The hindlimb unloading model of microgravity differently affects skeletal muscle properties of young and adult mice

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Abstract

Disuse induces atrophy and slow-to-fast transition of skeletal muscle fibers leading to an impaired contractile function. In the hindlimb unloading (HU) rat model, anti-gravitational muscles, such as the slow-twitch soleus muscle, undergo a severe atrophy, with a reduction of muscle weight and fiber diameter, and a slow-to-fast fiber type transition accompanied by profound changes in ion channel expression/function and Ca2+ homeostasis. Little is known about the disuse-induced alterations of skeletal muscle when hindlimb unloading is applied to mice. In the present study we measured atrophy, the resting chloride conductance of sarcolemma (gCl) and the resting cytosolic calcium concentration (restCa) in soleus (Sol) muscle of young (two-three months old) and adult (six-seven months old) mice after 14 days of HU. In adult mice HU induced alterations of muscle functional parameters resembling those observed in the HU rat model, including a significant atrophy of Sol muscle with a reduction of the muscle-to-body weight ratio, an increase of gCl and a concomitant decrease of restCa. Surprisingly, in young mice the atrophy indices and Ca2+ homeostasis, but not the gCl, resulted differently affected by HU with respect to the adult mice. We suggest that growth signals in young and still-growing mice may somewhat counteract HU effects. In conclusion, the HU adult mouse is a useful animal model to gain insight into the cellular/molecular mechanisms responsible for disuse-induced muscle impairment, at the aim to evaluate the possible benefits of drugs in protecting muscle contractile function, and to prepare future spaceflight missions.

Key Words: muscle disuse, age, hindlimb unloading, mouse, muscle atrophy

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Exposure to microgravity during space flights induces atrophy and slow-to-fast transition of skeletal muscle fibers contributing to impaired motor activity and postural maintenance of the astronauts upon return to Earth [7]. Moreover it is widely acknowledged that the fragility of atrophied muscles may expose individuals to dramatic muscle damage in response to normal activity. These effects may be quite similar to those observed in conditions of muscle disuse encountered under normal gravity during prolonged immobilization due to bed rest, trauma, or chronic invalidating diseases. The hindlimb unloading (HU) rat model was developed in the seventies to mimic the effects of space flights on bone homeostasis, and was further widely used to investigate the effects of disuse on skeletal muscle function [14]. In this model, anti-gravitational muscles, such as the slow-twitch soleus muscle, undergo a severe atrophy, with a reduction of muscle weight and fiber diameter, and a slow-to-fast fiber type transition characterized by an increased expression of fast isoforms of myosin heavy chain (MHC). In previous studies we demonstrated that in parallel to these phenomena, soleus muscle of HU rats show profound changes in ion channel expression/ function and Ca2+ homeostasis [5, 6, 8, 18, 19]. For instance, in accord with fiber type transition, the macroscopic chloride conductance (gCl) of sarcolemma at rest, a determinant of muscle excitability, increases, while the resting cytosolic calcium concentration (restCa) decreases in Sol muscle of HU rats, thereby reaching values more similar to those of a fast-twitch muscle. Such alterations likely contribute to functional impairment of postural muscles in the normal gravity environment and their molecular correlates may represent promising targets for effective countermeasures.

Recently, due to the possibility to easily manipulate mouse genome as well as to include mice in future space missions, there has been a growing interest toward the murine HU model. Nevertheless, in contrast

to rats, little is known about the disuse-induced alterations of skeletal muscle when hindlimb suspension is applied to mice. In the present study we measured atrophy, the gCl and the restCa in soleus (Sol) muscle of young (two-three months old) and adult (six-seven months old) mice after 14 days of hindlimb suspension. Such an experimental approach allowed us to characterize the HU mouse model, compared to the HU rat, and at the same time to explore the influence of the age period on muscle disuse mechanisms. Indeed it is noteworthy that both gCl and restCa are sensitive indices of the differentiated state of the muscle. For instance, the gCl dramatically increases during early postnatal development up to adult age [3, 4], whereas it is significantly lowered during aging process [17] in concomitance to an increase of restCa [9].

The results show that HU effects in adult mice were very similar to those previously observed in HU rats. In contrast, HU differently affected muscle weight loss and restCa, but not the gCl, in the young, still growing mice, probably due to counteracting effects of growth signaling. In conclusion, the adult HU mouse appears as a valuable model for the study of disuse-induced muscle impairment.

Materials and Methods

Animal care and hindlimb unloading

Experiments were approved by the Italian Health Department and complied to the Italian guidelines for the use of laboratory animals, which conform with the European Community Directive published in 1986 (86/609/ECC). Two-three month and 6-7 month-old male C57BL mice, weighting 21-22 g and 28-29 g respectively (Charles River Laboratories, Calco, Italy), were randomly assigned to control (CTRL) and hindlimb unloading (HU) groups within each age-related group. The CTRL mice at both ages taken in consideration, were maintained free in single cages for 14 days. To induce muscle unloading, the animals of HU group were suspended individually in special cages for 2 weeks using a method similar to that used previously for HU rats [19]. A thin string was linked at one extremity to the tail by sticking plaster and at the other extremity to the top of the cage. The length of the string was adjusted to allow the animals moving freely on the forelimbs, while the body was inclined at 30-40° from the horizontal plane. All mice had water ad libitum and received 8 g a day of standard rodent chow. The food remaining on the day after was weighted to calculate daily food consumption. At the end of suspension, the mice were unfastened from the string and deeply anesthetized by intraperitoneal injection of urethane (1.2 g/kg body weight) to allow removing of soleus (Sol) and extensor digitorum longus (EDL) muscles. Muscles were used immediately for the electrophysiological experiments or frozen in liquid nitrogen and stored at -80°C for other studies. After surgery, animals were killed by an overdose of urethane.

Ex vivo electrophysiological studies

Muscles tied at the end of each tendon were placed on a glass rod located in a 25-ml bath chamber maintained at 30°C and perfused with 95% O2/5% CO2 in normal and/or chloride-free physiological solution [18]. The normal physiological solution had the following composition (in mM): NaCl 148, KCl 4.5, CaCl2 2.0, MgCl2 1.0, NaHCO3 12.0, NaH2PO4 0.44, and glucose 5.5. The chloride-free solution was prepared by equimolar substitution of methylsulfate salts for NaCl and KCl and nitrate salts for CaCl2 and MgCl2. The pH of all the solutions was carefully maintained between 7.2 and 7.3 during each experiment. Using a computer-assisted two-intracellular-microelectrodes technique in current-clamp mode, the resting membrane potential, cable parameters, component conductances and excitability characteristics of Sol and EDL muscle fibers were measured in vitro. Cable parameters, in both normal and chloride-free solutions, were calculated from the electrotonic potential elicited by square-wave hyperpolarizing current pulse (100-ms duration) injected by the current electrode filled with 2 M potassium citrate. The membrane voltage responses were monitored by the voltage electrode, filled with 3 M KCl, at two distances from the current electrode. The cable parameters, including fiber diameter and membrane resistance (Rm), were done in real time under computer control as described elsewhere [2]. The reciprocal of Rm measured in normal physiological solution gives a value of the total membrane conductance (Gm) and the same parameter measured in chloride free solution is assumed to be the potassium conductance (gK). The mean chloride conductance gCl is calculated as the mean Gm minus the mean gK. The data are expressed as mean ± S.E.M. The estimates for S.E.M. and number of fibers (n) of gCl were obtained from the variance of, assuming no covariance, and from the number of fibers sampled for Gm and gK, respectively [2]. Comparison of mean values was performed by using unpaired Student's t-test (considering $P < 0.05$ as significant).

Determination of the cytosolic calcium concentration at rest (restCa)

The resting cytosolic Ca2+ concentration (restCa) was determined in freshly, mechanically-dissected muscle fibers using a QuantiCell 900 fluorescence imaging system (Visitech International, Sunderland, UK), as previously described [8]. Briefly, small bundles of 5-10 fibers arranged in a single layer were dissected longwise, tendon to tendon, from Sol muscles, by using microscissors. The bundles were incubated with the fluorescent Ca2+ probe fura-2 for

Table 1. *Effects of HU on muscle atrophy parameters in young and adult mice*

				Soleus		*Extensor Digitorum Longus*	
		n	Change in body weight over 14 days (g)	Muscle weight (mg)	Muscle to body weight (mg/g)	Muscle weight (mg)	Muscle to body weight (mg/g)
Young	CTRL	12	1.3 ± 0.3	10.3 ± 0.6	0.44 ± 0.02	11.6 ± 0.4	0.49 ± 0.02
	HU	8	0.3 ± 0.4*	9.2 ± 1.0	0.41 ± 0.05	11.1 ± 0.4	0.49 ± 0.02
Adult	CTRL	8	0.3 ± 0.4	10.9 ± 0.2	0.38 ± 0.01	15.3 ± 0.7	0.53 ± 0.03
	HU	8	-0.9 ± 0.6	9.0 ± 0.4*	0.32 ± 0.02*	15.3 ± 0.7	0.54 ± 0.02

Young (2-3 month-old) and adult (6-7-month-old) mice were hindlimb-unloaded for 14 days and compared to age-matched control mice maintained in standard condition. Each value is expressed as mean ± SEM calculated from the number of mice indicated (n). * indicates significant differences ($P<0.05$, unpaired Student's t-test) between HU mice and relative controls.

60-90 minutes at 30°C in a physiological (NP) solution containing in mM, 148 NaCl, 4.5 KCl, 2.5 CaCl2, 1 MgCl2, 12 NaHCO3, 0.44 NaH2PO4, and 5.5 glucose. The solution was supplemented with 5 μM of acetoxymethyl ester of the dye mixed to 10% (v/v) pluronic F-127 (molecular probes, Leiden, The Netherlands) and the pH was adjusted to 7.3-7.4 by bubbling the solution with 95% O2/5% CO2. After incubation, the cells were washed with NP solution and mounted in a modified RC-27NE recording chamber (Warner Instruments, Hamden, CT). Tendons of the bundles were attached by hair loops, one extremity to a fixed tube, the other to a mobile one.

Fiber integrity was verified by assessing visible contractile activity under x400 magnification in response to a depolarizing solution containing 100 mM K+. Next the mean sarcomere length was adjusted to ~2.5 μm by moving the mobile tube fixed to one muscle tendon. The fluorescence ratio was converted off-line to restCa by using the calibration parameters determined in each muscle, using the equation restCa = (R-Rmin)/(Rmax-R)•KD•β, where R is the 340-to-380 nm fluorescence ratio; KD is the affinity constant of fura-2 for Ca2+ given by the manufacturer, i.e. 145 nM (Molecular probes), and β, Rmin, and Rmax are calibration parameters determined in ionomycin-permeabilized fibers bathed in NP solution for the calculation of Rmax or in Ca2+-free solution for the calculation of Rmin [11]. The β value was calculated as the ratio of fluorescence intensities emitted by the fibers excited at 380 nm in Ca2+-free and NP solutions. Data are expressed as mean ± S.E.M. from N mice/n fibers. Statistical analysis was performed with unpaired Student's t-test.

Results

According to the different ages, the ground-based control young mice significantly grew over 14 days, whereas adult control animals showed no variation in body weight (Table 1). HU slightly but significantly affected body weight evolution during the same period, since young mice showed no more growth and adult mice lost ~3% of body weight. As an useful index of muscle atrophy, we determined for each group of animals the muscle mass of Sol and EDL expressed as absolute muscle weight and as muscle weight normalized to the body weight. At the end of 14-days period, the Sol muscle weight of HU young mice was 10.7 % minor than that of relative CTRL. Atrophy was greater in adult mice, since HU mice had Sol muscle weight 17 % minor with respect to CTRL mice. The different age-dependent degree of HU-induced atrophy was further confirmed by the muscle-to-body weight values. Indeed, relative muscle weight of suspended muscle was reduced by 7 % in young mice and 16 % in adult mice when compared to muscles of their corresponding ground-based control mice. In contrast to Sol muscle, no sign of atrophy was found in EDL muscles (Table 1).

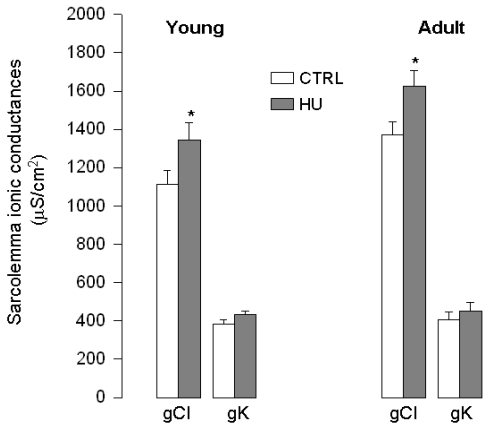

Fig. 1 *Effect of HU on the sarcolemma chloride (gCl) and potassium (gK) conductances at rest in young (2-3 month-old) and adult (6-7-month-old) mice. Each bar represents the mean value ± SEM of gCl and gK measured in at least 11 fibers from at least 2 mice.*
** indicates significant differences (P<0.05, unpaired Student's t-test) between HU mice and relative controls.*

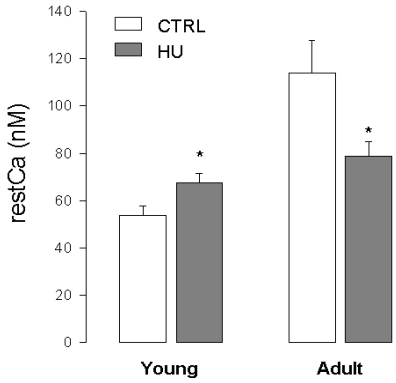

Fig. 2 *Effect of HU on the resting cytosolic calcium concentration (restCa) in young (2-3 month-old) and adult (6-7-month-old) mice. The restCa was determined by Fura-2 fluorescence imaging in single myofibers mechanically dissociated from Sol muscle of control (CTRL) and hindlimb-unloaded (HU) mice at each age period. Each bar represents the mean value ± SEM of restCa measured in at least 18 fibers from at least 4mice.*
** indicates significant differences. (P<0.05, unpaired Student's t-test) between HU mice and relative controls.*

In control mice, the gCl of Sol muscle resulted an electrical parameter influenced by the age, being 1114 ± 70 μS/cm2 and 1372 ± 66 μS/cm2 in young and adult mice, respectively (P<0.01 with unpaired Student's t-test). At both ages, hindlimb suspension induced a significant increase of gCl by ~20% with respect to the related ground-based animals. In contrast to what observed for gCl, both the age and the HU had no influence on the sarcolemma potassium conductance at rest (gK). Indeed, each group of animals showed a gK value within the range of 380-450 μS/cm2 (Fig. 1).

In control mice, the restCa values of Sol muscle fibers of adult animals was ~2-fold that measured in Sol muscle of young mice (P<0.01 with unpaired Student's t-test). A such marked difference in calcium homeostasis between the young and adult control animals was accompanied by a very different effect of HU, which induced a restCa change in opposite direction in the two groups. Indeed, in young mice the restCa was 53.9 ± 3.8 nM and 67.4 ± 4.2 nM in control and HU condition, respectively. On the contrary, in the case of adult animals, hindlimb suspension produced a significant ~30 % reduction of restCa, showing control and HU mice at this age period a restCa value of 113.8 ± 13.9 nM and 78.7 ± 6.2 nM respectively (Fig. 2).

Discussion

The hindlimb-unloading model has long been used to investigate the effects of inactivity on skeletal muscle, especially using rats [14]. In previous studies, using the HU rat model, we found a profound changes in ion channel expression/function and Ca2+ homeostasis [5, 6, 8, 18, 19]. Here, we characterize the effects of HU on mouse muscles focusing our attention also on the influence of the age period on this murine model of skeletal muscle inactivity. In adult mice HU induced alterations of muscle functional parameters resembling those observed in the HU rat model. Indeed, in accordance with previous findings obtained in rats, 14 days of HU in mice induced a significant atrophy of Sol muscle with a reduction of the muscle-to-body weight ratio, an increase of gCl and a concomitant decrease of restCa. Importantly, as shown in the HU rat model [19], the gCl increment could alter sarcolemma excitability, probably contributing to impairment of resistance to fatigue of the postural soleus muscle as proposed by others [15, 16]. In addition, the gCl increase may contribute to the mechanisms leading to soleus fiber type slow-to-fast transition, since pharmacological or genetic lowering of the gCl can trigger the opposite fast-to-slow shift of fast-twitch muscle properties [20-22]. Also calcium ions play a pivotal role in regulating muscle physiology [1]. At short term, they trigger contraction, while at longer term, they modulate gene expression. Indeed it has been shown that alteration of calcium homeostasis can induce fiber type transition [12-21]. In the HU rat, the reduction of restCa in soleus muscle fibers is in accord with the slow-to-fast transition of fiber type [8]. The same mechanism likely applies to mice subjected to

HU. Thus, the HU mouse results a useful animal model to gain insight into the cellular/molecular mechanisms responsible of muscle disuse as well as to evaluate the possible benefits of drugs in protecting muscle contractile function. The advantages of using mice rather than rats as the HU model include the availability of transgenic mice, the possibility to modify mouse genome, and the possibility to compare HU to actual microgravity effects within the same animal strain since the lower dimension of mice may be suitable for space flights.

Surprisingly, these results show that atrophy as well as restCa, were differently affected by HU in young mice with respect to the adult mice. Indeed, in young mice, the muscle weight loss was not significant after HU and calcium homeostasis was altered in the direction of an increase of restCa. Importantly, the minor muscle weight, restCa, and gCl in young mice with respect to adult mice indicate that these parameters are still evolving in growing, 2-3 month-old mice. It is striking that HU and mouse growing are expected to produce opposite effects on muscle weight and restCa, thus suggesting that "growth" signals may somewhat compensate for the effects of disuse on these parameters. On an other hand, we can not exclude that growing mice may show molecular signaling and hormonal status different from adult mice, with possible effects on muscle properties and growth. In contrast, the HU effect on gCl in young mice completely overlapped that observed in adult mice, resulting at both ages in a 20%-increase of gCl with respect to their relative controls. As already observed in rat fast-twitch muscles, the soleus muscle gCl was higher in adult compared to young mice, probably due to transcriptional and post-transcriptional mechanisms [3, 4, 21]. Thus, the results show that both HU and growing signals result in gCl increase in soleus muscle and may thus not counteract each other.

In conclusion, our study demonstrated that adult HU mice may represent a useful model for the study of the effects of simulated microgravity on skeletal muscle properties, and that the effects of muscle inactivity may be however strongly influenced by the animal age.

Abbreviations

restCa, resting cytosolic calcium concentration; gCl, resting chloride conductance; gK, resting potassium conductance; Sol, soleus muscle; EDL, extensor digitorum longus muscle.

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