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Role of NO-Synthase in Regulation of Protein Metabolism of Stretched Rat *m. soleus* Muscle during Functional Unloading

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Abstract—Gravitational unloading causes atrophy of muscle fibers and can lead to destruction of cytoskeletal and contractile proteins. Along with the atrophic changes, unloaded muscle frequently demonstrates significant shifts in the ratio of muscle fibers expressing fast and slow myosin heavy chain isoforms. Stretching of the *m. soleus* during hindlimb suspension prevents its atrophy. We supposed that neuronal NO-synthase (NOS) (which is attached to membrane dystrophin–sarcoglycan complex) can contribute to maintenance of protein metabolism in the muscle and prevent its atrophy when *m. soleus* is stretched. To test this hypothesis, we used Wistar rats (56 animals) in experiments with hindlimb suspension during 14 days. The group of hindlimb suspended rats with stretched *m. soleus* was injected with L-NAME to block NOS activity. We found that *m. soleus* mass and its protein content in hindlimb-suspended rats with stretched *m. soleus* were preserved due to prevention of protein degradation. NOS is involved in maintenance of expression of some muscle proteins. Proliferation of satellite cells in stretched *m. soleus* may be due to nNOS activity, but maintenance of muscle mass upon stretching is regulated not by NOS alone.

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The long-term functional unloading of skeletal muscle is known to be responsible for their atrophy and results in destruction of cytoskeletal and contractile proteins [1, 2]. The extent of destruction differs for different cytoskeletal, membrane, and contractile proteins, but already after 14 days of unloading, *m. soleus* atrophy reaches 40-45%. Contractile proteins make the largest contribution to the muscle mass reduction [1, 2]. Besides, expression in *m. soleus* of fast myosin heavy chain (MHC) isoforms increases, which alters its locomotor functions. However, *m. soleus* stretching upon unloading prevents development of these processes [3, 4]. Cell factors triggering these changes are still poorly studied.

We supposed that neuronal NO synthase (nNOS) may contribute to maintenance of muscle protein metabolism and to prevention of atrophy in the case of *m. soleus* stretching. Our hypothesis was based on some facts found in previous works. Thus, it was shown in 2000 in an experiment on C2C12 cell culture that the NO donor nitroprusside can prevent destruction of the muscle cell cytoskeleton by inhibition the proteolysis of thalline protein by calpain [5]. It is known that accumulation of calcium in muscle fibers occurs upon muscle functional unloading [6, 7]. In some works, a correlation between change in calcium content and calpain activation [8], resulting in protein degradation, was shown. We paid attention to these investigations because along with increased calcium concentration, a decreased content of nNOS during long-term decrease in muscle motor

Abbreviations: atrogin-1/MAFbx, atrogin-1/Muscle Atrophy F-box; CSA, cross section area; DETC, diethyldithiocarbamate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HS, hindlimb-suspended rats; HSS, hindlimb-suspended rats with stretched *m. soleus*; HSSN, hindlimb-suspended rats with stretched *m. soleus* and L-NAME injection; Hsp90β, 90β heat shock proteins; L-NAME, N-nitro-L-arginine methyl ester hydrochloride; MF, muscle fiber; MHC, myosin heavy chain(s); mTOR, mammalian target of rapamycin; MuRF-1, muscle-specific RING finger protein 1; (n)NOS, (neuronal) NO synthase; p70S6K, p70S6 kinase; P-p70S6K, phosphorylated form of p70S6 kinase.

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activity and muscle protein destruction was shown [9, 10].

We showed earlier that during muscle unloading NO level decreases, and NO may be a signal molecule involved in regulation of protein metabolism [11]. The activation of NOS and increased NO production may be a mechanism preventing atrophy of unloaded muscle fiber when it is stretched. Nitrogen oxide prevents many effects of calcium ionophores on C2C12 muscle cells, including decreasing destruction of vinculin, proteins of intercellular contacts, and total protein content. Koh and Tidball [5] were the first to show that NO inhibits proteolysis caused by calcium ionophores in muscle cell culture. NO is produced endogenously in skeletal muscle. Pye et al. [12] determined that upon contraction of a single fiber the NO content in it increases by 48%. This increase is not observed after introduction of a NOS inhibitor. It was found that NO is produced in C2C12 skeletal muscle cells upon their static or dynamic stretching and regulates calpain-activated degradation of cytoskeletal proteins [5]. Zhang et al. [13] repeated this experiment with some modifications and found that in the case of 10% stretching of C2C12 cells, the NO content and NOS activity increase along with synthesis of thalline, vinculin, and desmin with simultaneous increase in the fiber rigidity. These effects are enhanced by introduction of the NO donor L-arginine or by inhibition of calpain and are blocked by introduction of the NOS inhibitor N-nitro-L-arginine methyl ester hydrochloride (L-NAME). The blocking of NOS by introduction of L-NAME was also studied in *in vivo* experiment on rats, and prevention of hypertrophy of overloaded *m. plantaris* was found [14].

All these works demonstrate a clear connection between metabolism of cytoskeletal proteins, NO production, and NOS activity in muscle fibers. Previous investigations were carried out using tissue culture, but their results make it possible to draw some analogy with *in vivo* effects observed in muscle upon stretching.

The goal of this work was to test the hypothesis that NOS is involved in prevention of unloaded muscle atrophy upon its stretching by blocking NOS with L-NAME. For this, we tested the state of the most important cytoskeletal (desmin, dystrophin, and α-actin) and contractile (myosin heavy chains (MHC)) proteins in unloaded muscle, both stretched and stretched with administration of NOS inhibitor. These proteins provide for integration of intracellular structures of muscle fiber and the muscle contractile function. It is interesting to note that muscle stretching upon its unloading completely prevents MHC type I transformation to type II [1]. Perhaps NOS is involved in the regulation of this process. To estimate the state of muscle protein metabolism, we tested the system of proteasome degradation (E3 ligase) and protein synthesis (Akt-mTOR-S6K signal pathway). Atrogin-1 and MuRF1 were chosen as proteolysis markers because earlier growth of just these E3-ligases was registered upon muscle functional unloading [15]. Some authors associate changes in muscle mass with the presence of myonuclei and myosatellites [16, 17].

We demonstrated earlier a decrease in NO level upon muscle unloading [11]. The decrease in NO concentration decreases the proliferation of myosatellites [18]. To test the hypothesis concerning the relationship between NOS activation upon stretching, myosatellite proliferation, and muscle mass maintenance, we identified myosatellites. If the hypothesis is valid, blocking the NOS activity of unloaded muscle upon its stretching should not prevent muscle atrophy.

MATERIALS AND METHODS

Experiments were carried out in accordance with the rules of biomedical ethics (protocol No. 264, March 5, 2009, was certified by the Russian Academy of Sciences Committee on Bioethics). The animals were kept at 22°C with free access to water and food. Fifty six 2.5-month-old Wistar rat males of 220-240 g were divided into four groups (14 animals in each): intact control (group C, body mass 253.0 ± 9.2 g, *m. soleus* mass 101.3 ± 4.0 mg); the second group of rats was hindlimb-suspended for 14 days in such a way that the forelimbs were touching the floor (group HS, body mass 246.0 ± 10.4 g, *m. soleus* mass 55.0 ± 2.1 mg) [19]. The two other groups were also hindlimb-suspended for 14 days, but with *m. soleus* stretching (both hindlimbs were immobilized at the ankle joint with an angle of 35°): group 3 was hindlimb-suspended with *m. soleus* stretching (group HSS, body mass 255.3 ± 6.4 g, *m. soleus* mass 93.2 ± 6.1 mg); group 4 was hindlimb-suspended with *m. soleus* stretching (similarly to group 3) and L-NAME administration (L-NAME daily with drinking water, 90 mg/kg rat body mass) (group HSSN, body mass 233.3 ± 12.3 g, *m. soleus* mass 101.2 ± 3.4 mg). Stretching of *m. soleus* during hindlimb suspension was performed as described earlier [20]. The rats were sacrificed by intraperitoneal Nembutal overdose (75 mg/kg), and the *soleus* muscles were immediately frozen in liquid nitrogen. Seven animals from each group were used to determine the NO relative content in *m. soleus* (samples were kept in liquid nitrogen), while *m. soleus* of the other seven animals were used for other investigations (samples were kept at −85°C). Cross sectional areas (CSA) of type I and II muscle fibers were determined by revealing MHC along with immunohistochemical detection of dystrophin on the muscle cross sections; desmin and α-actin were determined by immunoblotting; nNOS (neuronal NO synthase) was determined by immunoblotting and real-time PCR; the levels of atrogin-1 and MuRF1 mRNA as well as mRNA of type I, IIa, IIb, and IId/x MHC and of Hsp90β were determined by real-time PCR.

Determination of relative intramuscular nitrogen oxide content. Relative intramuscular nitrogen oxide (NO) was determined using a standard spin trap and electron paramagnetic resonance technique (EPR) [21, 22]. We used diethyldithiocarbamate (DETC) as a spin trap that forms in tissues nitrosyl paramagnetic complexes with iron, which are in equilibrium with stationary NO concentration within the tissue and have a characteristic EPR spectrum. The spin trap was introduced into the rat at the rate of 500 mg/kg body weight. Immediately after introduction of DETC, aqueous solution of 29 mM $FeSO_4$ mixture with 116 mM sodium citrate (2 ml/kg body weight) was injected intramuscularly. After 30 min, the animals were decapitated, *m. soleus* was frozen in liquid nitrogen, and the EPR signal was registered on a Bruker EMX-8 EPR spectrometer. The EPR signal is a superposition of signal of the $NO\text{-}Fe^{2+}(DETC)_2$ complex as well of other paramagnetic centers identified in tissues. The EPR signal in *m. soleus* is shown in our previous paper [11].

Detection of dystrophin and MHC. Sections of muscle tissue 10-μm thick prepared using a cryostat microtome were incubated with primary antibodies to dystrophin or type I MHC or type II MHC (primary antibodies were diluted 1 : 20 (Novocastra, NCL-DYSI, USA), 1 : 60 (NCL-MHCs, DSMZ, Germany), 1 : 40 (sc-75)) and with secondary antibodies (1 : 1000 (GAM, IMTEK, Russia), Alexa 546, and Alexa 350 (Molecular Probes, Switzerland)). All samples were incubated for 1 h at room temperature. A negative control (without primary antibodies) was carried out for each reaction to reveal nonspecific staining. The percentage of fibers with damaged dystrophin layer was counted on the section. Myonuclei were identified using DAPI (4,6-diamidino-2-phenylindole-dihydrochloride) from MP Biomedicals, USA (dilution 1 : 700). The numbers of nuclei per muscle fiber were counted. Myosatellite cells were revealed using satellite marker M-cadherin (Santa Cruz, USA, sc6740, dilution 1 : 200; secondary FITC-conjugated antibodies, IMTEK, Russia, dilution 1 : 150). The numbers of myosatellites per muscle fiber were counted.

Polyacrylamide gel electrophoresis. Sections 20-μm thick were made from each *m. soleus* sample (10-15 mg) and immediately homogenized for 25 min in 100 μl of buffer. We described detailed buffer composition for nNOS, desmin, α-actin, p70S6K, and P-p70S6K analysis in a previous paper [11]. Samples for nNOS, desmin, and actin were heated at 95°C for 2 min. Part of the supernatant was taken for determination of total protein concentration using the RC DC Protein Assay (Bio-Rad Laboratories, USA); the remaining volume was used for analysis by electrophoresis. All samples were stored at −84°C. Electrophoresis was carried out in 8% polyacrylamide gel for nNOS and in 12% polyacrylamide gel for desmin and α-actin; for p70S6K 10% separating and 5% concentrating polyacrylamide gels were used. Samples were applied at the rate of 40 μg for nNOS, 20 μg for desmin and α-actin, and for p70S6K 15 μg total protein in each lane. Samples of each group were applied to the gel with control samples. Electrophoresis was carried out at 15 mA/gel in a Bio-Rad mini-system at room temperature.

Western blotting. Protein was transferred as described in the earlier paper [11]. Primary polyclonal anti-nNOS antibodies (BD Biosciences, England; dilution 1 : 250) and primary anti-desmin (Novocastra, NCL-L-DES-DERII; 1 : 200), anti-α-actin (Sigma; 1 : 500), anti-p70S6K (Santa Cruz; 1 : 10,000), and anti-P-p70S6K (Abcam, USA; phosphorylation site T389, 1 : 10,000) monoclonal antibodies were used. The following secondary antibodies were used: horseradish-conjugated for nNOS (GAR, IMTEK; 1 : 1000), biotinylated for desmin and α-actin (GAM, IMTEK; 1 : 500), biotinylated for p70S6K and P-p70S6K (GAR, Sigma; 1 : 200,000). Then blots with desmin and α-actin were incubated with avidin–peroxidase conjugate (Sigma; 1 : 1000) for 30 min and developed in 3% hydrogen peroxidase solution in TBST (Tris-buffered saline, pH 7.4, and 0.1% Tween 20) with diaminobenzidine; p70S6K and P-p70S6K were incubated with streptavidin–peroxidase conjugate (Sigma; 1 : 10,000) for 1 h; nNOS, p70S6K, and P-p70S6K were developed on film using the Immun-Star Substrate Kit (Bio-Rad). Protein bands were analyzed using a GS-800 densitometer (Quantity-One software; Bio-Rad). All image densities were measured in the linear region of developing reagent, scanner, and X-ray film. Optical density of control group band on the analyzed membrane was taken as 100%, whereas bands of other groups were compared with the control group bands on the same membrane (Fig. 1a). Changes expressed in percent of control were calculated for each group as M ± m (mean ± standard error).

Analysis of gene expression. Reverse transcription. Total RNA was extracted from 10 mg frozen *m. soleus* using the RNeasy Micro Kit (Qiagen, Germany). All RNA samples were treated with proteinase K and DNase I. RNA concentration was determined at 260 nm. For storage, aqueous solution of isolated RNA was frozen to −84°C. For reverse transcription, 1 μg RNA, $oligo(dT)_{15}$, random hexanucleotides $d(N)_6$, and reverse transcriptase MMLV (37°C for 60 min according to standard protocol) were used. cDNA samples for real-time PCR were kept at −84°C. All samples were analyzed at least three times; all reactions were measured using an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). β-Actin and GAPDH were used as reference genes. Primers used for real-time PCR are given in the previous paper [11]. To estimate relative changes in specific gene expression levels in a sample, the following formula was used:

$$\Delta Ct = Ct(ref) - Ct(test),$$

where Ct(ref) is the point of the base line intersection with the reference gene amplification graph in the sam-

ple, while Ct(test) is the point of the base line crossing with the studied gene amplification graph in the same sample. Then the ΔCt mean value and its standard error in each group were determined. The change in the analyzed gene expression level in experimental groups was estimated compared to control level using the formula:

$$\Delta\Delta Ct = \Delta Ct(group) - \Delta Ct(control).$$

Statistical single-factor analysis ANOVA was carried out. All results are given as M ± m (mean ± standard error). The reliability of difference between groups was determined by the unmatched Student *t*-criterion at null hypothesis probability $p < 0.05$.

RESULTS AND DISCUSSION

Rat mass in all groups did not differ from the control, which is indicative of the absence of stress in the animals during the experiments. The administration of L-NAME to the hindlimb-suspended rats with *m. soleus* stretching (HSSN group) resulted in the same deep decrease in NO content compared to the control group (C) ($p < 0.05$) and group HS (table). No such pronounced change was observed in *m. soleus* of group HSS.

The *m. soleus* mass in group HS was decreased by 46% relative to group C as well as NO concentration in this muscle (table). Despite the fact that NO content in *m. soleus* of the group with stretching and nNOS blocking (HSSN group) was decreased compared to group C, the muscle mass did not differ from that in group HSS (table). Results similar to those with *m. soleus* mass were also obtained for MF I and MF II CSA. In group HS the MF I and MF II CSA values decreased by 45 and 28%, respectively ($p < 0.05$), whereas the MF CSA in both groups of suspended animals with *m. soleus* stretching did not differ from that in group C. The percentage of fibers with ruptures in the dystrophin layer was 21 ± 6 ($p < 0.001$), 4 ± 1, and 8 ± 1 ($p < 0.01$) in HS, HSS, and HSSN groups, respectively, compared to 4 ± 1% in the control group. Although in groups with stretching (HSS group) and NOS blocking (HSSN) percentage of fibers with ruptures in dystrophin layer was significantly below that in group HS, these groups (HSS and HSSN) differed significantly from each other ($p < 0.05$). Desmin level in all experimental groups did not differ from that in control (Fig. 1b). However, α-actin content in the HS and HSSN groups was much decreased compared to the HSS group (Fig. 1c). The number of myosatellites, revealed using the M-cadherin marker, was less by 29% in group HS compared to group C (0.25 ± 0.01 against 0.35 ± 0.02 myosatellites/fiber ($p < 0.05$)), whereas in animals of group HSS it exceeded the control value by 68% (0.59 ± 0.06) ($p < 0.05$). Number of myosatellites in the HSSN group did not increase but remained at control level (0.46 ± 0.06).

In the group with *m. soleus* stretching and L-NAME administration (HSSN) the same decrease in nNOS mRNA level compared to control was registered ($p < 0.05$), like in group HS (Fig. 2). This parameter in groups HS and HSSN was also significantly decreased compared to group HSS. It should be noted that NO concentration in both groups (HSSN and HS) had the same dynamics (table). It is interesting that relative content of nNOS protein in group HS was lower, while in group HSSN significantly higher than in control ($p < 0.05$) (Fig. 3).

Concentration of Hsp90 protein mRNA in both groups of hindlimb-suspended rats with *m. soleus* stretching (HSS and HSSN) was reliably higher than that in the group of hindlimb-suspended rats without muscle stretching (HS) (Fig. 4). It should be noted that even in the case of nNOS activity blocking (HSSN group), the Hsp90 mRNA level exceeded that in the HSS group (Fig. 4).

Mass of *m. soleus* and cross sectional areas of muscle fibers (MF CSA)

Parameter	Group			
	C	HS	HSS	HSSN
m. soleus mass, g	101 ± 4	55 ± 2*	93 ± 6	101 ± 3
CSA, μm²				
MF I	2085 ± 159	1154 ± 60*	2264 ± 586	2377 ± 168
MF II	1737 ± 174	1249 ± 80*	1527 ± 351	1540 ± 75
NO concentration (signal amplitude), relative units	1.01 ± 0.15	0.45 ± 0.03*#	0.70 ± 0.17	0.44 ± 0.05*#
Myosatellites/fiber	0.35 ± 0.02	0.25 ± 0.01*	0.59 ± 0.06*	0.46 ± 0.06

* Differences from C group, $p < 0.05$.
\# Differences from HSS group, $p < 0.05$.

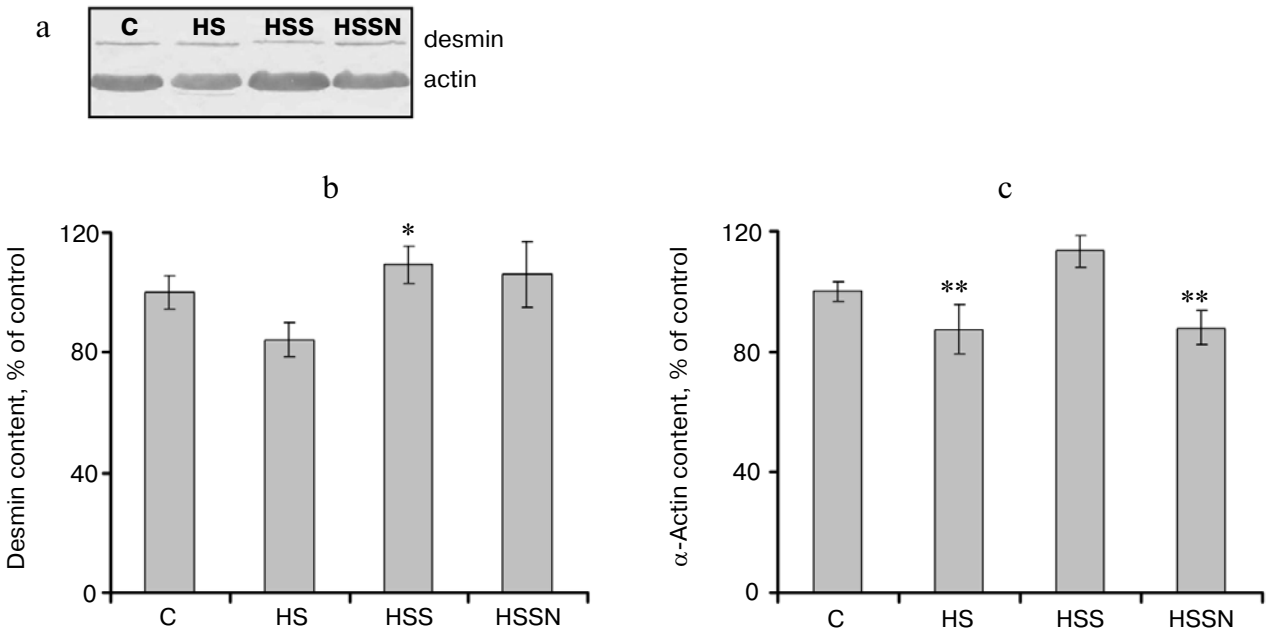

Fig. 1. a) Immunoblot example for desmin and α-actin. The content of cytoskeletal protein desmin (b) and myofibrillar protein α-actin (c) in *m. soleus* of hindlimb-suspended rats (HS), hindlimb-suspended rats with *m. soleus* stretching (HSS), and hindlimb-suspended rats with *m. soleus* stretching and L-NAME administration (HSSN) was estimated relative to the level of corresponding proteins in control (C). Results are given as M ± m (n = 7 for each group). * Differences from HS are significant at $p < 0.02$; ** differences from HSS are significant at $p < 0.02$.

In group HS, the level of mRNA of E3-ligases involved in proteasomal utilization of proteins atrogin-1/MAFbx and MuRF-1 was significantly increased compared to both control (Fig. 5) and groups HSS and HSSN. In this case, relative concentration of mRNA marker of intensity of ribosomal p70S6 kinase protein synthesis in group HS was decreased relative to the control group (Fig. 6). Simultaneously, there were no signifi-

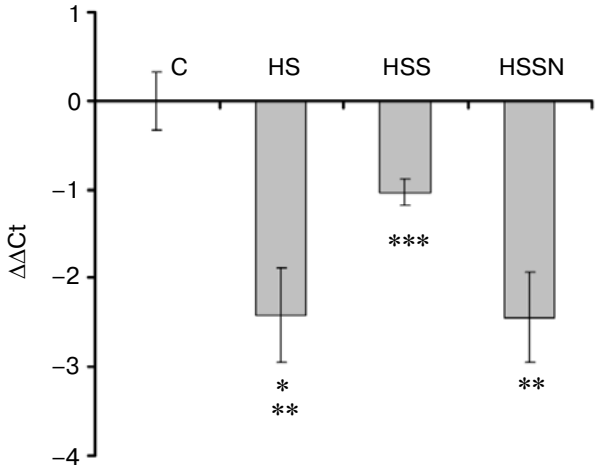

Fig. 2. Change in nNOS mRNA content in *m. soleus* of hindlimb-suspended rats (HS), hindlimb-suspended rats with *m. soleus* stretching (HSS), and hindlimb-suspended rats with *m. soleus* stretching and L-NAME administration (HSSN) was estimated relative to control level (C) by the standard Livak technique (the ΔΔCt method). Results are given as M ± m (n = 7 for each group). * Differences from C are significant at $p < 0.01$; ** differences from HSS are significant at $p < 0.05$; *** differences from C are significant at $p < 0.05$.

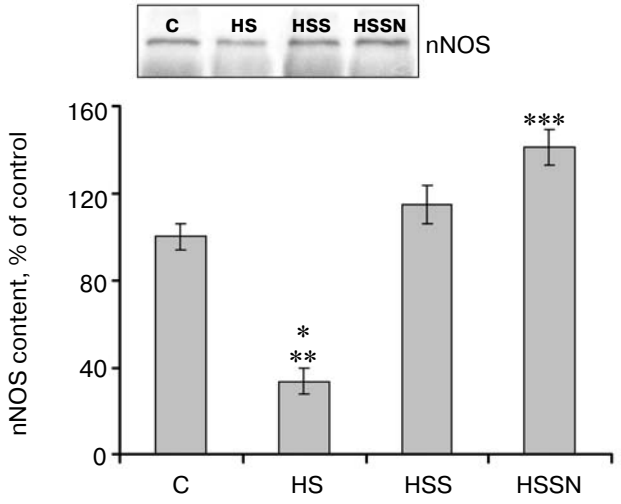

Fig. 3. Content of nNOS protein in *m. soleus* of hindlimb-suspended rats (HS), hindlimb-suspended rats with *m. soleus* stretching (HSS), and hindlimb-suspended rats with *m. soleus* stretching and L-NAME administration (HSSN) was estimated relative to that in control (C). Results are given as M ± m (n = 7 for each group). * Differences from C are significant at $p < 0.001$; ** differences from HSS are significant at $p < 0.001$; *** differences from C are significant at $p < 0.01$.

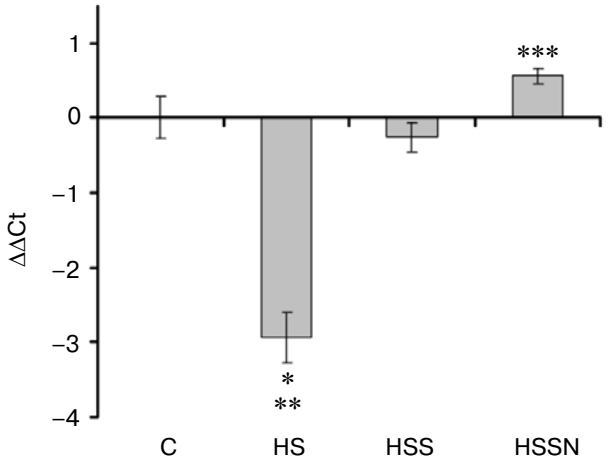

Fig. 4. Change in Hsp90β mRNA content in *m. soleus* of hindlimb-suspended rats (HS), hindlimb-suspended rats with *m. soleus* stretching (HSS), and hindlimb-suspended rats with *m. soleus* stretching and L-NAME administration (HSSN) was estimated relative to control level (C) by the Livak method (ΔΔCt method). Results are given as M ± m (n = 7 for each group). * Differences from C are significant at $p < 0.001$; ** differences from HSS are significant at $p < 0.001$; *** differences from HS are significant at $p < 0.001$.

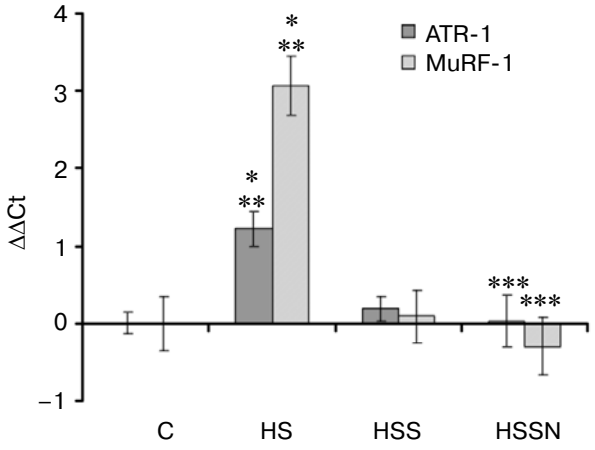

Fig. 5. Change in atrogin-1 (ATR-1) and MuRF-1 mRNA content in *m. soleus* of hindlimb-suspended (HS), hindlimb-suspended with *m. soleus* stretching (HSS), and hindlimb-suspended with *m. soleus* stretching and L-NAME administration (HSSN) rats was estimated relative to control level (C) by the Livak technique. Results are given as M ± m (n = 7 for each group). * Differences from C are significant at $p < 0.001$; ** differences from HSS are significant at $p < 0.001$; *** differences from HS are significant at $p < 0.001$.

cant differences between HSS and HSSN groups concerning contents of atrogin-1/MAFbx, MuRF-1, or ribosomal p70S6 kinase.

Relative level of MHC I mRNA in HS group was significantly decreased relative to control group (Fig. 7). In the HSS group this decrease was prevented. However, in the case of suspension with *m. soleus* stretching and L-NAME introduction (group HSSN), the MHC I mRNA concentration was reliably lower than that in both HSS group ($p < 0.05$) and C group ($p < 0.05$). Expression of MHC IId/x mRNA in the HS group significantly exceeded that in control ($p < 0.001$) (Fig. 8). In groups HSS and HSSN the content of the MHC IId/x mRNA was significantly lower than that in the HS group ($p < 0.005$) but reliably higher than that in the control ($p < 0.005$). No changes relative to control were registered in the MHC IIa mRNA expression during the 14-day-long experiment.

The decrease in NO content upon L-NAME administration in the HSSN group by a value comparable with that in the HS group (relative to group C) indicates adequate effect of the inhibitor on NOS activity (table). In the HSS group no significant differences from control group by this parameter were observed. The decrease in NO content in *m. soleus* after two weeks of hindlimb suspension was found earlier [11]. Some authors also showed the increase in NO production upon muscle stretching and a relationship between NOS activity and expression of some cytoskeletal proteins [23]. However, despite sig-

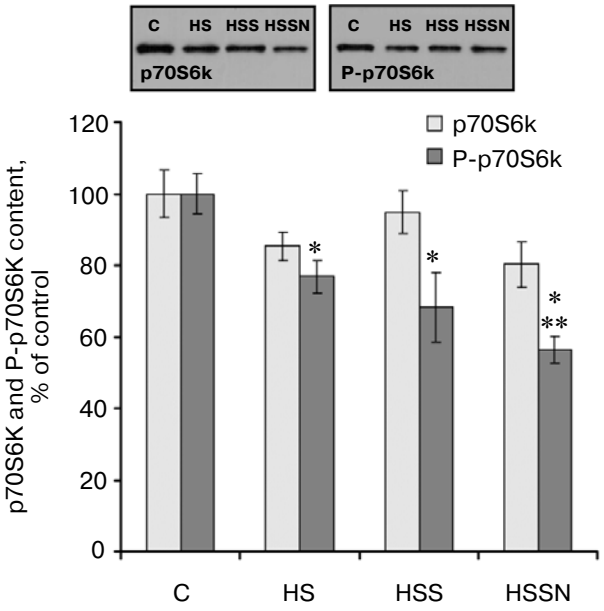

Fig. 6. Content of non-phosphorylated p70S6K and phosphorylated p70S6K in *m. soleus* of hindlimb-suspended (HS), hindlimb-suspended with *m. soleus* stretching (HSS), hindlimb-suspended with *m. soleus* stretching and L-NAME administration (HSSN) rats was estimated relative to level of the proteins in control (C). Immunoblots of p70S6K (total content) and phosphorylated p70S6K (P-p70S6K) are shown. Results are given as M ± m (n = 7 for each group). * Differences from C are significant at $p < 0.05$; ** differences from HS are significant at $p < 0.05$.

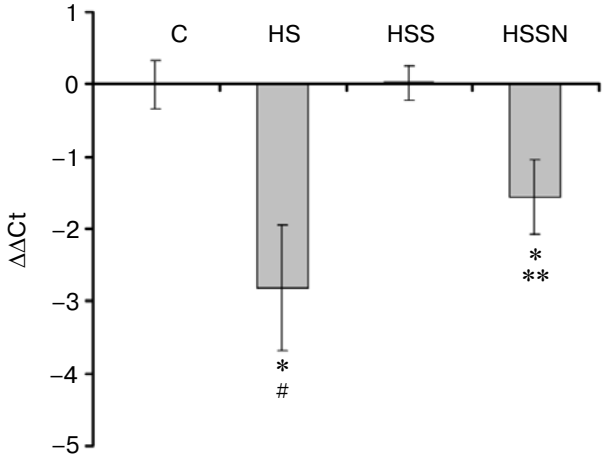

Fig. 7. Change in MHC I mRNA content in *m. soleus* of hindlimb-suspended (HS), hindlimb-suspended with *m. soleus* stretching (HSS), and hindlimb-suspended with *m. soleus* stretching and L-NAME administration (HSSN) rats was estimated relative to control (C) level by the Livak method. Results are given as M ± m (n = 7 for each group). * Differences from C are significant at $p < 0.05$; ** differences from HSS are significant at $p < 0.05$; # differences from HSS are significant at $p < 0.02$.

nificant decrease in NO concentration in the rat *m. soleus* in the group with NOS inhibitor administration (HSSN) relative to control, the muscle mass did not differ from that in group HSS. The parameters of muscle fibers (MF CSA) had the same dynamics (table).

Thus, we have found that atrophy was prevented in both groups of experimental rats with hindlimb suspension and *m. soleus* stretching. Atrophy prevention upon *m. soleus* stretching during animal hindlimb suspension correlates with results of previous investigations [3, 4], but we have still not revealed its relationship with NOS activity. Also, no differences were found between HSSN, HSS, and C groups in desmin content, although its content in the HS group was significantly lower than that in the HSS group (Fig. 1b). Dynamics of desmin content in *m. soleus* changes depending on the time of hindlimb suspension, and the most pronounced decrease is observed after the first week [24, 25]. By the third week of hindlimb suspension its complete recovery occurs [26, 27].

There are no differences in muscle mass, MF CSA, and desmin content between rat groups with and without NOS inhibitor administration, while the percentage of fibers with breaks in the dystrophin layer varied significantly in these groups. In the HS group the increase in percentage of such fibers was most pronounced (+21%). Dystrophin disruption in *m. soleus* upon hindlimb suspension was earlier shown by some authors [26, 28]. In the group with inhibited NOS activity (HSSN) there were also significantly more fibers with disrupted dystrophin layer than in group HSS.

The α-actin content was equally decreased in groups HS and HSSN compared to groups C and HSS (Fig. 1c). A decrease in dystrophin concentration upon animal hindlimb suspension was noted earlier [28], but possible NOS involvement in maintenance of muscle protein metabolism upon muscle stretching was not discussed. It can be concluded that NOS does not significantly contribute to prevention of atrophy during *m. soleus* stretching upon hindlimb suspension, but it is involved in maintenance of expression of some cytoskeletal muscle proteins. How can this protection be achieved? We found that in the case of NOS blocking by L-NAME during muscle stretching, both NO (table) and nNOS mRNA contents decrease relative to those in groups C and HSS (Fig. 2). The level of these changes is comparable to that in group HS. However, it was unexpected for us that we did not observe a decrease in nNOS level in the HSSN group (Fig. 3). Simultaneously, the Hsp90 mRNA content in the group with NOS blocking (HSSN) was the highest among all groups (Fig. 4). It is known that Hsp90 fulfills several intracellular functions. Hsp90 forms a complex with NOS and only in this state it retains its complete catalytic activity and stability. Chaperons form stable complexes with many proteins, and thus they prevent processes of chaperon-dependent ubiquitination [29, 30]. Hsp90 stimulates NO formation by enhancement of nNOS catalytic function in intact cells *in vivo* proportionally to its concentration [29]. In this case efficiency of nNOS protection is proportional to the level of intracellular Hsp90 expression [29]. In particular, nNOS complexed with Hsp90 is more resistant to proteolytic degradation by calpain, whose concentration significantly increased in *m. soleus* of animal upon unloading [31].

Thus, despite our results concerning decrease in nNOS mRNA expression, it seems that the nNOS protein proper was protected from degradation by Hsp90.

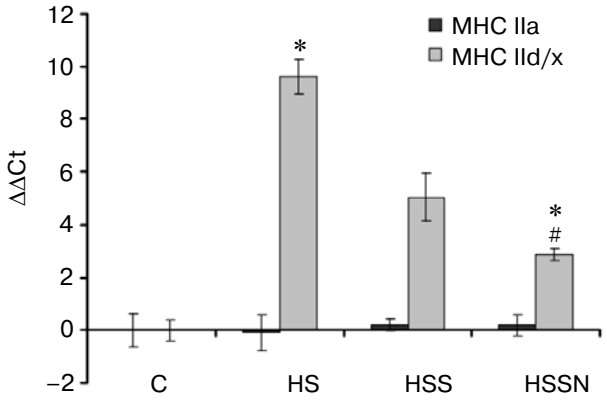

Fig. 8. Change in MHC IIa and IId/x mRNA content in *m. soleus* of hindlimb-suspended (HS), hindlimb-suspended with *m. soleus* stretching (HSS), and hindlimb-suspended with *m. soleus* stretching and L-NAME administration (HSSN) rats was estimated relative to control (C) by the Livak method. Results are given as M ± m (n = 7 for each group). * Differences from C are significant at $p < 0.001$; # differences from HS are significant at $p < 0.001$.

However, this cannot assure preservation of the NOS activity proper at the previous level because it was shown earlier that preservation of nNOS concentration during long-term increase in muscle contractile activity can be accompanied by increase in the enzyme activity measured by the rate of L-[^{14}C]arginine transformation to L-[^{14}C]citrulline [32]. Besides, the increase in Hsp90 concentration could have a protective function for some cytoskeletal proteins in the rat group with stretching and nNOS blocking (HSSN group). There are reports that NO is able to both activate synthesis of some cytoskeletal proteins [5, 13] and inhibit activity of muscle μ-calpains [5]. Judging by the fact that α-actin level in *m. soleus* of group HSS is significantly higher and the number of fibers with damaged dystrophin layer is significantly lower than that in the group with the nNOS blocking (HSSN group) (Fig. 2), it appears that the nNOS activity upon stretching can be important for protection against destruction of some *m. soleus* cytoskeletal proteins.

A question arises, what prevents *m. soleus* atrophy upon stretching, a decrease in protein destruction or enhancement of anabolic processes? To answer this question, we tested the work of units of proteasomal degradation (E3 ligases atrogin-1/MAFbx and MuRF-1) and the protein synthesis (Akt-mTOR-S6K signal pathway) system. It is known that the first disorders in skeletal muscle upon unloading are due to decrease in protein synthesis followed by accelerated proteolysis [33]. Atrogin-1 and MuRF-1 were chosen as proteolysis markers because earlier significant growth of just these E3-ligases was noted upon functional unloading of muscle [33-35], whereas MuRF-1 removal prevents development of atrophy [15]. This is not surprising because MuRF-1 is involved in ubiquitination of thick myofilament components [15], while contractile proteins occupy up to 75% of the muscle fiber volume. Unlike the HS group, no increase in the atrogin-1/MAFbx and MuRF-1 content was observed in the groups with hindlimb suspension and *m. soleus* stretching (HSS and HSSN) (Fig. 5) along with decrease in Hsp90 mRNA also involved in proteasomal degradation. They form stable complexes with many "client" proteins and thus prevent further chaperon-dependent ubiquitination [36]. We checked whether protein synthesis increased in HSS and HSSN groups by detection of phosphorylated and non-phosphorylated p70S6K. Akt-mTOR-S6K is well known as the main pathway of protein synthesis regulation at the level of translation initiation [37]. We found a decrease in S6K phosphorylation in *m. soleus* on the 14th day of hindlimb suspension, which was already shown earlier [38] (Fig. 6). Since the p-S6K/S6K ratio was the same in all groups, we supposed that the decrease in S6K phosphorylation level was the result of decrease in total kinase content after intensification of proteolysis in muscle fibers. However, in both groups with *m. soleus* stretching (HSS and HSSN) the decrease in S6K phosphorylation level was as significant as in group HS (Fig. 6), although no *m. soleus* atrophy in these groups was found. It can be concluded that the muscle mass and the content of some proteins in groups with *m. soleus* stretching are maintained due to prevention of protein degradation. At the same time, protein synthesis regulated by Akt-mTOR-S6K signaling was decreased in this group. It should be noted that in addition to Akt-mTOR-S6K, there are different factors involved in regulation of protein synthesis, like those at the level of translation or elongation. Besides, prevention of atrophy in *m. soleus* upon its stretching could be due to increased myosatellite proliferation (table).

Some authors believe that muscle atrophy can be due to lowering the number of myosatellites in them [16, 17] similarly to that observed in our work. Some authors showed that the nNOS activity is stimulated upon muscle stretching, which results in increase in NO concentration [39, 40]. The increase in NO concentration can activate hepatocyte growth factor that interacts with myosatellite c-met receptor related to proliferation of the latter [40, 41]. We supposed that NO upon stretching activates myosatellites that can be involved in the muscle mass maintenance. In fact, in the HSS group the number of myosatellites exceeded that in control by 68% ($p < 0.05$), whereas in the group with blocked nNOS activity (HSSN group) the number of myosatellites in *m. soleus* did not increase but remained at control level (table). This shows that NOS blocking prevents myosatellite regeneration. However, in this group *m. soleus* atrophy also was not observed like in the HSS group (table). It can be concluded that proliferation of satellite cells upon *m. soleus* stretching is associated with nNOS activity, but it does not make a decisive contribution to muscle mass maintenance upon stretching.

Both in hindlimb-suspended animals (group HS) and in the group with L-NAME administration (HSSN) a decrease in MHC I mRNA expression was observed. This was not registered upon muscle stretching without NOS inhibition (Fig. 7). The increase in MHC IIb and IId/x mRNA expression compared to control group C was observed in all hindlimb-suspended groups (Fig. 8). Redistribution of MHC isoforms towards higher content of type II myosins upon *m. soleus* unloading was described previously [42]. However, we have shown for the first time the involvement of NOS in regulation of slow MHC mRNA expression upon muscle stretching. Earlier we also found prevention of decrease in MHC I mRNA level in rats after administration of NO donor upon hindlimb suspension [11]. Lori et al. noted that introduction of the NOS blocker L-NAME upon muscle functional overload prevents muscle fiber "transformation" towards higher content of type I fibers [43]. Phenotypic muscle plasticity can be regulated by endogenous NO via calcium/calmodulin-dependent NOS [44, 45].

Neuronal NOS does not significantly contribute to prevention of atrophy during *m. soleus* stretching upon rat

hindlimb suspension but is involved in maintenance of expression of some muscle proteins, in particular, in regulation of MHC I mRNA expression upon muscle stretching. The increase in myosatellite number in the muscle upon its stretching is NO-dependent. Simultaneously, no direct association was detected between number of myosatellites and maintenance of muscle mass and cross sectional area of muscle fibers upon muscle stretching.

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