Research Square

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Calpain-dependent degradation of cytoskeletal proteins as a key mechanism for a reduction in intrinsic passive stiffness of unloaded rat postural muscle

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Research Article

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Abstract

In mammals, prolonged mechanical unloading results in a significant decrease in passive stiffness of postural muscles. The nature of this phenomenon remains unclear. The aim of the present study was to investigate possible causes for a reduction in rat soleus passive stiffness after 7 and 14 days of unloading (hindlimb suspension, HS). We hypothesized that HS-induced decrease in passive stiffness would be associated with calpain-dependent degradation of cytoskeletal proteins or a decrease in actomyosin interaction. Wistar rats were subjected to HS for 7 and 14 days with or without PD150606 (calpain inhibitor) treatment. Soleus muscles were subjected to biochemical studies and *ex vivo* measurements of passive tension with or without blebbistatin treatment (an inhibitor of actomyosin interactions). Passive tension of isolated soleus muscle significantly reduced after 7- and 14-day HS compared to control values. PD150606 treatment during 7- and 14-day HS induced an increase in alpha-actinin-2 and − 3, desmin contents compared to control, partly prevented a decrease in intact titin (T1) content and prevented a decrease in soleus passive tension. Incubation of soleus muscle with blebbistatin did not affect HS-induced reductions in specific passive tension in soleus muscle. Our study suggests that calpain-dependent breakdown of cytoskeletal proteins, but not a change in actomyosin interaction, significantly contributes to unloading-induced reductions in intrinsic passive stiffness of rat soleus muscle.

Introduction

It is well-known that elimination of axial loading and ground reaction force under real or simulated microgravity (mechanical unloading) leads to a significant decrease in muscle tone of postural/antigravity muscles, resulting in impaired locomotor and postural functions [20, 15]. To quantify muscle tone, it is customary to use indicators of muscle stiffness. Muscle stiffness is defined as an increment of the tensile force per cross-sectional area in response to the relative elongation of muscle fibers [34]. Muscle stiffness can serve as an indicator of the structural and functional state of skeletal muscle. Intrinsic skeletal muscle stiffness is determined by both an active component, represented by actomyosin interactions, and by the state of the intracellular cytoskeleton, represented by protein molecules exhibiting elastic properties (titin) that are capable of mechanical resistance in response to muscle stretching/contraction. Previously, it has been demonstrated that mechanical unloading (rat hindlimb suspension for 2 or 3 weeks) results in a significant decrease in passive properties of both isolated rat soleus muscles [5] and single muscle fibers [36]. However, there is a problem in determining muscle stiffness independent of actomyosin interactions. The active stiffness component can be eliminated by using blebbistatin, a highly specific inhibitor of myosin II, which can freely enter the cell through the sarcolemma, bind to myosin and block its transition to a state of strong binding to actin [9, 1]. Earlier, in our laboratory, the contribution of actomyosin bonds and cytoskeletal proteins to the passive stiffness of rat soleus after 3-day HS was evaluated. It was found that in both control and HS animals, the use of blebbistatin had an identical effect on the intrinsic stiffness of soleus muscle. These data

suggest that the state of the actomyosin complex does not contribute to a decrease in passive stiffness of rat soleus after 3-day HS [27].

Of particular interest is the giant sarcomeric protein titin, which is known to significantly contribute to the passive stiffness of skeletal muscles [14, 21]. A significant reduction in titin content in soleus muscle has been previously shown following 14 days of HS [18, 36]. It has been also shown that there is an increase in the content of proteolytic fragment of titin (T2) in rat soleus of rats after 14-day HS [32]. At the same time, 3-day mechanical unloading did not affect the content of intact titin in rat soleus muscle [12]. It is possible that the mechanisms underlying a reduction in soleus muscle passive stiffness at later stages of unloading would differ from those that operate during the first three days of unloading. In this regard, the purpose of the present work was to elucidate possible mechanisms responsible for a decrease in intrinsic passive stiffness of rat postural soleus muscle after 7 and 14 days of mechanical unloading.

Materials And Methods

Animal care and experimental protocol. The research involved male Wistar rats weighing 209 ± 21 g (mean ± SD) that were randomly divided for two HS experiments. The rats were divided into three groups for each of the experiments (n = 16/group). In each group, soleus muscles from 8 animals were collected for the measurement of passive tension with or without blebbistatin incubation. Soleus muscles from the remaining 8 animals were subjected to biochemical analysis to assess the abundance of cytoskeletal proteins.

In *Experiment 1*, rats were randomly assigned to the following 3 groups: 1) vivarium control (C), 2) hindlimb suspension for 7 days (7HS); 3) hindlimb suspension for 7 days with daily injections of calpain inhibitor PD150606 (7HS + PD). In *Experiment 2*, rats were randomly assigned to the following groups: 1) vivarium control (C), 2) hindlimb suspension for 14 days (14HS); 3) hindlimb suspension for 14 days with daily injections of calpain inhibitor PD150606 (14HS + PD). The calpain inhibitor PD150606 (Sigma-Aldrich, USA) at a dose of 3 mg/kg (diluted in 1% DMSO) was daily administered via intramuscular injections. The C and 7HS groups were treated with the equivalent amount of the vehicle.

Temperature and humidity in the vivarium room were maintained at 24°C and 50%, respectively, with 12/12 hour light/dark cycle. All rats had access to a standard diet and water *ad libitum*. The animals were anesthetized with an intraperitoneal injection of tribromoethanol (240 mg/kg) prior to all surgical manipulation. The animals were sacrificed with an additional tribromoethanol injection (750 mg/kg).

Hindlimb suspension. Mechanical unloading was carried out using a standard hindlimb suspension (HS) model [24]. Briefly, a strip of adhesive tape was applied to the animal's tail, which was suspended by passing the tape through a swivel that was attached to a metal bar on the top of the cage. After that, the hindlimbs of the rats were lifted slightly off the floor of the cage (head-down tilt posture). The suspension height was adjusted to prevent the hindlimbs from touching any supporting surface.

In vitro **muscle preparation and stimulation.** *Ex vivo* force measurements of rat soleus muscle were carried out as previously described [37]. The isolated muscle optimal length was estimated with digital caliper *in situ*. Then each muscle was dissected and placed in a cooled Ringer-Krebs solution (138 mM NaCl, 5 mM KCl, 1 mM NaH2PO4, 2 mM CaCl2, 2 mM MgCl2, 24 mM NaHCO3, 11 mM glucose) with constant perfusion with carbogen (95% O2 + 5% CO2) and incubated for 15 minutes. One of the muscles was incubated for 15 min in saline with 75 μM blebbistatin (Apexbio Technology, USA), a specific inhibitor of actomyosin interactions [28]. Double knots were tied around the distal and proximal ends of the muscle near the musculotendinous junction. After that, the muscle was attached to the lever arm/force transducer from one end and to the fixed hook from the other end in the temperature-controlled (28ºC) water bath (Aurora Scientific Bath 809C). Optimal muscle length (L_0) was determined with a series of twitch contractions (0.5ms, 10V). After that, the soleus muscle was set to the slack length (Ls) or the length from which the beginning of tension development was measurable. Then the muscle was stretched by 25% of Ls at a speed of 50 mm/s. The muscle was stretched for two minutes, after which the length was returned to Ls [3]. The maximum force was recorded at the end of the stretch. The tension obtained as a result of 3 repetitions for each muscle was used for all calculations. To normalize the parameters, muscle physiological cross-sectional area (CSA) was calculated as a muscle wet weight divided by the product of muscle optimal length and density [29, 17]. Force measurements were performed by using Aurora Scientific Dual Mode Lever System 305C-LR, with a data acquisition frequency of 10 kHz. Data processing was carried out by using Aurora Scientific 615A Analysis Software Suite.

Determination of the abundance of cytoskeletal proteins. Western blotting was carried out as previously described [37]. The total protein fraction was isolated and the content of desmin, α-actinin-2, α-actinin-3, and telethonin was subsequently assessed. The RIPA reagent kit (Santa Cruz, USA) was used for protein extraction. The samples were diluted in a 2X sample electrophoresis buffer (5.4 mM Tris-HCl (pH 6.8), 4%-Ds-Na, 20%-glycerin, 10%-2-mercaptoethanol, 0.02%- bromphenol blue). Electrophoresis was performed in 10% separation PAGE. Following electrophoresis, the total protein fraction was transferred to nitrocellulose membrane via western blotting. The detection of the protein of interest was performed with the following primary antibodies: desmin (Abcam, ab8592, 1:1000, USA), GAPDH (ABM, G041, USA, 1:10000), α-actinin-2 (Santa Cruz, sc-17829, USA, 1:1000), α-actinin-3 (MERCK, MABT143, USA, 1:1000) and telethonin (Abcam, ab210773, 1:1000, USA). After rinsing the membrane to remove unbound primary antibody, secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase (Santa Cruz, USA) were used at a dilution of 1:50000. The blots were visualized by using the Clarity Western ECL Substrate (BioRad Laboratories, USA). Western blot data were processed by using Image Studio Digits Ver4.0 software (LI-COR Biotechnology, USA).

Electrophoresis and detection of the giant proteins (titin and nebulin) were previously described [37]. Changes in titin and nebulin contents were carried out using the technique of SDS-electrophoresis in 2.2% polyacrylamide gel with 0.5–0.6% agarose [35], with modifications aimed at improving the focusing of the studied protein bands in the gel [41]. To ensure equal loading, samples from the control and

experimental groups were all run on the same gel. SDS-PAGE was performed using the Helicon VE–10 system (Moscow, Russia) at 8 mA. Following SDS-PAGE, the gels were stained with Coomassie Brilliant Blue (G-250 and R-250, 1:1). Titin and nebulin contents were normalized to the content of myosin heavy chains (MyHC).

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). Since the normal distribution of the sample was not confirmed in all cases, a nonparametric Kruskal-Wallis test was used to compare the groups with each other. The differences were accepted as statistically significant at $p < 0.05$.

Results

Muscle weight. We did not observe any significant changes in rat body weight in the experimental groups in comparison with the control group. Soleus muscle weight significantly decreased after 7 and 14 days of HS by 31% and 33%, respectively. The administration of PD150606 partially prevented soleus weight loss only after 7 days of HS (Tables 1, 2). Changes in mechanical properties of the isolated soleus muscle from *Experiment 1* and *Experiment 2* are presented in Table 1 and Table 2, respectively.

Table 1
Soleus weight and mechanical properties after 7-day HS.

	C	7HS	7HS + PD
body weight, g	195.4 ± 4.9	190.2 ± 6.3	188.6 ± 2.9
soleus weight, mg	98.1 ± 8.2	69.2 ± 2.3*	82.3 ± 5.5$
soleus weight / body weight, mg/g	5.1 ± 0.5	3.7 ± 0.1*	4.33 ± 0.2$
muscle length, mm	19.8 ± 0.5	18.7 ± 0.6	21.14 ± 0.6
CSA, mm^2	4.7 ± 0.5	3.3 ± 0.1*	3.58 ± 0.3*
twitch tension, mN	77.8 ± 6.5	51.1 ± 1.9*	74.25 ± 3.1$
passive tension, mN	117.7 ± 11.6	68.5 ± 3.2*	99.5 ± 3.1$
passive tension/CSA, mN/ mm^2	25.4 ± 1.2	21.0 ± 1.4*	28.4 ± 1.5$
passive tension + blebbistatin, mN	77.4 ± 7.1#	36.1 ± 2.1#*	59.1 ± 7.8#$
passive tension/CSA + blebbistatin, mN/mm^2	17.4 ± 1.1#	12.3 ± 0.7#*	18.6 ± 2.7#$

*CSA – muscle physiological cross-sectional area. C, control, 7HS, hindlimb suspension for 7 days, 7HS + PD, hindlimb suspension for 7 days + PD150606. * - significant difference from the C group ($p < 0.05$), $ – significant difference from the 7HS group ($p < 0.05$), # – significant difference of blebbistatin-treated muscles form blebbistatin-untreated muscles ($p < 0.05$). Data are shown as mean ± SEM.*

Table 2
Soleus weight and mechanical properties after 14-day HS.

	C	14HS	14HS + PD
body weight, g	241.2 ± 4.8	218.6 ± 6.6	223.6 ± 4.5
soleus weight, mg	116.1 ± 6.1	68.3 ± 3.1*	61.5 ± 3.2*
soleus weight / body weight, mg/g	4.8 ± 0.3	3 ± 0.1*	2.7 ± 0.1*
muscle length, mm	22.1 ± 0.7	19.7 ± 6.1	21.4 ± 5.3
CSA, mm^2	4.5 ± 0.2	3.0 ± 0.3*	3.3 ± 0.2*
twitch tension, mN	86.8 ± 7.1	56.8 ± 4.2*	92.4 ± 7.3$
passive tension, mN	129.7 ± 12.5	62.8 ± 7.4*	123.9 ± 10.1$
passive tension /CSA, mN/ mm^2	26.5 ± 2.2	18.8 ± 2.8*	50.5 ± 3.7*$
passive tension + blebbistatin, mN	67.7 ± 6.1#	31.75 ± 3.6*#	98.1 ± 10.5#*$
passive tension/CSA + blebbistatin, mN/mm^2	13.8 ± 0.9#	10.6 ± 0.9#*	35.9 ± 4.1#*$

*CSA, muscle physiological cross-sectional area. C, control, 14HS, hindlimb suspension for 14 days, 14HS + PD, hindlimb suspension for 14 days + PD150606. * - significant differences from the C group ($p < 0.05$), $ – significant differences from the 14HS group ($p < 0.05$), # – significant difference of blebbistatin-treated muscles form blebbistatin-untreated muscles ($p < 0.05$). Data are shown as mean ± SEM.*

Passive tension of rat soleus muscle. We observed that specific passive tension of the intact (without blebbistatin treatment) soleus muscle after 7 days of HS decreased by 18% compared with the control group (Fig. 1A). Specific passive tension of the isolated rat soleus in the presence of blebbistatin was significantly lower for all experimental groups compared to the intact (untreated) muscle (Fig. 1A). However, the magnitude of a decrease in specific passive tension in the blebbistatin-treated and intact (untreated) soleus muscle did not differ between the control and 7HS groups (Fig. 1A). As shown in Fig. 1A, administration of PD150606 (a calpain inhibitor) during 7-day HS prevented a decrease in specific passive tension of rat soleus muscle (Fig. 1A).

In the 14HS group, specific passive tension of the intact (blebbistatin untreated) soleus muscle significantly decreased by 35% compared with the C group (Fig. 2B). As in the 7-day HS experiment, specific passive tension in blebbistatin-treated soleus muscle in all experimental groups was significantly lower than that in soleus from the blebbistatin-untreated groups (Fig. 2B). At the same time, the magnitude of a decrease in specific passive tension in the blebbistatin-treated and untreated soleus muscle did not differ between the control and 14HS groups (Fig. 1B). Thus, as in case with 7-day HS, no contribution of actomyosin bonds to the 14-day HS-induced decrease in soleus passive tension was revealed. As shown in Fig. 1B, inhibition of calpains with PD150606 treatment during 14-day HS resulted in a significant increase in soleus muscle passive tension compared to the C group.

Abundance of cytoskeletal proteins in rat soleus muscle. There were no statistically significant changes in the content of alpha-actinin-2 and − 3 between the C and 7HS groups (Fig. 2A). However, there was a trend towards a decrease in the contents of desmin and telethonin after 7 days of HS (Fig. 2A). In the 7HS + PD group, the content of alpha-actinin-2 and alpha-actinin-3 increased by 70% and 78%, respectively, compared with the C group (Fig. 2A). In the 7HS + PD group, the content of desmin significantly increased by 70% compared to the C group (Fig. 2A). The content of telethonin in the 7HS + PD group did not differ from the C group (Fig. 2A).

After 14 days of HS, there were no significant differences in the content of alpha-actinin-2 and alpha-actinin-3 between experimental groups (Fig. 2B). However, we observed a downward trend in alpha-actinin-2 levels in the 14HS + PD group (Fig. 2B). The content of desmin in the 14HS and 14HS + PD groups was significantly higher compared to the C group (Fig. 2B). There was also a significant increase in the content of telethonin in the 14HS + PD group compared to the C and 14HS groups (Fig. 2B).

Abundance of giant proteins titin and nebulin. One-week unloading induced a significant 43% decrease in the content of intact titin (T1) compared to the C group (Fig. 3A). In the 7HS + PD group there was a partial restoration of titin T1 content by 22% compared with the 7HS group (Fig. 3A). In addition, an increase in the content of the proteolytic fragment of titin (T2) by 57% in the 7HS group was partially prevented by PD150606 administration. There was a decrease in the content of nebulin in the 7HS and 7HS + PD groups by 38% and 36%, respectively, compared with the C group (Fig. 3A).

Two-week HS resulted in a significant 43% decrease in the content of intact titin (T1) compared with the C group (Fig. 3B). The content of titin T1 in the 14HS + PD group was higher than in the 14HS group, but still significantly less than in the C group (Fig. 3B). A significant 93% increase in the content of titin proteolytic fragment (T2) in the 14HS group was partially prevented by treatment of rats with PD150606 (Fig. 3B). In the 14HS and 14HS + PD groups, there was a significant decrease in the content of nebulin by 51% and 37%, respectively, compared with the C group (Fig. 3B).

Discussion

Our study was aimed at identifying the potential contribution of either actomyosin interactions or cytoskeletal elements (titin, nebulin, desmin, alpha-actinins, and telethonin) to unloading-induced decline in intrinsic soleus muscle stiffness. A significant reduction in specific passive tension of isolated rat soleus muscle in response to 7- or 14-day mechanical unloading, observed in the present study, is in a good agreement with previous reports on the effect of hindlimb unloading on passive tension of single soleus muscle fibers of rats [36]. In a previous work of our laboratory, a similar reduction in passive tension was demonstrated after 3-day HS in both blebbistatin-treated and intact (without blebbistatin treatment) soleus muscles [27]. In this regard, it has been concluded that actomyosin interactions do not contribute to the changes of intrinsic soleus muscle stiffness at the early stage (3 days) of mechanical unloading [27]. In the present study, similar results were obtained: a significant decrease in passive tension of the isolated rat soleus muscle after 7- and 14-day HS appeared not to be associated with a

decrease in actomyosin interactions. Additionally, the administration of a calpain inhibitor PD150606 prevented an unloading-induced decrease in the content of titin T1 and an increase in the content of the proteolytic fragment of titin (T2), and also prevented a HS-related decrease in isolated soleus passive tension. These findings suggest that calpain-dependent degradation of titin may play a significant role in the mechanical unloading-induced reduction in the intrinsic soleus muscle stiffness. The obtained data on the decreased content of titin in rat soleus under unloading conditions are consistent with previously published reports [39, 38, 32, 36]. It is well-established that a giant protein titin significantly contributes to the intrinsic passive stiffness/tension of muscle fibers [36, 13]. It is also suggested that giant proteins can affect the formation of actomyosin bonds, which, in turn, impacts the ability of muscle fibers to provide active resistance to stretching [10]. It is known that giant proteins (titin and nebulin) undergo partial degradation under conditions of real or simulated weightlessness in both animals and humans [19, 33, 39, 40]. Degradation of titin could be associated with the activation of calcium-dependent proteases known as calpains. Murphy et al. (2006) have previously shown on single muscle fibers that Ca-induced activation of μ-calpain is involved in titin proteolysis and reduction in the peak passive force in response to a stretch [25].

We have also assessed the impact of calpain inhibition on the abundance of several cytoskeletal proteins in rat soleus muscle following 7 and 14 days of unloading. We observed a trend towards a decrease in both desmin and telethonin contents after 7-day HS compared to the control rats. At the same time, no changes in alpha-actinins' content were observed in response to 7-day HS. Calpain inhibitor administration during 7-day unloading led to a significant increase in the contents of desmin and alpha-actinins vs. the C group. Increased contents of desmin and telethonin were also observed in the 14HS + PD group compared to the C group. The results concerning the reduced protein abundances of the cytoskeletal proteins in rat postural muscle in response to 7-day mechanical unloading generally agree with previously published reports [26, 22, 37]. Furthermore, Enns et al. (2007) demonstrated that as early as 2–3 days of HS induce a decrease in desmin content in rat mixed vastus muscles with subsequent restoration of desmin content to control levels by the 9th day of HS [8]. The reason for an increased desmin content in rat soleus observed in the present study after 14 days of unloading is not clear, but previous reports showed that at longer periods of unloading (2, 3 or 6 weeks) the relative content of desmin in skeletal muscles remains unaffected [6, 22]. Aweida et al. (2018) have recently proposed a model for the destruction of desmin filaments in skeletal muscle under atrophic conditions [4]. According to this model, first, desmin filaments are get phosphorylated by glycogen synthase kinase-3β (GSK-3β), then get ubiquitinated by TRIM32 and undergo depolymerization by μ-calpains, ultimately resulting in the loss of myofibrils and muscle atrophy [4]. Thus, this model clearly links desmin degradation with the activation of μ-calpain. To date, it is established that intermediate filaments (desmin), as well as giant sarcomeric proteins (titin, nebulin) can serve as calpain substrates [11, 23]. Of note, it has been shown that total calpain activity in rat soleus muscle is upregulated following 1, 3 and 9 days of mechanical unloading [7]. Furthermore, activation of calpains during HS might be associated with increased concentration of Ca ions in the sarcoplasm of soleus muscle fibers, which was earlier demonstrated in both rats [2] and mice [16]. In addition, it is known that nitric oxide II (NO), the production of which is

decreased in skeletal muscle under unloading conditions, can also be involved in the regulation of calpain-dependent degradation of cytoskeletal proteins [31, 37].

The results obtained in the present study are in good agreement with previously published report by Salazar et al. (2010) showing that that inhibition of calpain (via muscle-specific calpastatin overexpression) prevents a HS-induced disruption of sarcomere structure and decreased maximum isometric specific force in murine soleus muscle [30]. Moreover, in calpastatin-overexpressing mice, peak passive force of isolated soleus muscles was unaffected by 14-day unloading and was accompanied by the maintenance of a uniformity of thick filament lengths [30]. These data by Salazar et al. (2010) and findings of the present study provide evidence for a model in which HS induces calpain-mediated cleavage of a number of cytoskeletal/sarcomeric proteins, leading to changes that promote a significant reduction in force-generating capacity and passive stiffness of rodent soleus muscle.

Conclusion

Inhibition of calpains during 7- and 14-day hindlimb unloading prevents degradation of a giant sarcomeric protein titin and contributes to the preservation of the specific passive tension in rat soleus muscle. Our study also suggests that calpain-dependent breakdown of cytoskeletal proteins, but not changes in actomyosin interaction, is likely to contribute to unloading-induced reductions in intrinsic passive stiffness of rat soleus muscle.

Declarations

Ethical Approval. This animal study was carried out in accordance with the recommendations of the European Convention for the protection of Vertebrate Animals used for Experimental and Scientific purposes (Council of Europe number 123, Strasbourg, 1986). All procedures with the animals were approved by the Bioethical Commission of the Institute of Biomedical Problems of the Russian Academy of Sciences/Physiology section of the Russian Bioethics Committee (protocol no. 421, 14.04.2016).

Human and Animal Ethics. Not applicable

Consent for publication. Not applicable

Availability of supporting data. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions. Author contributions: I. Melnikov and K. Sharlo performed Western Blot analysis. A. Ulanova and I. Vikhlyantsev performed titin and nebulin electrophoresis. S. Tyganov performed isolated muscle mechanical measurements. S. Tyganov, T. Mirzoev and B. Shenkman contributed to manuscript writing, image processing and interpretation of the data. B. Shenkman contributed to the conception of the study and supervised the project. All the authors contributed to the final version of the manuscript.

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Figures

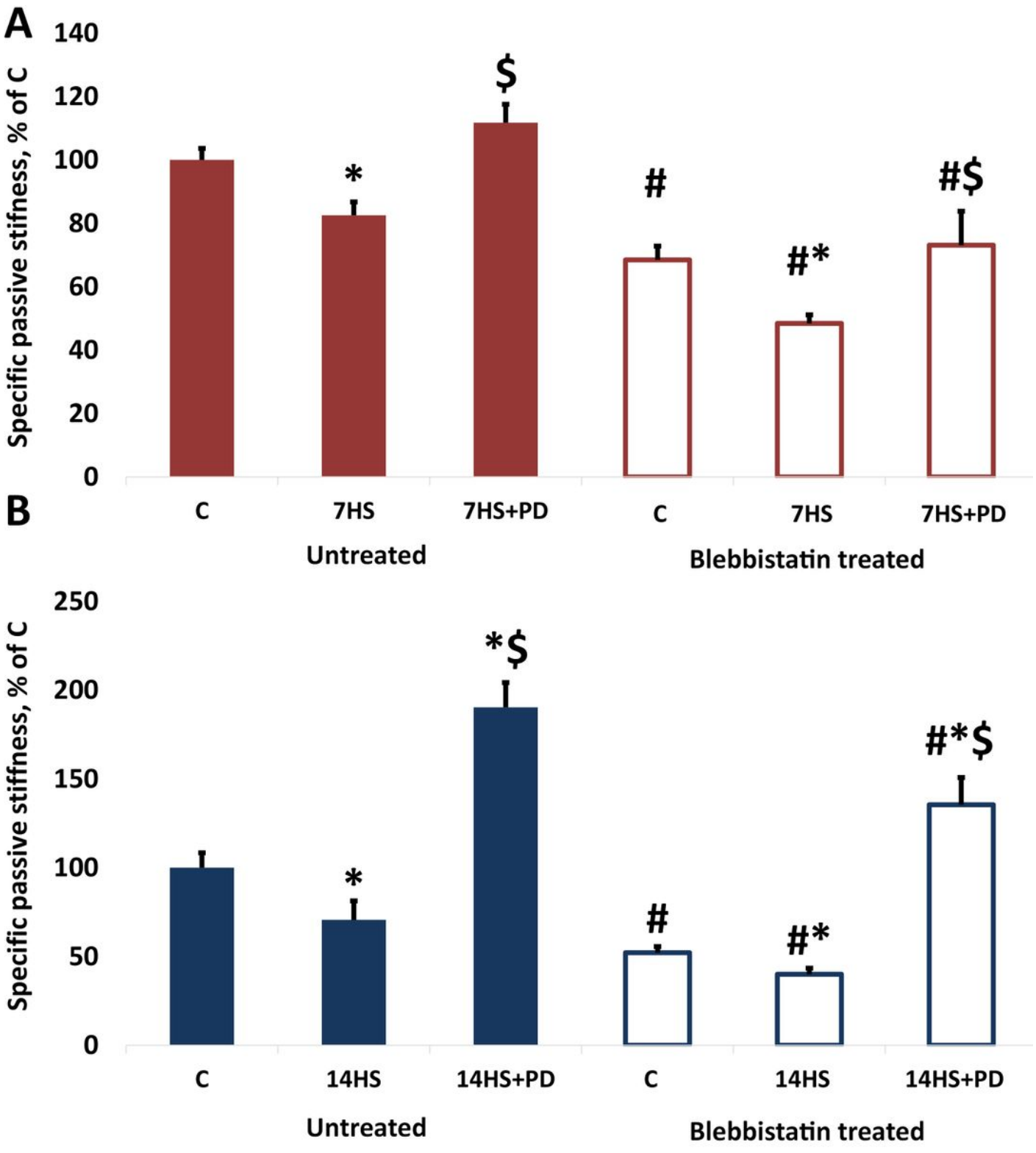

Figure 1

Specific passive tension of rat soleus muscle after 7-day HS **(A).** Specific passive tension of rat soleus muscle after 14-day HS **(B).** Data shown as % of the C group (Mean±SEM), n=8 per group. * – significant difference from the C group, $p<0.05$; $ – significant difference from the 7HS group ($p<0.05$); # – significant difference of blebbistatin-treated muscles from blebbistatin-untreated muscles, $p<0.05$. C,

control group; HS, hindlimb suspension group; HS+PD, hindlimb suspension + treatment with calpain inhibitor (PD150606).

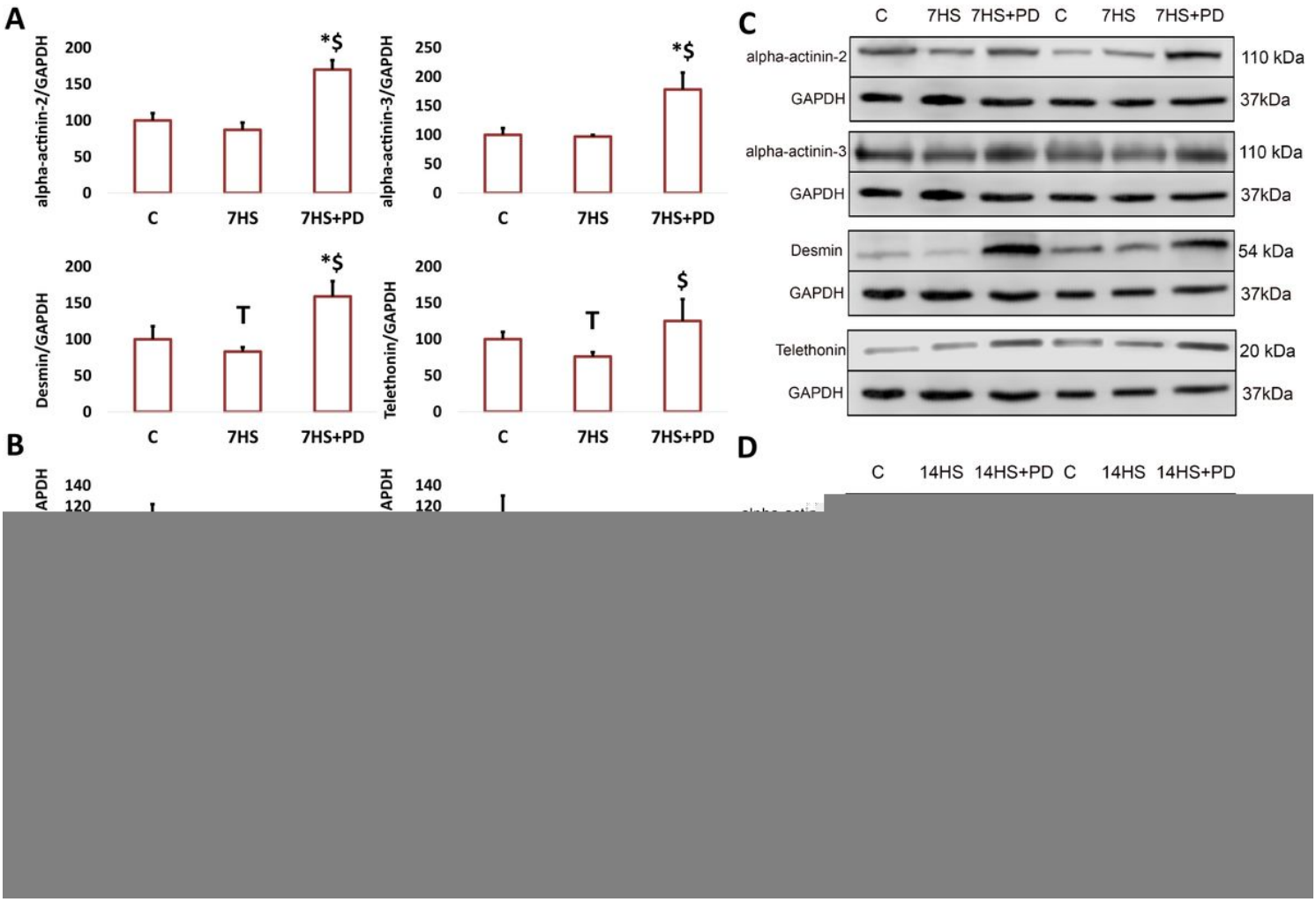

Figure 2

Quantification of desmin, α-actinin-2, α-actinin-3, and telethonin in rat soleus after 7-day HS **(A)**. Quantification of desmin, α-actinin-2, α-actinin-3, telethonin in rat soleus after 14-day HS **(B)**. Representative immunoblots for the studied proteins in the 7-day experiment **(C)**. Representative immunoblots for the studied proteins in the 14-day experiment **(D)**. Data shown as % of the C group (Mean±SEM), n=8 per group. * – significant difference from the C group, $p<0.05$; $ – significant differences from the 7HS group ($p<0.05$); T – downward trend compared to the C group, $p<0.07$. C, control group; HS, hindlimb suspension group; HS+PD, hindlimb suspension + treatment with calpain inhibitor (PD150606).

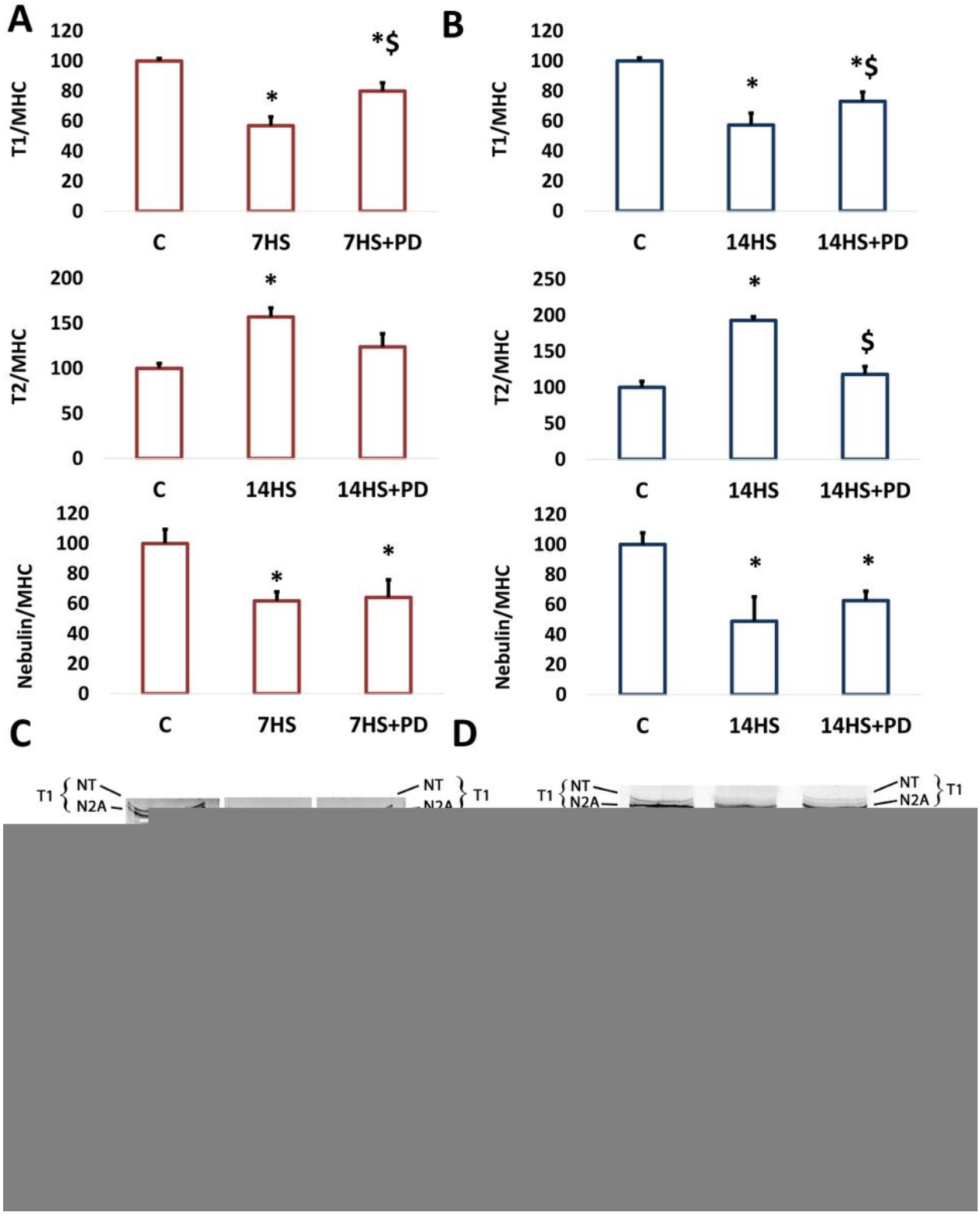

Figure 3

Quantification of intact titin (T1), proteolytic fragment of titin (T2) and nebulin in rat soleus muscle after 7-day HS **(A)**. Quantification of intact titin (T1), proteolytic fragment of titin (T2) and nebulin in rat soleus after 14-day HS **(B)**. Representative images for the studied proteins in the 7-day experiment **(C)**. Representative images for the studied proteins in the 14-day experiment **(D)**. Data shown as % of the C group (Mean±SEM), n=8 per group. * – significant difference from the C group, $p<0.05$; $ – significant

difference from the 7HS group ($p<0.05$). C, control group; HS, hindlimb suspension group; HS+PD, hindlimb suspension + treatment with calpain inhibitor (PD150606).