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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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 analysis and ex vivo measurements of passive tension with or without blebbistatin treatment (an inhibitor of actomyosin interactions). Passive tension of isolated soleus muscle was significantly reduced after 7- and 14-day HS compared to the 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.

Keywords Soleus muscle · Calpains · PD150606 · Passive stiffness · Cytoskeletal proteins · Blebbistatin

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, 27]. 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 [51]. 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 a passive component, determined by the extracellular matrix (ECM) and 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 [9] and single muscle fibers [55]. 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 [1, 14]. 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 [41].

Of particular interest is the giant sarcomeric protein titin, which is known to significantly contribute to the passive stiffness of skeletal muscles [19, 29]. A significant reduction in titin content in soleus muscle has been previously shown following 14 days of HS [25, 55]. 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 [47]. At the same time, 3-day mechanical unloading did not affect the content of intact titin in rat soleus muscle [17]. It is possible that the mechanisms underlying a reduction in soleus muscle passive stiffness at later stages of unloading would differ from those mechanisms that are involved during the first 3 days of unloading. One of the key proteolytic systems existing in skeletal muscles is the calpain system (please see an excellent review by Hyatt and Powers (2020) for further reading on the calpain system in skeletal muscles) [22]. Calpains represent calcium-activated cysteine proteases that are known to be activated in skeletal muscles under unloading conditions [12, 13]. Calpains exert their function via cleaving target substrates at specific sites, forming fragmented proteins that can be further degraded by other proteolytic systems such as the ubiquitin–proteasome system [22]. To date, a significant role of calpains in the proteolytic cleavage of sarcomeric and cytoskeletal proteins (titin, nebulin, and desmin and others) is well established [16, 21]. Several previous studies have shown that inhibition of calpains is able to obviate such unloading-induced changes as decreased muscle mass, reduced maximum specific force, disruption of sarcomere structure, increased protein ubiquitination, and slow to fast myosin transformation [32, 44, 49, 54]. However, in the current study, we for the first time attempted to elucidate the possible role of calpain-dependent proteolysis in a reduction in soleus muscle passive stiffness related to alterations in the key cytoskeletal/sarcomeric proteins (titin, nebulin, desmin) under different stages of mechanical unloading. We hypothesized that a HS-induced decrease in passive stiffness would be associated with calpain-dependent degradation of cytoskeletal proteins or a decrease in actomyosin interaction. 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 eight animals were collected for measurements of passive tension with or without blebbistatin incubation. Soleus muscles from the remaining eight animals were subjected to biochemical analysis to assess the abundance of cytoskeletal proteins.

In experiment 1, the rats were randomly assigned to the following three 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, the 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 route of administration and PD150606 dosage was selected based on previously published reports [28, 49]. 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 h light/dark cycle. All rats had access to a standard diet and water ad libitum. Prior to soleus muscle collection, the animals were anesthetized with an intraperitoneal injection of tribromoethanol (400 mg/kg). The animals were euthanized by decapitation under deep anesthesia.

Hindlimb suspension

Mechanical unloading was carried out using a standard hindlimb suspension (HS) model [37]. 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 [57]. 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 min. One of the muscles was incubated for 15 min in saline at 80°C. Optimal muscle length (L₀) was determined with a series of twitch contractions (0.5 ms, 10 V). 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 2 min, 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 three 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 [24, 43]. 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.

RT-PCR analysis

RT-PCR analysis was performed as previously described [56]. In brief, reverse transcription was performed by incubation of 0.5 μg of RNA, random hexamers d(N)₆, dNTPs, RNase inhibitor, and MMLV reverse transcriptase for 60 min at 42 °C. The samples to be compared were run under similar conditions (template amounts, duration of PCR cycles). Relative quantification was performed based on the threshold cycle (CT value) for each of the PCR samples [30]. RPL19 mRNA expression was not significantly altered in any of the experimental groups compared to the control; thus, RPL19 mRNA expression was chosen for normalization of all PCR data in the current study. PCR primers used for RNA analysis are shown in Table 1.

Determination of the abundance of cytoskeletal proteins

Western blotting was carried out as previously described [57]. 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 2× sample electrophoresis buffer (5.4 mM Tris–HCl (pH 6.8), 4% Ds-Na, 20% glycerin, 10% 2-mercaptoethanol, 0.02% bromophenol 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, 1:10,000), 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 1:50,000. 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 [57]. 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 [52], with modifications aimed at improving the focusing of the studied protein bands in the gel [61]. To ensure equal

| Gene          | Primer sequence                  |
|---------------|----------------------------------|
| RPL19         | 5′-GTACCCCTCTCCTCTCCATGTC-3′      |
| collagen Ia   | 5′-ATCAAGCCCAAACCCAAAGGAG-3′      |
| collagen IIIa | 5′-TGATGGATCAATGAGGAGA-3′         |
| collagen IV   | 5′-TTCCCTGTAACCAACCTGGTATG-3′     |
| collagen Vla2 | 5′-ACGCTACGGAGACTGTACA-3′         |
| fibronectin   | 5′-GCCGCACCTTCTTCTGACAC-3′        |
| ubiquitin     | 5′-ACCTCCAGTGATGTCCTGC-3′         |
| LC3B          | 5′-GAGAACGAGCTCCTCGTGTT-3′        |

Table 1 List of primers used for PCR
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 deviation (SD). 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 (Table 2). 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 2 and 3). Changes in mechanical properties of the isolated soleus muscle from experiment 1 and experiment 2 are presented in Tables 2 and 3, respectively.

| Table 2 | Soleus weight and mechanical properties after 7-day HS |
|---------|-----------------------------------------------|
| C       | 7HS                                           | 7HS + PD                      |
| Body weight, g                | 195.4 ± 12.8                                | 190.2 ± 16.7                  | 188.6 ± 7.8                |
| Soleus weight, mg              | 98.1 ± 20.8                                  | 69.2 ± 4.67                   | 82.3 ± 13.55               |
| Soleus weight/body weight, mg/g | 5.1 ± 1.3                                    | 3.7 ± 0.37*                   | 4.33 ± 0.65*               |
| Muscle length, mm              | 19.8 ± 1.0                                   | 18.7 ± 1.2                    | 21.14 ± 1.1                |
| CSA, mm²                       | 4.7 ± 1.0                                    | 3.3 ± 0.3*                    | 3.58 ± 0.7*                |
| Twitch tension, mN             | 77.8 ± 17.3                                  | 51.1 ± 4.7*                   | 74.25 ± 6.2$               |
| Passive tension, mN            | 117.7 ± 23.2                                 | 68.5 ± 6.37                   | 99.5 ± 8.3$                |
| Passive tension/CSA, mN/mm²    | 25.4 ± 2.4                                   | 21.0 ± 2.8*                   | 28.4 ± 3.9$                |
| Passive tension + blebbistatin, mN | 77.4 ± 14.2*                           | 36.1 ± 4.5$*                   | 59.1 ± 20.8*              |
| Passive tension/CSA + blebbistatin, mN/mm² | 17.4 ± 2.9#                              | 12.3 ± 1.8*#                   | 18.6 ± 5.9*#            |

Data are shown as mean ± SD. 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 (\( p < 0.05 \)), 5 significant difference from the 7HS group (\( p < 0.05 \)), 4 significant difference of blebbistatin-treated muscles form blebbistatin-untreated muscles (\( p < 0.05 \)).

| Table 3 | Soleus weight and mechanical properties after 14-day HS |
|---------|-----------------------------------------------|
| C       | 14HS                                          | 14HS + PD                      |
| Body weight, g                | 241.2 ± 12.6                                | 218.6 ± 18.8                  | 223.6 ± 12.6                |
| Soleus weight, mg              | 116.1 ± 15.1                                 | 68.3 ± 6.8*                   | 61.5 ± 8.4*                 |
| Soleus weight/body weight, mg/g | 4.8 ± 0.8                                    | 3 ± 0.2*                      | 2.7 ± 0.4*                  |
| Muscle length, mm              | 22.1 ± 1.5                                   | 19.7 ± 1.6                    | 21.4 ± 1.7                  |
| CSA, mm²                       | 4.5 ± 0.6                                    | 3.0 ± 0.5*                    | 3.3 ± 0.5*                  |
| Twitch tension, mN             | 86.8 ± 18.7                                  | 56.8 ± 11.0*                  | 92.4 ± 19.4$               |
| Passive tension, mN            | 129.7 ± 25.0                                 | 62.8 ± 14.4*                  | 123.9 ± 26.8$              |
| Passive tension/CSA, mN/mm²    | 26.5 ± 5.5                                   | 18.8 ± 2.8*                   | 50.5 ± 9.8$*               |
| Passive tension + blebbistatin, mN | 67.7 ± 12.2*                         | 31.75 ± 7.2*#                   | 98.1 ± 27.6*#            |
| Passive tension/CSA + blebbistatin, mN/mm² | 13.8 ± 2.4#                              | 10.6 ± 2.6*#                   | 35.9 ± 10.8*#            |

Data are shown as mean ± SD. 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 (\( p < 0.05 \)), 5 significant differences from the 14HS group (\( p < 0.05 \)), 4 significant difference of blebbistatin-treated muscles form blebbistatin-untreated muscles (\( p < 0.05 \)).
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 in 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, the 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 the 14HS group (Fig. 1b). Thus, as in the 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, the 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.

**Fig. 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 ± SD), 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). Circles represent individual data points.
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 toward 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.
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 the 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.
The content of titin T1 in the 14HS + PD group was higher than in the 14HS group, but still significantly lower 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 the 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).

Expression levels of the ECM markers and markers of proteolysis

One-week unloading induced a significant 64% decrease in the mRNA expression of collagen Ia. Other types of collagen were not significantly changed after 7-day HS (Fig. 4a). In the 7HS + PD group, there was a significant increase in the collagen Ia, IIIa, and IV mRNA expression compared to the 7HS group (Fig. 4a). Collagen IV and fibronectin mRNA expression levels in the 7HS + PD group were even higher than in the C group (Fig. 4a). One-week unloading also led to a significant 113% increase in ubiquitin mRNA expression compared to the C group, which was prevented by PD150606 administration (Fig. 4c). We observed no changes in LC3B mRNA expression (a well-known autophagy marker) in soleus muscle in the studied groups of rats (Fig. 4c).

Two weeks of HS resulted in a significant reduction in collagen Ia and IV mRNA expression by 55% and 50%, respectively (Fig. 4b). PD150606 administration during 14-day HS prevented a reduction in collagen IV mRNA expression (Fig. 4b). We also observed a 58% decrease in collagen Vla2 mRNA expression in the 14HS + PD group compared to the C group (Fig. 4b). Ubiquitin mRNA expression significantly increased by 72% after 14-day HS, and this increase was prevented by PD150606 administration (Fig. 4d). No changes in LC3B mRNA expression were observed in the 14-day experiment (Fig. 4d).

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

![Figure 4](https://example.com/fig4.png)

**Fig. 4** Collagen Ia, IIIa, IV, and Vla2 mRNA content after 7-day HS (a). Collagen Ia, IIIa, IV, and Vla2 mRNA content after 14-day HS (b). Fibronectin, ubiquitin, and LC3B mRNA content after 7-day HS (c). Fibronectin, ubiquitin, and LC3B mRNA content after 14-day HS (d). Data are shown as % of the C group (mean ± SD), 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). Circles represent individual data points
the isolated rat soleus muscle in response to 7- or 14-day mechanical unloading, observed in the present study, is in good agreement with previous reports on the effect of hindlimb unloading on the passive tension of single soleus muscle fibers of rats [55]. 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 [41]. 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 [41]. In the present study, similar results were obtained: a significant decrease in the 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. In addition, the administration of 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 the 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 [47, 55, 58, 60]. It is well established that a giant protein titin significantly contributes to the intrinsic passive stiffness/tension of muscle fibers [18, 55]. 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 [15]. It is known that giant proteins (titin and nebulin) undergo partial degradation under conditions of real or simulated weightlessness in both animals and humans [26, 48, 59, 60]. Degradation of titin could be associated with the activation of calcium-dependent proteases known as calpains. Murphy et al. 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 [39]. Interestingly, in the current study, we observed that the contents of titin fragments between day 7 and day 14 of HS showed only minute changes between the HS + PD groups. At both time points (7 and 14 days), there was no significant difference between the C and the HS + PD groups. At the same time, there was a substantial change in the soleus muscle specific passive stiffness between day 7 and day 14, especially in the 14HS + PD group which was almost twofold as stiff as under control conditions. One possible explanation for this phenomenon is that this significant increase in the soleus muscle–specific passive stiffness in the HS + PD group compared to the control values could be associated with changes in extracellular components of passive tension such as proteins of the ECM. Indeed, Marcucci et al. have recently shown that ECM significantly contributes to the whole passive structural stiffness within a bundle of fibers taken from human vastus lateralis muscle [33]. However, it should be noted that the muscle bundles were taken from elderly subjects and ECM stiffness in this case could be greater than in younger subjects, as suggested by data obtained in murine skeletal muscles [62]. In our study, RT-PCR revealed that calpain inhibition during 7-day HS resulted in a significant upregulation in collagen Ia, collagen IIIA, collagen IV, and fibronectin mRNA expression compared to the 7HS group. We speculate that this increase in the gene expression of the key ECM proteins could lead to enhanced synthesis of different types of collagen and fibronectin between day 7 and day 14 of HS ultimately resulting in a greater ECM stiffness by day 14 of HS, thereby contributing to the overall passive stiffness of the soleus muscle in the 14H + PD group.

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 toward 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 versus 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 [35, 40, 57]. Furthermore, Enns et al. demonstrated that as early as after 2–3 days of HS occurs a decrease in desmin content in rat mixed vastus muscles with subsequent restoration of desmin content to control levels by the ninth day of HS [13]. The reason for the 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 rat mixed vastus muscles with subsequent restoration of desmin content to control levels by the ninth day of HS [13]. The reason for the 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 rat mixed vastus muscles with subsequent restoration of desmin content to control levels by the ninth day of HS [13]. The reason for the 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 rat mixed vastus muscles with subsequent restoration of desmin content to control levels by the ninth day of HS [13].
in rat soleus muscle is upregulated following 1, 3, and 9 days of mechanical unloading [12]. Furthermore, since calpains are calcium-dependent proteases, activation of calpains during HS is likely associated with increased concentration of calcium ions in the sarcoplasm of soleus muscle fibers, which was earlier demonstrated in both rats [2] and mice [23]. Emerging evidence suggests several potential mechanisms that could explain an increase in cytosolic levels of calcium in skeletal muscle fibers during mechanical unloading/muscle disuse. The first mechanism of increased calcium levels may be associated with a flux of extracellular calcium due to opening of calcium ion channels. It was shown that under muscle denervation transient receptor potential, subfamily V, member 2 (TRPV2) and purinergic channels (P2X7R) become activated [10]. Thus, skeletal muscle inactivity could lead to the activation of extracellular calcium channels resulting in increased cytosolic calcium levels and calpain activation. Another mechanism of increased calcium levels with unloading could be associated with ROS-induced calcium leak from the sarcoplasmic reticulum due to dysfunction of ryanodine receptor 1 (RyR1) [34]. Indeed, a significant increase in a passive calcium leakage from the SR in single muscle fibers of rat soleus muscle was previously showed after 14-day hindlimb unloading [64]. In support of the RyR1-related mechanism, leaky RyR1 was suggested to play a potential role in muscle fatigue [7], muscle dystrophy [5], and sarcopenia [4] and this could be associated with cysteine nitrosylation or oxidation resulting in the weak binding of 12-kDa FK506-binding protein (FKBP12) with RyR1 [63]. Evidence also suggests that dihydropyridine receptors (L-type calcium channels) can also be involved in the accumulation of intracellular calcium ions in skeletal muscle fibers under unloading conditions [38]. Furthermore, an elevation of cytosolic calcium levels could be also attributed to a decreased function of sarco(endo)plasmic reticulum calcium ATPase (SERCA), a pump, which is responsible for lowering cytosolic calcium by bringing it into the SR. A recent study by Braun et al. demonstrated an impaired SERCA-related calcium uptake in mouse soleus muscle after 35–37 days of spaceflight (unloading) [8]. Moreover, Thom et al. observed a significant reduction in the maximal rate of SR calcium uptake in human vastus lateralis muscle after 10-day lower limb cast immobilization [53].

In addition, it is known that nitric oxide II (NO), the production of which is known to be decreased in soleus muscle under unloading conditions [31, 45, 46], could also be involved in the regulation of calpain-dependent degradation of cytoskeletal proteins [50]. It is also important to note that, in the present study, treatment of rats with PD150606 during both 7-day and 14-day HS completely prevented an increased level of ubiquitin mRNA expression in rat soleus muscle. This finding is in agreement with a previous study involving 3-day HS rats [49] and suggests that the preservation of cytoskeletal proteins in soleus muscle of PD150606-treated rats could be partly attributed to downregulation of the ubiquitin–proteasome system.

The results obtained in the present study are in good agreement with a previously published report by Salazar et al. showing that the 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 [44]. 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 [44]. These data by Salazar et al. 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 the intrinsic passive stiffness of rat soleus muscle.

**Author contribution** I.M., K.S., and S.T. performed animal experiments. I.M. and K.S. performed Western blot analysis. A.U. and I.V. performed titin and nebulin electrophoresis. S.T. performed isolated muscle mechanical measurements. K.S. performed PCR analysis. S.T., T.M., and B.S. contributed to manuscript writing, image processing, and interpretation of the data. B.S. 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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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.
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).

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no competing interests.

References

1. Allingham JS, Smith R, Rayment I (2005) The structural basis of blebbistatin inhibition and specificity for myosin II. Nat Struct Mol Biol 12:378–379. https://doi.org/10.1038/nsmb908
2. Altaeva EG, Lysenko LA, Kantserova NP, Nemova NN, Shenkman BS (2010) The basal calcium level in fibers of the rat soleus muscle under gravitational unloading: the mechanisms of its increase and the role in calpain activation. Dokl Biol Sci 433:241–243. https://doi.org/10.1134/S0012496610040010
3. Anderson J, Li Z, Goubel F (2001) Passive stiffness is increased in soleus muscle of desmin knockout mouse. Muscle Nerve 24:1090–1092.
4. Andersson DC, Betzenhauser MJ, Reiken S, Meli AC, Umanskaya A, Shiomi T, D’Armiento J, Marks AR (2012) Leaky ryanodine receptors in beta-sarcoglycan deficient mice: a potential common defect in muscular dystrophy. Skelet Muscle 2:9. https://doi.org/10.1186/2044-5040-2-9
5. Aweida D, Rudesky I, Volodin A, Shimko E, Cohen S (2018) GSK3-beta promotes calpain-1-mediated desmin filament depolymerization and myofibrillosis in atrophy. J Cell Biol 217:3698–3714. https://doi.org/10.1083/jcb.201802018
6. Bellinger AM, Reiken S, Dura M, Murphy PW, Deng SX, Landry DW, Nieman D, Leharte SR, Samara M, LaCampagne AR, Marks AR (2008) Remodeling of ryanodine receptor complex causes “leaky” channels: a molecular mechanism for decreased exercise capacity. Proc Natl Acad Sci USA 105:2198–2202. https://doi.org/10.1073/pnas.0711074105
7. Braun JL, Gromerella MS, Hamstra SI, Messner HN, Fajardo VA (2021) Characterizing SERCA function in murine skeletal muscles after 35–37 days of spaceflight. Int J Mol Sci 22. https://doi.org/10.3390/ijms222111764
8. Canon F, Goubel F (1995) Changes in stiffness induced by hindlimb suspension in rat soleus muscle. Pflugers Arch 429:332–337. https://doi.org/10.1007/bf00374147
9. Cea LA, Cisterna BA, Puebla C, Frank M, Figueroa XF, Cardozo C, Willocq K, Latorre R, Szczesny C (2013) De novo expression of connexin hemichannels in denervated fast skeletal muscles leads to atrophy. Proc Natl Acad Sci USA 110:16229–16234. https://doi.org/10.1073/pnas.1312331110
10. Chopard A, Pons F, Marini JP (2001) Cytoskeletal protein contents before and after hindlimb suspension in a fast and slow rat skeletal muscle. Am J Physiol Regul Integr Comp Physiol 280:E323–E330. https://doi.org/10.1152/ajpregu.2001.280.2.E323
11. Enns DL, Belcastro AN (2006) Early activation and redistribution of calpain activity in skeletal muscle during hindlimb unweighting and reweighting. Can J Physiol Pharmacol 84:601–609. https://doi.org/10.1139/y06-013
12. Enns DL, Raastad T, Ugelstad I, Belcastro AN (2007) Calpain/calpastatin activities and substrate depletion patterns during hindlimb unweighting and reweighting in skeletal muscle. Eur J Appl Physiol 100:445–455. https://doi.org/10.1007/s00421-007-0445-4
13. Farman GP, Tachampa K, Mateja R, Cazorla O, Lacampagne A, de Tombe PP (2008) Blebbistatin: use as inhibitor of muscle contraction. Pflugers Arch 455:995–1005. https://doi.org/10.1007/s00424-007-0375-3
14. Gault M, Djinov-Carugo K (2016) The sarcomeric cytoskeleton: from molecules to motion. J Exp Biol 219:135–145. https://doi.org/10.1242/jeb.124941
15. Goll DE, Thompson VF, Li H, Wei W, Cong J (2003) The calpain system. Physiol Rev 83:731–801. https://doi.org/10.1152/physrev.00029.2002
16. Goto K, Okuyama R, Honda M, Uchida H, Akema T, Ohira Y, Yoshioka T (2003) Profiles of connectin (titin) in atrophied soleus muscle induced by unloading of rats. J Appl Physiol 94:897–902. https://doi.org/10.1152/japphysiol.00408.2002
17. Granzier H, Labeit S (2007) Structure-function relations of the giant elastic protein titin in striated and smooth muscle cells. Muscle Nerve 36:740–755. https://doi.org/10.1002/mus.20886
18. Hyatt HW, Powers SK (2020) The role of calpains in skeletal muscle remodeling with exercise and inactivity-induced atrophy. Int J Sports Med. https://doi.org/10.1055/a-1199-7662
19. Ingalls CP, Wenke JC, Armstrong RB (2001) Time course changes in [Ca2+]i, force, and protein content in hindlimb-suspended mouse soleus muscles. Aviat Space Environ Med 72:471–476
20. Kanzaki K, Watanabe D, Kuratani M, Yamada T, Matsunaga S, Wada M (2017) Role of calpain in eccentric contraction-induced proteolysis of Ca(2+)-regulatory proteins and force depression in rat fast-twitch skeletal muscle. J Appl Physiol 122:396–405. https://doi.org/10.1152/japplphysiol.00270.2016
21. Kasper CE (1995) Sarcolemmal disruption in reloaded atrophic skeletal muscle. J Appl Physiol 79:607–614
22. Kasper CE, Xun L (2000) Expression of titin in skeletal muscle varies with hind-limb unloading. Biol Res Nurs 2:107–115. https://doi.org/10.1177/109980040020010204
23. Kozlovskaya I, Dmitrieva I, Grigorieva L, Kirenskaya A, Kreydich Y (1988) Gravitational mechanisms in the motor system. Stud Physiol Rev 337. https://doi.org/10.1007/bf00374147
24. Kanzaki K, Watanabe D, Kuratani M, Yamada T, Matsunaga S, Wada M (2017) Role of calpain in eccentric contraction-induced proteolysis of Ca(2+)-regulatory proteins and force depression in rat fast-twitch skeletal muscle. J Appl Physiol 122:396–405. https://doi.org/10.1152/japplphysiol.00270.2016
25. Kasper CE (1995) Sarcolemmal disruption in reloaded atrophic skeletal muscle. J Appl Physiol 79:607–614
30. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔ Ct) method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

31. Lomonosova YN, Kalamarov GR, Bugrova AE, Shevchenko TF, Kartashkina NL, Lysenko EA, Shvets VI, Nemirovskaya TL (2011) Protective effect of L-arginine administration on proteins of unloaded m. soleus. Biochem Biokhimia 76:571–580. https://doi.org/10.1134/S0006297911050075

32. Ma XW, Li Q, Xu PT, Zhang L, Li H, Yu ZB (2011) Tetanic contractions impair sarcomeric Z-disc of atrophic soleus muscle via calpain pathway. Mol Cell Biochem 354:171–180. https://doi.org/10.1007/s11010-011-0816-3

33. Marcucci L, Bondi M, Randazzo G, Reggiani C, Natali AN, Pavan PG (2019) Fibre and extracellular matrix contributions to passive forces in human skeletal muscles: An experimental based constitutive law for numerical modelling of the passive element in the classical Hill-type three element model. PLoS ONE 14:e0224232. https://doi.org/10.1371/journal.pone.0224232

34. Matecki S, Dridi H, Jung B, Saint N, Reiken SR, Scheurmann V, Mrozek S, Santulli G, Umanskaya A, Petrof BJ, Saber S, Marks AR, Lacampagne A (2016) Leaky ryanodine receptors contribute to diaphragmatic weakness during mechanical ventilation. Proc Natl Acad Sci USA 113:9069–9074. https://doi.org/10.1073/pnas.1609707113

35. Mirzoev TM, Shenkman BS, Ushakov IB, Ogneva IV (2012) Desmin and alpha-actinin-2 content in rat soleus muscle in the dynamics of gravitational unloading and subsequent reloading. Dokl Biochem Biophys 444:144–146. https://doi.org/10.1134/S1607672912030052

36. Mohrhauser DA, Underwood KR, Weaver AD (2011) In vitro degradation of bovine myofibrils is caused by μ-calpain, not caspase-3. J Anim Sci 89:798–808. https://doi.org/10.2527/jas.2010-3149

37. Morey-Holter ER, Globus RK (2002) Hindlimb unloading rodent model: technical aspects. J Appl Physiol 92:1367–1377. https://doi.org/10.1152/japplphysiol.00969.2001

38. Mukhina AM, Altueva EG, Nemirovskaya TL, Shenkman BS (2008) The role of L-type calcium channels in the accumulation of Ca2+ in soleus muscle fibers in the rat and changes in the ratio of myosin and sera isoforms in conditions of gravitational unloading. Neurosci Behav Physiol 38:181–188. https://doi.org/10.1007/s11055-008-0027-x

39. Murphy RM, Verburg E, Lamb GD (2006) Ca2+ activation of muscle. Muscle Nerve 26:404–412. https://doi.org/10.1002/mus.10219

40. Ogneva IV (2010) Transversal stiffness of fibrils and desmin content in leg muscles of rats under gravitational unloading of various durations. J Appl Physiol 109:1702–1709. https://doi.org/10.1152/japplphysiol.00793.2010

41. Petrao IO, Tyaganov SA, Mirzoev TM, Tsaturyan AK, Kozlovskaya IB, Shenkman BS (2018) Early decline in rat soleus passive tension with hindlimb unloading: inactivation of cross-bridges or activation of calpains? Dokl Biochem Biophys 481:205–207. https://doi.org/10.1134/S1607672918040075

42. Roman BI, Verhasseti S, Mangodi CW, De Wever O, Stevens CV (2018) Synthesis of C-ring-modified blebbistatin derivatives and evaluation of their myosin II ATPase inhibitory potency. Bioorg Med Chem Lett 28:2261–2264. https://doi.org/10.1016/j.bmcl.2018.05.041

43. Roy RR, Zhong H, Monti RJ, Vallance KA, Edgerton VR (2002) Mechanical properties of the electrically silent adult rat soleus muscle. Muscle Nerve 26:404–412. https://doi.org/10.1002/mus.10219

44. Salazar JJ, Michele DE, Brooks SV (2010) Inhibition of calpain prevents muscle weakness and disruption of sarcomere structure during hindlimb suspension. J Appl Physiol 108:120–127. https://doi.org/10.1152/japplphysiol.01080.2009

45. Sharlo KA, Paramonova II, Lvova ID, Mochalova EP, Kalashnikov VE, Vilchinskaya NA, Tyaganov SA, Konstantinova TS, Shevchenko TF, Kalamarov GR, Shenkman BS (2021) Plantar mechanical stimulation maintains slow myosin expression in disused rat soleus muscle via NO-dependent signaling. Int J Mol Sci 22. https://doi.org/10.3390/ijms22031372

46. Sharlo KA, Paramonova II, Lvova ID, Vilchinskaya NA, Bugrova AE, Shevchenko TF, Kalamarov GR, Shenkman BS (2020) NO-Dependent mechanisms of myosin heavy chain transcription regulation in rat soleus muscle after 7-days hindlimb unloading. Front Physiol 11:814. https://doi.org/10.3389/fphys.2020.00814

47. Shenkman BS, Nemirovskaya TL, Belozerova IN, Vikhlyantsev IM, Matveeva OA, Staroverova KS, Podlubnaya ZA (2002) Effects of Ca2+(-) binding agent on unloaded rat soleus: muscle morphology and sarcomeric titin content. J Gravit Physiol 9:P139-140

48. Shenkman BS, Podlubnaya ZA, Vikhiliantsev IM, Litvinova KS, Udal’tsov SN, Nemirovskaya TL, Lemesheva IU, Mukhina AM, Kozlovskaya IB (2004) Human soleus fibers contractile characteristics and sarcomeric cytoskeletal proteins after gravitational unloading. Contribution of support stimulus. Biofizika 49:881–890

49. Shenkman BS, Belova SP, Lomonosova YN, Kostrominova TY, Nemirovskaya TL (2015) Calpain-dependent regulation of the skeletal muscle atrophy following unloading. Arch Biochem Biophys 584:36–41. https://doi.org/10.1016/j.abb.2015.07.011

50. Shenkman BS, Nemirovskaya TL, Lomonosova YN (2015) NO-dependent signaling pathways in unloaded skeletal muscle. Front Physiol 6:298. https://doi.org/10.3389/fphys.2015.00298

51. Shenkman BS, Tsaturyan AK, Vikhlyantsev IM, Kozlovskaya IB, Grigoriev AI (2021) Molecular mechanisms of muscle tone impairment under conditions of real and simulated space flight. Acta Naturae 13:13–25

52. Tatsumi R, Hattori A (1995) Detection of giant myofibrillar proteins connectin and nebulin by electrophoresis in 2% polyacrylamide slab gels strengthened with agarose. Anal Biochem 224:28–31. https://doi.org/10.1016/0003-2697(95)10044-4

53. Thom JM, Thompson MW, Ruel PA, Bryant GJ, Fonda JS, Harmer AR, Janse de Jonge XA, Hunter SK (2001) Effect of 10-day cast immobilization on sarcoplasmic reticulum calcium regulation in humans. Acta Physiol Scand 172:141–147. https://doi.org/10.1046/j.1365-201x.2001.00853.x

54. Tidball JG, Spencer MJ (2002) Expression of a calpastatin transgene slows muscle wasting and obviates changes in myosin isoform expression during murine muscle disuse. J Physiol 545:819–828. https://doi.org/10.1113/jphysiol.2002.024935

55. Tourse T, Stevens L, Granzier H, Moumier Y (2002) Passive tension of rat skeletal soleus muscle fibers: effects of unloading conditions. J Appl Physiol 92:1465–1472. https://doi.org/10.1152/japplphysiol.00621.2001

56. Tyaganov SA, Mochalova EP, Belova S, Sharlo K, Rozhkov S, Kalashnikov V, Turitikova O, Mirzoev T, Shenkman B (2021) Plantar mechanical stimulation attenuates protein synthesis decline in disused skeletal muscle via modulation of nitric oxide level. Sci Rep 11:Art9 8906. https://doi.org/10.1038/S41598-021-89362-6

57. Tyaganov SA, Mochalova EP, Melnikov YI, Vikhlyantsev IM, Ulanova AD, Sharlo KA, Mirzoev TM, Shenkman BS (2021) NOS-dependent effects of plantar mechanical stimulation on mechanical characteristics and cytoskeletal proteins in rat soleus.
58. Udaka J, Ohmori S, Terui T, Ohotsuki I, Ishiwata S, Kurihara S, Fukuda N (2008) Disuse-induced preferential loss of the giant protein titin depresses muscle performance via abnormal sarcomeric organization. J Gen Physiol 131:33–41. https://doi.org/10.1085/jgp.200709888

59. Ulanova A, Gritsyna Y, Vikhlyantsev I, Salmov N, Bobylev A, Abdusalamova Z, Rogachevsky V, Shenkman B, Podlubnaya Z (2015) Isoform composition and gene expression of thick and thin filament proteins in striated muscles of mice after 30-day space flight. Biomed Res Int 2015:104735. https://doi.org/10.1155/2015/104735

60. Ulanova A, Gritsyna Y, Salmov N, Lomonosova Y, Belova S, Nemirovskaya T, Shenkman B, Vikhlyantsev I (2019) Effect of L-arginine on titin expression in rat soleus muscle after hindlimb unloading. Front Physiol 10:1221. https://doi.org/10.3389/fphys.2019.01221

61. Vikhlyantsev IM, Podlubnaya ZA (2017) Nuances of electrophoresis study of titin/connectin. Biophys Rev 9:189–199. https://doi.org/10.1007/s12551-017-0266-6

62. Wood LK, Kayupov E, Gumucio JP, Mendias CL, Claflin DR, Brooks SV (2014) Intrinsic stiffness of extracellular matrix increases with age in skeletal muscles of mice. J Appl Physiol 117:363–369. https://doi.org/10.1152/japplphysiol.00256.2014

63. Xia R, Stangler T, Abramson JJ (2000) Skeletal muscle ryanodine receptor is a redox sensor with a well defined redox potential that is sensitive to channel modulators. J Biol Chem 275:36556–36561. https://doi.org/10.1074/jbc.M007613200

64. Yoshioka T, Shirota T, Tazoe T, Yamashita-Goto K (1996) Calcium movement of sarcoplasmic reticulum from hindlimb suspended muscle. Acta Astronaut 38:209–212. https://doi.org/10.1016/0094-5765(96)00010-0

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