and MuRF-1 ($p < 0.05$) were found. No difference in the IGF-1 mRNA content between all three groups was found. Atrophy was significantly less pronounced in the group with unloading and L-arginine administration compared to that without the amino acid, and no destruction of cytoskeletal proteins was observed. We conclude that administration of L-arginine upon functional unloading decreases the extent of *m. soleus* atrophy, prevents the decrease in it of type I MHC mRNA, and blocks destructive changes in some cytoskeletal proteins. Such effect can be due to the absence of increase in this group of the content of some ubiquitin ligases and decreased intensity of the p70S6 kinase synthesis marker.

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Key words: *m. soleus* atrophy, L-arginine administration, cytoskeletal proteins, NO, nNOS, Hsp90, E3 ligases, P70/S6k, IGF-1, MHC

Cytoskeletal and contractile proteins are known to undergo degradation upon functional unloading of muscle [1-3]. Various cytoskeletal, membrane, and contractile proteins exhibit different dynamics of their degradation. However, within 14 days after unloading, *m. soleus* atrophy reaches 40-45%. Contractile proteins make the largest contribution to the muscle mass decrease [1, 3]. Also, the expression of myosin heavy chain (MHC) fast isoforms increases in *m. soleus*, which changes their locomotor functions. Desmin and α-actin are the most important cytoskeletal proteins providing for integration of the muscle fiber intracellular structures and for muscle contractile function [1]. Therefore, investigation of destruction of these proteins in the case of atrophy is of particular interest. Cellular factors launching the atrophy process are still poorly studied. The role of calcium in launching proteolysis of cytoskeletal proteins is known [4, 5]. At the same time, more and more data appear showing that NO may also be involved in regulation of protein metabolism in skeletal muscle. Thus, previous experiments with mdx mice showed that administration of NO

Abbreviations: atrogin-1/MAFbx, atrogin-1/Muscle Atrophy F-box; CSA, cross sectional area; DETC, diethyldithiocarbamate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hsp90β, 90β heat shock proteins; IGF-1, insulin-like growth factor 1; MF, muscle fiber; MHC, myosin heavy chain; mTOR, mammalian target of rapamycin; MuRF-1, muscle-specific RING finger protein 1; nNOS, neuronal NO synthase; p70S6K, p70S6 kinase; P-p70S6K, phosphorylated form of p70S6 kinase.

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precursor L-arginine results in increased content of utrophin and some cytoskeletal muscle proteins [6, 7]. However, addition of NO to muscle cell C2C12 culture partially prevents cytoskeletal protein decomposition stimulated by administration of calcium ionophores [8], which is indicative of a relationship of these processes. We paid attention to these studies because in a number of works, along with increase in calcium concentration, a decreased content of neuronal NO synthase was also demonstrated in the case of long-term retardation of moving activity and destruction of muscle proteins [9, 10]. We supposed that the NO level can also decrease upon functional unloading of muscle, and it might serve as a signal molecule related to the regulation of protein metabolism. To test this hypothesis, we introduced into animals the NO precursor L-arginine during functional unloading, and on one side, we estimated the level of expression and content of contractile and some cytoskeletal muscle proteins, while on the other side, we tested the work of the proteasome degradation (E3 ligases atrogin-1/MAFbx and MuRF-1) system and protein synthesis (Akt-mTOR-S6K signal pathway) units and insulin-like growth factor. Atrogin-1 and MuRF-1 were chosen as markers of proteolysis, because earlier significant growth of just these E3 ligases was registered upon functional unloading of muscle, and removal of MuRF-1 prevented development of atrophy [11]. This is not surprising because MuRF-1 is involved in ubiquitination of thick myofilament components [11], and contractile proteins occupy up to 75-80% of the volume of muscle fibers. If this hypothesis is correct, then L-arginine administration might prevent or decrease atrophy in *m. soleus* and decrease destruction of some cytoskeletal proteins.

MATERIALS AND METHODS

The experiments were conducted according to the rules of biomedical ethics (protocol No. 264 of March 5, 2009 was approved by the Russian Academy of Sciences Committee on Bioethics). Rodents were kept at 20°C with food and water *ad libitum*. Forty-two 2.5-month-old male Wistar rats of 220-240 g were assigned to three groups (14 animals in each). The first was the control (C) group. The rats of the second group were hindlimb suspended during 14 days so that hindlimbs being suspended and forelimbs touching the floor (group HS) [12]. The third group was hindlimb suspended with L-arginine administration with drinking water (HSL group, L-arginine concentration 500 mg/kg rat mass). The rats were sacrificed by Nembutal overdose (75 mg/kg), and the *m. soleus* was immediately frozen in liquid nitrogen. Seven animals from each group were used for determination of relative NO content in *m. soleus* (specimens were frozen and kept in liquid nitrogen), while *m. soleus* of the other seven animals were used for all the other investigations. These specimens were frozen in liquid nitrogen and kept at −85°C. An additional experiment was carried out in advance with administration during 14 days of the same dose of L-arginine to seven rats kept, like the control group, in cages. The NO content in muscle of these animals and the muscle mass remained the same as in rats that did not get the L-arginine supplement (data not shown). The absence of changes in NO concentration in this group (unlike previously obtained increase after administration of preparation for three days [13]) can be due to inverse substrate regulation of neuronal NO synthase (nNOS) activity. Cross sectional areas (CSA) of type I and II muscle fibers were determined by revealing myosin heavy chains (MHC), and the content of dystrophin (immunohistochemical assay on muscle cross sections), desmin (immunoblotting), nNOS (immunoblotting and real time PCR), mRNA of atrogin-1 and MuRF-1, mRNA of MHC type I, IIa, IIb, and IId/x, insulin-like growth factor (IGF-1), and of heat shock proteins 90β (Hsp90β) (real time PCR) were determined.

Determination of relative NO content in muscle. Relative NO content in muscle was determined by the standard spin trapping technique and electron paramagnetic resonance (EPR) [14, 15]. Diethyldithiocarbamate (DETC) was used as the trap, which forms in tissues nitrosyl paramagnetic complexes with iron that are in equilibrium with stationary NO concentration in the tissue and have a characteristic EPR spectrum. Spin trap was introduced into the rat to 500 mg/kg. DETC binds NO by formation of paramagnetic complexes. Immediately after DETC treatment, aqueous solution of the 29 mM $FeSO_4$ mixture with 116 mM sodium citrate (2 ml/kg) was introduced intramuscularly. Thirty minutes later animals were decapitated, *m. soleus* was frozen in liquid nitrogen, and EPR signal was registered at liquid nitrogen temperature on an EMX-8 EPR spectrometer (Bruker, Germany). It was shown in preliminary experiments that DETC is accumulated in muscle at concentrations sufficient for measurements. To do this, muscle preparation after DETC treatment was bubbled with nitrogen oxide, and the EPR spectrum was registered. Spectrum amplitude after tissue saturation with nitrogen oxide was one order of magnitude higher, which shows that the trapping concentration in the preparation is sufficient for correct measurements and the signal is not saturated. The EPR signal is a superposition of the signal of $NO-Fe^{2+}(DETC)_2$ complex and other paramagnetic centers detected in tissues (Fig. 1). However, there are components in the registered signal that coincide in signal shape and position with EPR signal of iron nitrosyl complexes with dithiocarbamate. In this case, the low-field spectrum component is well expressed and is not in superposition with other signals. This makes it possible to estimate the relative NO content by the amplitude of first component of triplet ultrafine structure of this complex.

Fig. 1. Relative NO content in *m. soleus*. Examples of EPR spectra of NO–$Fe^{2+}(DETC)_2$ complex in skeletal muscle of control (a) and suspended (b) rats. Spin trap was introduced 30 min before start of measurement. Measurement of amplitude of the first component (arrow) of triplet superfine structure of the complex allows estimation of the relative NO concentration.

Detection of dystrophin and MHC. Ten micrometer thick cross sections of muscle tissue prepared using a cryostat were incubated with primary monoclonal antibodies against dystrophin or MHC type I or MHC type II (primary antibodies were diluted 1 : 20, NCL-DYSI (Novocastra, USA), 1 : 60, NCL-MHCs (Novocastra), and 1 : 40, sc-75 (DSMZ, Germany)) and with secondary antibodies (1 : 1000, GAM (IMTEK, Russia), Alexa546, and Alexa350 (Molecular Probes, USA)). All specimens were incubated for 1 h at room temperature. The negative control was prepared for each reaction (without primary antibodies) to reveal unspecific staining, and percentage of fibers with damaged dystrophin layer was calculated relative total number of fibers on the section.

Electrophoresis in polyacrylamide gel. Twenty micrometer thick cross sections (10-15 mg) were prepared from each *m. soleus* specimen and immediately homogenized for 25 min in 100 μl buffer containing (*for nNOS*) 62.5 mM Tris-HCl, pH 6.8, 150 mM NaCl, 2% Triton X-100, 2% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, complete mini (cocktail of protease inhibitors (Roche, Germany)); (*for desmin and α-actin*) 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromphenol blue; (*for p70S6K*, *P-p70S6K*) 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 10 mM EDTA, 50 mM β-glycerophosphate, 0.5 mM DTT, 5 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml pepstatin, 0.1% Triton X-100. Then specimens were centrifuged at 20,000*g* for 25 min. Samples *for nNOS*, *p70S6K*, and *P-p70S6K* were diluted by 2× sample buffer (5.4 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.02% bromphenol blue). Samples *for nNOS*, *desmin*, *and actin* were boiled at 95°C for 2 min. An aliquot of supernatant was taken for total protein determination by RC DC Protein Assay (Bio-Rad Laboratories, USA), and the rest volume was used for analysis by PAGE. All samples were kept at –84°C. Electrophoresis *for nNOS* was carried out in 8%, *for desmin and α-actin* in 12%, and *for p70S6K* in 10% separating polyacrylamide gels (0.2% methylene-bis-acrylamide, 0.1% SDS, 375 mM Tris-HCl, pH 8.8, 0.05% ammonium persulfate, 0.1% TEMED) and in 5% concentrating polyacrylamide gel (0.2% methylene-bis-acrylamide, 0.1% SDS, 125 mM Tris-HCl, pH 6.8, 0.05% ammonium persulfate, 0.1% TEMED). The cathode (192 mM Tris-glycine, pH 8.6, 0.1% SDS) and anode (25 mM Tris-HCl, pH 8.6) buffers were used. Samples *for nNOS*, *desmin, and α-actin* as well as *for p70S6K* were loaded at the rate of 40, 20, and 15 μg total protein in each sample per lane. Samples of each group were loaded on the gel together with control samples. Electrophoresis was carried out at 15 mA/gel in a mini system (Bio-Rad Laboratories) at room temperature.

Western blotting. Electrotransfer of proteins was carried out in buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol, 0.04% SDS) onto nitrocellulose membrane at 350 mA and 4°C in the mini Trans-Blot system (Bio-Rad) *for nNOS* for 80 min and *for desmin*, *α-actin*, and *p70S6K* for 25 min. Membranes were blocked in 5% dry milk solution (Bio-Rad) in TBST (4 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20) overnight at 4°C. To reveal protein bands, primary polyclonal antibodies – *anti-nNOS* (BD Biosciences, USA; dilution 1 : 250), and primary monoclonal antibodies – *anti-desmin* (Novocastra; NCL-L-DES-DERII, 1 : 200), *anti-α-actin* (Sigma, USA; 1 : 500), *anti-p70S6K* (Santa Cruz, USA; 1 : 10,000), *anti-P-p70S6K* (Abcam, USA; T389 phosphorylation region, 1 : 10,000) were used. The secondary antibodies (GAR, IMTEK, 1 : 1000) conjugated with horseradish peroxidase were used *for nNOS*; biotinylated secondary antibodies (GAM, IMTEK, 1 : 500) and (GAR, Sigma; 1 : 200,000) were used *for desmin and α-actin* and *for p70S6K and P-p70S6K*, respectively. Then blots with desmin and α-actin were incubated with avidin–peroxidase conjugate (Sigma, 1 : 1000) for 30 min and following development using 3% hydrogen peroxide solution in TBST with diaminobenzidine; p70S6K, P-p70S6K were incubated with streptavidin–peroxidase conjugate (Sigma, 1 : 10,000) for 1 h. The *nNOS*, *p70S6K*, and *P-p70S6K* were revealed on the film using the Immun-Star™ Substrate Kit (Bio-Rad Laboratories). Incubations with antibodies were carried out for 1 h at room temperature. Blots were washed 6 times, 10 min each, in TBST. Western blotting was repeated at least three times. Protein bands were analyzed using a GS-800

densitometer (Quantity-One™ software; Bio-Rad). All image densities were measured in linear range of developing reagent, scanner, and XR-film. Optical absorption (OA) of the control group band on analytical membrane was taken as 100%, while OA of different groups was compared with that of control group bands localized on the same membrane. Changes expressed on a percentage basis relative to control were calculated for each group as $M \pm m$.

Analysis of gene expression. Total RNA was extracted from 10 mg frozen *m. soleus* using an RNeasy Micro Kit (Qiagen, Germany). All RNA samples were treated with proteinase K and DNase I. RNA concentration was determined at 260 nm. Then aqueous solution of isolated RNA was frozen at −84°C for subsequent reverse transcription. Aqueous solution of 1 μg RNA, oligo$(dT)_{15}$, random hexanucleotides $d(N)_6$, and MMLV reverse transcriptase were used for reverse transcription. Reverse transcription was carried out for 60 min at 37°C according to the standard protocol. Obtained cDNA samples were placed for storage at −84°C for real time PCR. All samples were analyzed no less than three times and all reactions were measured using detecting amplificatory iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad), and β-actin and GAPDH were used as reference genes. Primers used for real time PCR are shown in Table 1. The following formula was used to estimate relative changes in specific gene expression level in the sample:

$$\Delta C_t = C_{t(\text{ref})} - C_{t(\text{test})},$$

where $C_{t(\text{ref})}$ is the point of the basic line and reference gene amplification graph intersection in the sample, while $C_{t(\text{test})}$ is the point of basic line and the studied gene amplification graph intersection in the same sample. Then we determined in each group mean ΔC_t value and its standard error. The change in the analyzed gene expression level in experimental groups was estimated relative to control level according to the formula:

$$\Delta\Delta C_t = \Delta C_{t(\text{group})} - \Delta C_{t(\text{control})}.$$

Statistical single-factor analysis ANOVA was carried out. All results are given as $M \pm m$. Authenticity of differences between groups was determined using the unpaired Student t-criterion at the null hypothesis probability $p < 0.05$.

RESULTS

Mass of rats in all groups did not differ from the control (Table 2), which is indicative of absence of stress in the animals during the experiment. The *m. soleus* mass was decreased in the suspended group HS by 46% ($p < 0.05$) relative to that in control group. At the same time, in the group with L-arginine administration (HSL), muscle atrophy was prevented in part and its mass by 21% exceeded that in group HS ($p < 0.05$) (Table 2). Similar results indicative of the efficiency of L-arginine administration were obtained for CSA of type I and II muscle fibers (MBI and MBII CSA). In group HS they were decreased by 45 and 28%, respectively ($p < 0.05$) compared to those in control group ($p < 0.05$, Table 2), whereas CSA of MBI and MBII fibers in the HSL group significantly exceeded the corresponding values in group HS (by 25 and 16% ($p < 0.05$), respectively). Note that MB II atrophy in HSL group was completely prevented (Table 2).

NO content in *m. soleus*. In the group of rats suspended with L-arginine administration the relative NO content in *m. soleus* did not differ from that in the control group, while in animals suspended without introduced arginine the NO content was 60% lower ($p < 0.05$) than in control animals (Table 2).

nNOS content. The nNOS protein content both in HS and in HSL groups was significantly lower than in control group (by 66.0 ± 6.0 and 57.0 ± 6.5%, respectively ($p < 0.05$)). Changes in its mRNA content showed a similar trend (Fig. 2), i.e. administration of L-arginine did not prevent the decrease in intramuscular nNOS content.

Content of Hsp90β mRNA. Hsp90β proteins fulfill several functions including protection of some proteins against destruction and involvement in their conformational changes [16]. After suspension for 14 days, the Hsp90β content in HS group was significantly lower than that in control group ($p < 0.001$). In contrast, in the group of animals with introduced NO precursor no decrease in

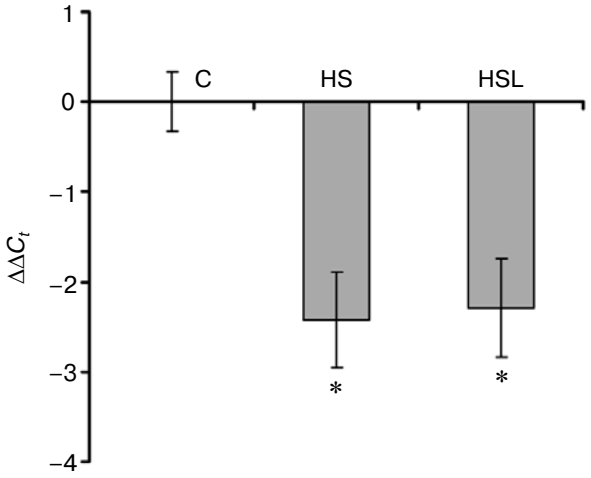

Fig. 2. nNOS mRNA content in *m. soleus* in suspended (HS) and suspended with L-arginine administration (HSL) rats relative to control (C) (standard Lebacque method ($\Delta\Delta C_t$ technique)). Here and in Figs. 3, 5-9 results are given as $M \pm m$ ($n = 7$ for each group). * Significant differences from control ($p < 0.01$).

Table 1. Characteristics of primers

Gene name	Sequence (5′→3′)	mRNA sequence from NCBI database	Position, bp	Product length, bp	T_m, deg
β-Actin	5-TCATGAAGTGTGACGTTGACATCC-3 5-GTAAAACGCAGCTCAGTAACAGTC-3	NM_031144.2	926-949 1213-1236	311	60
GAPDH	5-ACGGCAAGTTCAACGGCACAGTCAA-3 5-GCTTTCCAGAGGGGCCATCCACA-3	NM_017008.2	1001-1025 1404-1426	426	60
Type I MHC	5-ACAGAGGAAGACAGGAAGAACCTAC-3 5-GGGCTTCACAGGCATCCTTAG-3	NM_017240.1	5570-5594 5837-5857	288	60
Type IIa MHC	5-TATCCTCAGGCTTCAAGATTTG-3 5-TAAATAGAATCACATGGGGACA-3	NC_005109.2	5672-5693 5963-5984	310	60
Type IIb MHC	5-CTGAGGAACAATCCAACGTC-3 5-TTGTGTGATTTCTTCTGTCACCT-3	XM_340818.3	5761-5780 5957-5979	197	60
Type IId/x MHC	5-CGCGAGGTTCACACCAAA-3 5-TCCCAAAGTCGTAAGTACAAAATGG-3	XM_001078018.1	5890-5907 6009-6033	120	60
nNOS	5-AGTCCCCTGCTTCGTGAGAG-3 5-CACCCGAAGACCAGAACCAT-3	NM_052799.1	3872-3891 4051-4070	180	61.1
Atrogin-1/MAFbx	5-CTACGATGTTGCAGCCAAGA-3 5-GGCAGTCGAGAAGTCCAGTC-3	NM_133521.1	153-172 302-321	169	61.1
IGF-1	5-TCTGAGGAGGCTGGAGATGT-3 5-GTTCCGATGTTTTGCAGGTT-3	NM_178866.2	1018-1037 1257-1276	240	60
MuRF-1	5-GCCAATTTGGTGCTTTTTGT-3 5-AAATTCAGTCCTCTCCCCGT-3	NM_080903.1	1439-1458 1633-1652	214	60
Hsp90β	5-GAGGCAGAGGAAGAGAAAGG-3 5-ATGGGCTTCGTCTTATTCAG-3	NM_001004082.3	730-749 889-908	179	61.1

Note: β-Actin and GAPDH are reference genes; MHC, myosin heavy chains; nNOS, neuronal NO synthase; HSP90β, heat shock protein 90 (β isoform; IGF-1, insulin-like growth factor 1; upper line is forward primer; lower line is reverse primer.

Table 2. Characteristics of rats and their *m. soleus*

Parameter	C	HS	HSL
Mass of rat, g	253 ± 9	246 ± 10	249 ± 7
Mass of *m. soleus*, mg	101 ± 4	55 ± 2*	67 ± 5*#
Relative NO content	1.00 ± 0.15	0.40 ± 0.11*	0.89 ± 0.09
MFI CSA, μm^2	2085 ± 159	1154 ± 60*	1547 ± 59*#
MFII CSA, μm^2	1737 ± 174	1249 ± 80*	1495 ± 75

Note: MFI and MFII, type I and II muscle fibers. CSA, cross section area.
* Differences from control group are significant at $p < 0.05$.
# Differences from suspension group are significant at $p < 0.05$.

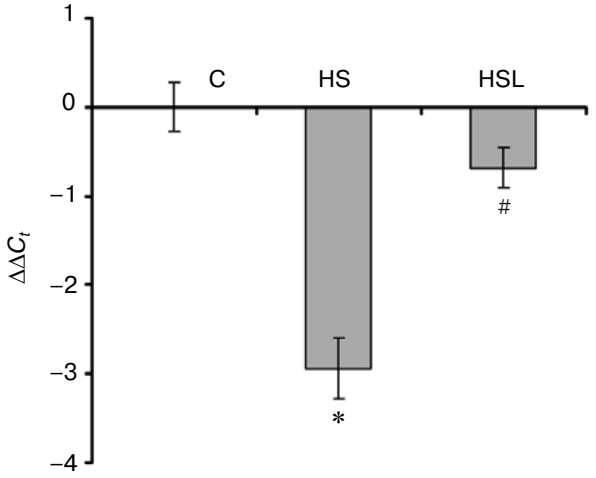

Fig. 3. Content of heat shock protein 90β (Hsp90β) mRNA in *m. soleus* in suspended (HS) and suspended with L-arginine administration (HSL) rats relative to control (C) (standard Lebacque method ($\Delta\Delta C_t$ technique)). * Significant differences from control ($p < 0.001$); # significant differences from HS ($p < 0.001$).

Hsp90β content was observed upon muscle functional unloading (Fig. 3).

Percentage of fibers having breaks in dystrophin layer. We detected significant increase in number of fibers with breaks in the dystrophin layer in suspended animals (39 ± 5%) compared to that in control group (9 ± 2%, $p < 0.05$). Administration of L-arginine completely prevented these changes, and the number of fibers with breaks in the dystrophin layer in these rats did not differ from that in control animals, being 14 ± 6% (Fig. 4).

Desmin and α-actin content in *m. soleus*. Desmin and α-actin content in *m. soleus* in the suspended groups (HS, HSL) did not differ from that in control group (Fig. 5). However, in *m. soleus* of animals with introduced NO precursor L-arginine the content of both proteins was significantly higher (by 24%) than in the HS group ($p < 0.05$).

Expression of atrogin-1 and MuRF-1 mRNA in *m. soleus*. The level of mRNA of E3 ligases atrogin-1 and MuRF-1 responsible for proteolytic degradation of proteins significantly increased in the HS group ($p < 0.001$) (Fig. 6) compared to control. In contrast, in rats with introduced L-arginine the content of mRNA of these ligases was the same as in control. This correlates with the above-mentioned data on degradation of cytoskeletal proteins and partial prevention of muscle atrophy.

Content of P70/S6k (phosphorylated and unphosphorylated P70S6 kinase). Ribosomal kinase p70 (p70S6K) regulates protein synthesis intensity at the level of translation initiation. Activated (phosphorylated) p70S6K kinase phosphorylates a number of translation initiation factors and ribosomal S6 protein, thus leading to activation of protein synthesis. The content of P70/S6k (phosphorylated and unphosphorylated) was significantly decreased in the HS group ($p < 0.05$) relative to that in both control and HSL group (Fig. 7). The p-S6K/S6k ratio was identical in all groups.

IGF-1 content in *m. soleus*. In the experimental groups intramuscular IGF-1 content did not differ from that in control.

Expression of mRNA of type I and IIa,b,d/x heavy myosin chains. L-Arginine administration for suspended animals prevented the decrease in MHC I mRNA expression (Fig. 8) observed in the HS group. Administration of L-arginine had no effect on expression level of MHC II isoform mRNAs, but in groups HS and HSL it was decreased (Fig. 9) compared to the control.

DISCUSSION

We discovered for the first time significant decrease in NO content in *m. soleus* after 2 weeks of hindlimb unloading (group HS) (Table 2). Earlier an increase in NO concentration in intact muscle upon contraction was reported [17], while other authors proved in experiments

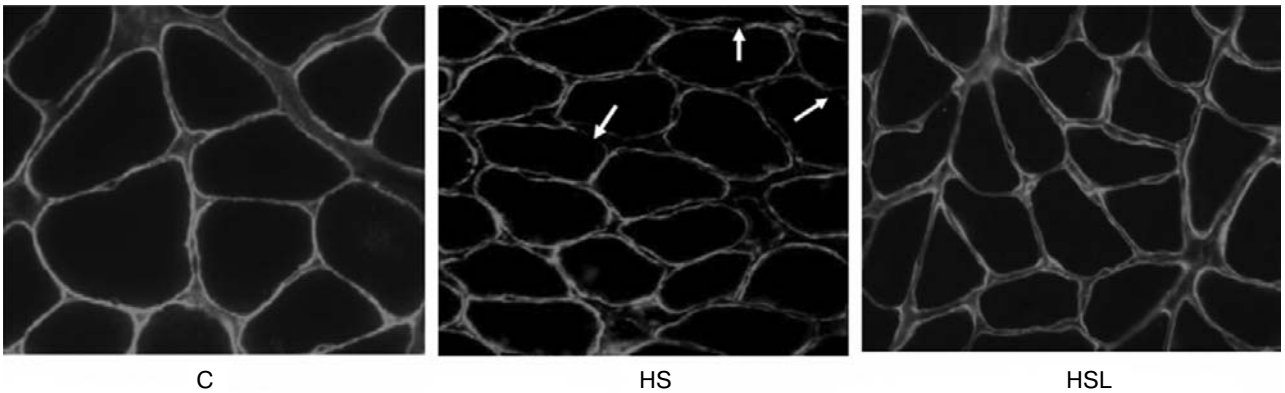

Fig. 4. Cross sections of *m. soleus* of control, suspended, and suspended with L-arginine administered rats. Breaks in dystrophin layer are shown by arrows.

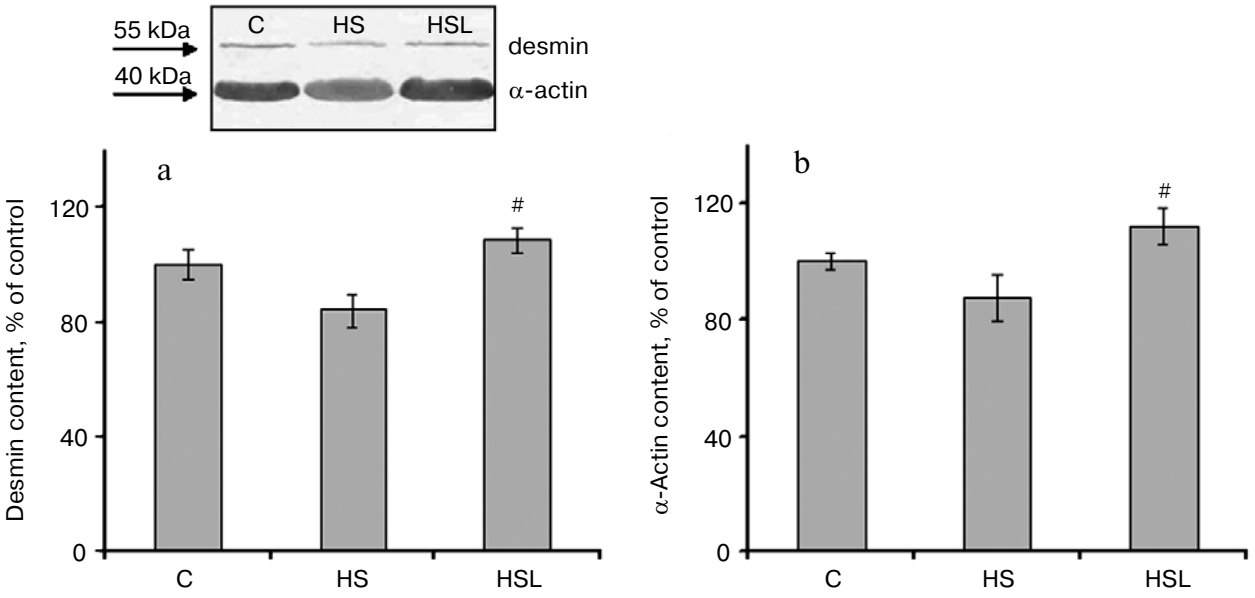

Fig. 5. Content of cytoskeletal protein desmin (a) and myofibrillar protein α-actin (b) in *m. soleus* in suspended (HS) and suspended with L-arginine administration (HSL) rats comparing to the content of the corresponding proteins in control (C). Aliquots of the *m. soleus* soluble fraction (20 μg/lane) were obtained, separated by electrophoresis, proteins on nitrocellulose membrane were revealed, and immunoblots were analyzed as described in "Materials and Methods". An example of an immunoblot for desmin and α-actin is shown. # Significant difference from HS ($p < 0.05$).

on single muscle fibers that NO can be produced inside the fiber (but not only in surrounding tissues) and regulated due to its contractile activity [18]. The administration of L-arginine prevented decrease in relative NO content in the HSL group (Table 2). Simultaneously with decrease in relative NO content in *m. soleus* of HS group rats, we detected a decrease in nNOS (both protein and mRNA) content (Fig. 2), which was quite expectable. Some authors have reported decrease in the level of human neuronal NO synthase in *m. soleus* upon hypokinesia [9, 10]. It was unexpected that the nNOS (both protein and mRNA) content in HSL group decreased like in the HS group. First, we believe that discrepancies in nNOS levels in HS and HSL groups and amounts of produced NO in them can be due to possible differences in enzyme activities and concentration in these groups. A similar situation was registered during the long-term increase in muscle contractile activity: nNOS concentration did not change, while enzyme activity measured by the rate of L-[^{14}C]arginine conversion to L-[^{14}C]citrulline was 1.5 times higher compared to that in the control group [19]. Second, the difference in NO and nNOS concentrations in HS and HSL groups might be due to differences in Hsp90 concentration in these groups. In animals of the HSL group, the content of Hsp90 mRNA did not differ from that in the control group, while in the HS group it was reliably lower (Fig. 3). Similar decrease in both Hsp90 protein and its mRNA concentrations after two-week-long functional unloading of *m. soleus* was detected earlier [20, 21]. It is known that nNOS forms a complex with Hsp90 chaperons and only in this state its complete catalytic activity and stability are preserved [22, 23]. Hsp90 increases NO formation via enhancement of nNOS catalytic function in intact cells *in vivo*, depending on its concentration [24]. Within the complex with Hsp90, nNOS is also more resistant to proteolytic degradation by calpain, whose concentration significantly increases in *m. soleus* of animals upon functional unloading [25]. The

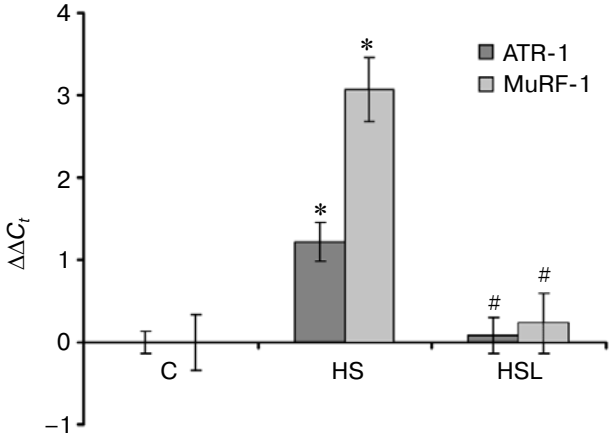

Fig. 6. Content of atrogin-1 (ATR-1) and MuRF-1 mRNA in *m. soleus* in HS and HSL rats relative to control (C) (standard Lebacque method ($\Delta\Delta C_t$ technique)). * Significant differences from control ($p < 0.001$); # significant differences from HS ($p < 0.005$).

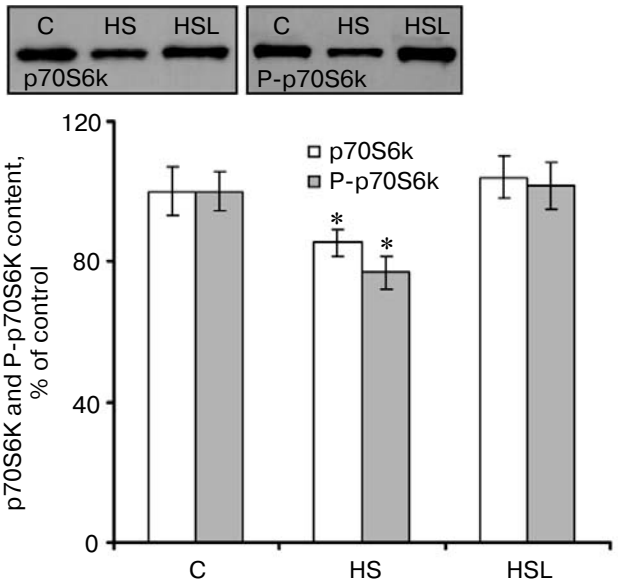

Fig. 7. Content of protein synthesis system components (unphosphorylated p70S6K and phosphorylated p70S6K) in *m. soleus* of suspended (HS) and suspended with L-arginine administration (HSL) rats relative to the content of corresponding proteins in control (C). Aliquots of *m. soleus* soluble fraction (15 μg per lane) were obtained, separated by electrophoresis, proteins on nitrocellulose membrane were detected, and immunoblots were analyzed as described in "Materials and Methods". Examples of immunoblots of p70S6K and phosphorylated p70S6K (P-p706K) are shown. * Significant differences from control ($p < 0.05$).

efficiency of nNOS protection is in proportion with the level of intracellular expression of Hsp90 [23].

In the group of rats (HSL) with L-arginine administration during suspension, a lower extent of muscle fiber atrophy and CSA is observed (Table 2). Koh and Tidball [26] found during investigation of muscle fiber culture that NO is involved in development of skeletal muscle hypertrophy via decrease in protein degradation and increase in protein synthesis and showed for the first time that NO inhibits proteolysis caused by calcium ionophores in muscle cell culture. This is due to the inhibition of m-calpain activity by NO after chemical modification of a cysteine residue in the enzyme active center. It is known that in unloaded muscle, calcium ions are accumulated in the cytoplasm [4]. The decreased atrophy in the HSL group could also be due to inhibition of calpain activities by NO. The decrease in the content of contractile proteins makes the most pronounced contribution to the development of atrophy, but destruction of cytoskeletal proteins significantly influences changes in muscle tonus and contractile properties of the muscle [1-3]. The effect of L-arginine administration on prevention of cytoskeletal protein destruction upon muscle unloading was not fully studied.

We found that the number of muscle fibers with destruction in the dystrophin layer decreased significantly in the HSL group, unlike those in the HS group (Fig. 4). The decrease in dystrophin content is usually accompanied by changes in membrane permeability. The extreme case, absence of dystrophin, is lethal in patients with Duchenne's childhood muscular dystrophy. Dystrophin destruction in *m. soleus* upon suspension was earlier shown by different authors [1, 27]. There are also reports concerning enhancement of utrophin (synthesized instead of dystrophin) synthesis upon L-arginine administration to mdx mice, which increases the extent of its binding to sarcolemma [6, 7]. We detected a similar

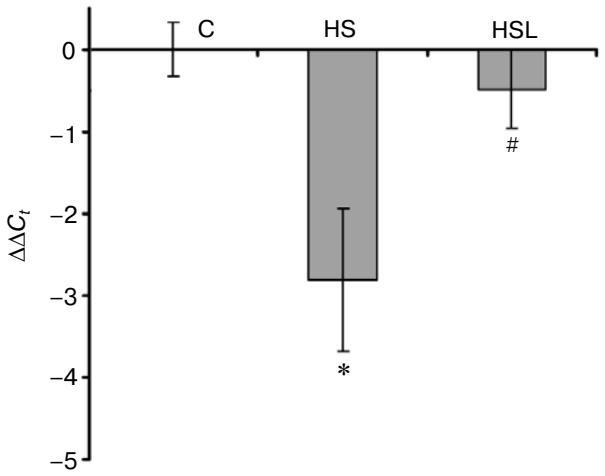

Fig. 8. Content of type I myosin heavy chain mRNA in *m. soleus* of suspended (HS) and suspended with L-arginine administration (HSL) rats relative to control (C) (standard Lebacque method ($\Delta\Delta C_t$ technique)). * Significant differences from control ($p < 0.05$); # significant differences from HS ($p < 0.05$).

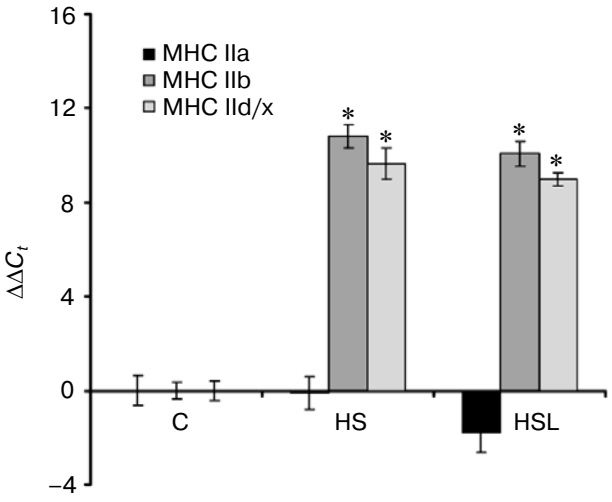

Fig. 9. Content of type IIa, IIb, IId/x myosin heavy chain mRNA in *m. soleus* of suspended (HS) and suspended with L-arginine administration (HSL) rats relative to control (C) (standard Lebacque method ($\Delta\Delta C_t$ technique)). * Significant differences from control ($p < 0.001$).

effect of L-arginine on dystrophin in unloaded muscle. The desmin and α-actin levels in the HS group were slightly lower than in the control group. Some authors report decrease in desmin and α-actin (both protein and mRNA) concentrations at early stages of suspension of rats (from 1 to 8 days) [28-31]. However, already after three and six weeks of suspension in rats (like after long-term hypokinesia in man) authors of works [1, 32] found no changes in the content of these substances. This suggests that the concentration of such cytoskeletal proteins as desmin and α-actin decreases during the first several days of muscle functional unloading and then, at later stages, is restored. Thus, we could not detect reliable decrease in their content after two-week-long rat suspension. At the same time, in animals of the HSL group the content of such proteins exceeded by 24% that in the HS group (Fig. 5). There are reports concerning the ability of NO to activate synthesis of some cytoskeletal proteins (talin and vinculin) in C2C12 cells [26]. This suggests that L-arginine administration (and probably, maintenance of NO level) upon unloading prevents decrease in concentration of some cytoskeletal proteins in *m. soleus* and decreases the extent of muscle atrophy.

To elucidate the mechanism of these processes in the HSL group, we tested the function of the proteasomal degradation system (content of E3 ligases atrogin-1 and MuRF-1) and protein synthesis (a unit of Akt-mTOR-S6K signal pathway), as well as the content of insulin-like growth factor in *m. soleus*. It is known that the first disturbances in unloaded muscle are caused by lowered protein synthesis followed by accelerated proteolysis [33], which plays an important role in the loss of muscle mass. Such E3 ligases as atrogin-1 and MuRF-1 involved in proteasomal degradation of proteins undergo especially intensive activation upon unloading of muscle [33-35]. Just for this reason, they were chosen as markers of proteasomal degradation in our work. Relative content of their mRNA increases in suspended rats (group HS) compared to control (Fig. 6). In contrast to this, it does not differ from that in animals administered L-arginine.

It has recently been shown that MuRF-1 is involved in ubiquitination of thick myofilament components by participation in their degradation [11]. The decrease in MuRF-1 expression in the HSL group could be due to lower extent of muscle atrophy in it. Hsp90 is also known to be involved in proteasomal degradation. They form stable complexes with many "client" proteins and thus prevent further chaperon-dependent ubiquitination [36]. In the HSL group, where partial prevention of atrophy was observed, the concentration of Hsp90 mRNA significantly exceeded that in the HS group. Hsp90 could also fulfill a protective function against proteasomal degradation of proteins in this group. It was previously shown that increase in NO concentration in unloaded muscle results in prevention of destruction of some cytoskeletal proteins, including decreased proteasomal degradation [8]. No doubt, one of reasons for prevention of atrophy and decrease in concentration of some cytoskeletal proteins in HSL group is their lowered degradation.

The intensity of anabolic signal processes in this group was checked in our laboratory by determination of the content of phosphorylated and unphosphorylated P70S6K. Akt-mTOR-S6K is well known as the main pathway of protein synthesis regulation at the level of translation initiation [37]. The decrease in S6K phosphorylation level in *m. soleus* on the 14th day of suspension was already shown before [38], whereas we detected the maintenance of its concentration upon L-arginine administration for the first time (Fig. 7). Since the p-S6K/S6K ratio was the same in all groups, we supposed that the decrease in S6K phosphorylation level upon suspension was due to decrease in total kinase content due to intensification of proteolysis in muscle fiber. This suggests that the decrease in protein synthesis upon suspension takes place at the level of translation initiation, whereas peroral administration of L-arginine prevents this process by preserving the total S6K content and phosphorylation at the level of control. We also studied intramuscular content of the IGF-1 mRNA upon muscle unloading (its anabolic properties are known) [39]. However, in most works the topic is IGF-1 of blood. Signal pathways launched by IGF-1 expressed in muscle upon unloading have not been studied. In our experiment, its concentration was the same in all groups. The ambiguous dynamics of IGF-1 was noted earlier. Thus after suspension of mice for two days its intramuscular concentration decreased, and by the eighth day it did not differ from the control [40]. The anabolic role of IGF-1 upon unloading requires further investigation. We conclude that differences in the muscle mass or content of cytoskeletal proteins in groups HS and HSL can be attributed both to the higher anabolic activity and decreased expression of proteolytic markers (atrogin-1/MAFbx, MuRF-1) after L-arginine administration during hindlimb suspension.

In animals of HS group, the decrease in MHC type I mRNA expression and increase in expression of type IIb and IId/x MHC compared to those in control was observed (Figs. 8 and 9). Similar redistribution of MHC isoforms towards higher content of type II myosins was described earlier [2]. Administration of L-arginine (the HSL group) completely prevented these changes (Figs. 8 and 9). It was noted in work [41] that administration of NO-synthase blocker (L-NAME) upon functional overloading of muscle prevents muscle fiber "transformation" towards higher content of type I fibers. Phenotypic plasticity of muscle can be regulated by endogenous NO via calcium-calmodulin-dependent NO synthase [42]. The Ca^{2+}/calmodulin pathway is one of the possible regulators of MHC I synthesis in skeletal muscle [43]. However, neuronal NOS also requires calmodulin for NO production [23, 44]. One of the stimuli causing changes in MHC expression in our experiment might be activation of the

Ca^{2+}/calmodulin pathway upon muscle functional unloading and its interaction with nNOS.

Thus, L-arginine administration upon hindlimb suspension decreases the extent of *m. soleus* atrophy and prevents decrease in the content of some cytoskeletal proteins and in MHC I mRNA. To some extent, the decrease in atrophy could be stimulated by the absence of increase in the level of proteasomal degradation components upon muscle unloading (atrogin-1/MAFbx, MuRF-1) and maintenance of intensity of protein synthesis by ribosomal p70S6 kinase.

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