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Differential regulation of myofilament protein isoforms underlying the contractility changes in skeletal muscle unloading

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Abstract

Weight-bearing skeletal muscles change phenotype rapidly in response to unloading. Using the hind limb-suspension rat model, we investigated the regulation of myofilament protein isoforms in correlation to contractility. Four weeks of continuous hind limb unloading produced progressive atrophy and contractility changes in soleus but not extensor digitorum longus (EDL) muscle. The unloaded soleus muscle also had decreased fatigue resistance. Together with the decrease of myosin heavy chain (MHC) isoform I and IIa and increase of MHC IIb and IIx, coordinated regulation of thin filament regulatory protein isoforms were observed: γ - and β -tropomyosin decreased and α -tropomyosin increased, resulting in an α/β ratio similar to that in normal fast twitch skeletal muscle; troponin I and troponin T (TnT) both showed decrease in the slow isoform and increases in the fast isoform. The TnT isoform switching began after 7 days of unloading and TnI isoform showed detectable changes at 14 days while other protein isoform changes were not significant until 28 days of treatment. Correlating to the early changes in contractility, especially the resistance to fatigue, the early response of TnT isoform regulation may play a unique role in the adaptation of skeletal muscle to unloading. When the fast TnT gene expression was up-regulated in the unloaded soleus muscle, alternative RNA splicing switched to produce more high molecular weight acidic isoforms, reflecting a potential compensation for the decrease of slow TnT that is critical to skeletal muscle function. The results demonstrate that differential regulation of TnT isoforms is a sensitive mechanism in muscle adaptation to functional demands.

Keywords

troponin T isoforms; muscle unloading; fatigue resistance; troponin I; tropomyosin; myosin; hind limb-suspended rat; Western blot protein quantification

Adult skeletal muscle retains plasticity to rapidly adapt to changes in activity and functional demands (48). The adaptations of skeletal muscle include changes in morphology, contractility, and protein contents (4,7). The structural and functional modifications can alter the performance of muscle to cope with changes in the environment or physical activities under physiological or pathological conditions. The high plasticity of skeletal muscle may also result in malfunction. An example is the effects of unloading on skeletal muscle function in astronauts during space flight (17) and in bedridden conditions (6,30,42). Muscle unloading due to long

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exposure to weightlessness or simulated weightlessness causes atrophy, loss of functional capacity, impaired locomotor coordination, and decreased resistance to fatigue in the antigravity muscles of the lower limbs (17). Besides reducing astronauts' mobility in space and upon returning to a gravity environment (9), the molecular mechanisms for the adaptation of skeletal muscle to unloading also has a medical importance in conditions such as disuse and paralysis (6,34,37).

The adaptation of skeletal muscle to unloading involves multiple changes in the muscle cells and the molecular mechanisms for unloading to alter muscle contractility remain to be determined. The contraction of skeletal muscle is initiated by the increase in intracellular $[Ca^{2+}]$. Relevant changes in the Ca^{2+} handling system in unloaded muscle cells include the increased expression of Ca^{2+} channel in the sarcolemma (7) and the increase in the maximal velocity of shortening due to changes in the Ca^{2+} -uptake and release by sarcoplasmic reticulum (43).

Downstream of the Ca^{2+} signaling pathway, the responsiveness of myofilaments is another determinant in skeletal muscle contractility. Ca^{2+} binding to troponin C (TnC) results in a series of allosteric changes in troponin I (TnI), troponin T (TnT) and tropomyosin (Tm), allowing the myosin head to form a strong cross-bridge with the actin thin filament to activate myosin ATPase and produce force (18). A reduction of myofilaments and the number of cross-bridges per cross-sectional area in soleus muscle was observed during unloading and may be related to the decrease in the contractile force (33). Previous studies have investigated the changes in myosin isoform expression during skeletal muscle unloading. In soleus muscle, unloading is known to result in decrease in the type I (slow) myosin heavy chain (MHC) and increase in the type II (fast) isoforms (11,17). A disproportional loss of thin filaments compared to the myosin thick filaments was observed in human soleus muscle after 17 days of bed rest and proposed to contribute to the increased velocity of contraction (42). Changes in TnI and TnT expression was reported in an early study of soleus muscle after 21 days of unloading although the isoform differentiation was not defined (13). Slow to fast isoform switches of TnT, TnI and TnC have been observed in unloaded soleus muscle (29,45) corresponding to fiber type and contractility changes (5). Further investigation is needed for a better understanding of the regulation and functional significance of thin filament regulatory protein isoforms in the maladaptations of slow skeletal muscle to unloading.

In the present study, we investigated the regulation of myofilament protein isoforms and functional implications using the hind limb-suspension rat model (55). Four weeks of continuous hind limb unloading produced progressive atrophy and contractility changes in soleus but not extensor digitorum longus (EDL) muscle. In addition to the decreases of MHC I and IIa and increases of MHC IIb and IIx as seen in previous studies, coordinated changes of thin filament regulatory proteins were observed. γ - and β -Tm decreased and α -Tm increased, resulting in an α/β ratio similar to that in normal fast skeletal muscle. TnI and TnT showed decreases of the slow isoform and increases of the fast isoform. Correlating to the early switching of TnT isoforms, soleus muscle showed decreased fatigue resistance early on during unloading. When the fast TnT gene expression was up-regulated in the unloaded soleus muscle, alternative RNA splicing switched to produce more high molecular weight acidic isoforms that may be a compensation for the decrease in slow TnT. The differential regulation of myofilament protein isoforms indicates their functions in adjusting contractility during adaptation and maladaptation to mechanical unloading.

MATERIALS AND METHODS

Hind Limb Suspension Rat Model of Continuous Skeletal Muscle Unloading

Male Sprague-Dawley rats weighing 180–210 g were randomly divided into control and hind limb suspension groups. The rats were housed in a $22\pm 2^{\circ}\text{C}$ environment, subjected to 12 h light/dark cycles, and fed with water and Rat Chow Ad Libitum. After individually caged for one week, continuous tail suspension was applied using a modified Morey-Holton method (55,57) for 3, 5, 7, 14 or 28 days. Care was taken to protect the tail tissue and the movement of the rats was not restricted during the treatment. Age-matched male rats were maintained without hind limb suspension and examined at the same schedule as controls. Muscle weight and body weight of the hind limb-suspended and control rats were recorded at the time of functional measurements.

Measurements of Muscle Contractility

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). Soleus or EDL muscles were rapidly excised before euthanasia. The muscle was rinsed in oxygenated Krebs-Henseleit solution, carefully split into two strips, and mounted horizontally in a continuously perfused myograph chamber according to the method described previously (57). The muscle was superfused with Krebs-Henseleit solution containing (in mM): 120.0 NaCl, 4.7 KCl, 1.2 MgSO_4 , 20.0 NaHCO_3 , 1.2 NaH_2PO_4 , 2.5 CaCl_2 and 10.0 glucose, pH 7.4, maintained at 37°C and bubbled with 95% O_2 /5% CO_2 . The distal tendon of the muscle was held by a spring clip and the proximal end was tied with 3-0 silk suture to a stainless steel hook connected to an isometric force transducer (TB-651, Kohden, Japan).

Via two platinum wire electrodes longitudinally flanking the muscle, the muscles were electrically stimulated at 6 V using a SEN-3301 pacer (Kohden, Japan) by 20 ms rectangle current pulses at 0.05 Hz. Isometric twitch tension was recorded after equilibration for 60 min. The length of the muscle was gradually increased to obtain the maximum developed force (L_{max}). Twitch contractile properties (peak tension per unit cross-sectional area (P_t), maximal rates of tension development and relaxation ($\pm dT/dt_{\text{max}}$), time to 50% peak tension (TP_{50}), time to peak tension (TPT), and time from peak tension to 75% relaxation (TR_{75}) were assessed under non-fatigued conditions.

Tetanic contractile force of the muscle strips was measured with stimulation of 20 ms rectangle pulses at 25 Hz for soleus and 5 ms rectangle pulses at 100 Hz for EDL. Continuous tetani (54) were carried out for 45 s in soleus and 35 s in EDL to examine the resistance to high-frequency fatigue.

On different muscle strips, tetanic contraction was examined in intermittent tetani according to an established protocol (35). The muscle was stimulated by a 0.5 ms rectangle current pulse-train (from 10 to 140 Hz) of 500 ms duration in every minute. The optimal frequency producing maximal tetanic tension was determined and used in the intermittent tetanic contraction measurements. The muscle strips were examined for fatigue resistance under the intermittent stimulation with 1 s intervals and 30% duty cycle for 5 min.

At the end of each experiment, the muscle was blotted dry and weighed. The cross-sectional area of the muscle was calculated from the muscle weight assuming the geometry of a cylinder with a specific gravity of 1.0 (14).

SDS-Polyacrylamide Gel Electrophoresis of MHC Isoforms

As described previously (10), total protein was extracted by homogenizing the rat skeletal muscle tissues in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer

containing 2% SDS. The skeletal muscle MHC isoforms were resolved by SDS-PAGE as described previously (47). The resolving gel contained 8% acrylamide with an acrylamide:bisacrylamide ratio of 50:1, 30% glycerol, 200 mM Tris base, 100 mM glycine (pH 8.8), and 0.4% SDS. The stacking gel contained 4% acrylamide, 70 mM Tris-HCl (pH 6.7), 4 mM EDTA, and 0.4% SDS. The gels were cast in the BioRad mini-Protean II system and run at 70 V in an icebox for 24 hrs. The gel was stained with Coomassie Brilliant Blue R250 to detect the protein bands.

Western Blot Analysis of MHC, Tm, TnI, and TnT Isoforms

The rat skeletal muscle SDS-gel samples were resolved by SDS-PAGE using 14% Laemmli gels with an acrylamide:bisacrylamide ratio of 180:1 cast using a BioRad mini-Protean II system. The resolved protein bands were electrically transferred to nitrocellulose membrane (0.45 μ m pore size) using a BioRad semi-dry transfer apparatus at 5 mA/cm² for 15 minutes. The membrane was blocked in Tris-buffered saline (TBS, 137 mM NaCl; 5 mM KCl, 25 mM Tris-HCl, pH 7.4) containing 1% BSA at room temperature for 1 h and incubated with monoclonal antibody (mAb) FA2 against MHC I (cardiac β -MHC) (26), mAb CH1 against α - and β -Tm or mAb CG3 against γ -Tm (32), mAb TnI-1 against TnI (28), mAb CT3 against cardiac/slow skeletal muscle TnT (25), or mAb T12 against an fast skeletal muscle TnT (31) in TBS containing 0.1% BSA at 4 °C overnight. After three washes with TBS containing 0.5% Triton X-100 and 0.05% SDS and two TBS rinses, the membrane was incubated with alkaline phosphatase-conjugated anti-mouse IgG second antibody (Sigma) in TBS containing 0.1% BSA at room temperature for 1.5 h. After washes as above, 5-bromo-4-chloro-3-indolyl phosphate /nitro blue tetrazolium substrate reaction was carried out as described previously (23) to visualize the MHC, Tm, TnI and TnT isoforms.

Quantification of Western Blots and Data Analysis

The Western blots were scanned at 600 dpi for densitometric quantification using the NIH Image 1.61 software. The Western blotting conditions for each of the antibodies were adjusted to provide a suitable range of quantitative detection as shown by the densitometry curves of serial dilutions of rat muscle protein extracts (Fig. 1). [To compare changes in a protein isoform, the Western blot densitometric values were normalized with that of the actin band in parallel SDS-gel. The ratios among Tm, TnI, or fast TnT isoforms were quantified by densitometry of the bands detected by the same mAb. Fig. 1B and 1C show that five folds of muscle protein loading produced ~2-fold change in Western blot intensity and ~3.5-fold change in the intensity of Coomassie Brilliant Blue R250 stained gels. The non-linear quantification of proteins by Western blot densitometry reflects the nature of an indirect measurement method. Although the differences in Western blot intensity do not directly reflect the actual amounts of the detected protein, these measurements provide informative comparisons for the relative amounts of the myofilament proteins, sufficient for monitoring the changes during muscle unloading. All values are presented as means $\pm$ standard error (SEM). The statistical significance of the contractile parameters and protein quantification was analyzed by Student's t test or one-way ANOVA. Differences with *P* values <0.05 were considered significant.

RESULTS

Muscle Atrophy and Changes in Contractility during Four Weeks of Hind Limb Unloading

The rat hind limb suspension model rapidly produced atrophy in the soleus but not EDL muscle (Table 1). The atrophy in unloaded soleus became significant after 7 days of hind limb suspension. Correspondingly, continuous hind limb unloading rapidly produced contractility changes in soleus but not EDL during the 4 weeks of treatment (Table 1). The different responses of soleus and EDL muscles are consistent with the established view that the rat model of simulated weightless selectively affects the weight bearing muscles (17).

The time course of unloading-induced changes in the twitch contractility of soleus muscle is shown in Fig. 2. Fig. 2A demonstrates the changes in maximum isometric twitch tension (P_t) during four weeks of hind limb suspension. As compared with control soleus muscle data from synchronous rats, the P_t showed a visible decrease after 7 days of unloading although statistic significance was not yet established ($P=0.079$ in two-tail t test). The decrease in P_t progressed in the unloaded soleus and showed statistic significance after 14 days of hind limb suspension ($P<0.05$) and became more obvious after 28 days of unloading ($P<0.01$).

Corresponding to the changes in twitch tension, the maximal rate of tension development ($+dT/dt_{\max}$) of soleus muscle increased after 1 week of unloading and progressed after 2 and 4 weeks of unloading as compared with the control values ($P<0.05$ and $P<0.01$, Fig. 2B). The maximal rate of relaxation ($-dT/dt_{\max}$) of soleus muscle also progressively increased with statistic significance detectable after 4 weeks of unloading ($P<0.01$) (Fig. 2C). Corresponding to the contractile and relaxation velocity changes, the time to develop peak tension (TPT, Fig. 2D) and the time for 75% relaxation (TR₇₅, Fig. 2F) of the unloaded rat soleus muscle showed progressive shortenings (Table 1). In contrast, the time to develop 50% peak tension (TP₅₀, Fig. 2E) was not significantly changed.

The changes in maximum tetanic tension (P_o) of the unloaded soleus muscle were illustrated in Fig. 3. Fig. 3A shows that after 7 days of hind limb suspension, the P_o of continuous tetani decreased ~33% although no statistic significance was established due to variations among individual animals. The decrease of continuous tetanic P_o of unloaded soleus progressed to ~60% ($P<0.01$) and ~78% ($P<0.01$), respectively, after 14 and 28 days of unloading. Different from the P_o in continuous tetani, Fig. 3B shows that the soleus P_o in intermittent tetani had no change after 7 or 14 days of unloading, but decreased by ~34% ($P<0.01$) after 28 days of unloading as compared with the synchronous controls.

Unloading-Induced MHC Isoform Switching

Four isoforms of MHC (I, IIa, IIb and IIx) are found in rat skeletal muscles (4,47). MHC I is the slow fiber specific isoform and MHC II are mainly expressed in fast fibers. The glycerol-SDS-PAGE run at low temperature clearly resolved the four MHC isoforms by a gel mobility order of MHC I > MHC IIb > MHC IIx > MHC IIa as shown in the control rat diaphragm muscle (Fig. 4A).

No significant change in MHC isoform expression was seen in the EDL muscle during 4 weeks of hind limb unloading (Fig. 4A). The normal rat soleus expresses two MHC isoforms, MHC I and IIa with MHC I being ~90% of the total MHC. Four weeks of unloading treatment resulted in a progressing decrease in MHC I in the soleus muscle (Fig. 4A). Western blots using mAb FA2 specific to MHC I and densitometry analysis confirmed the significant decrease in MHC I expression ($P<0.01$, Fig. 4B). During the switching of MHC isoforms in the unloaded soleus muscle, the down regulation of MHC I was accompanied by an up-regulation of MHC IIx that was detectable after 14 days of unloading and became the dominant isoform after 28 days when MHC IIa ceased expression and MHC IIb became significant. The results indicate a slow to fast transition of the fiber types in the soleus muscle during unloading that occurred significantly after 2 weeks of hind limb suspension.

Unloading-Induced Switching of Thin Filament Regulatory Protein Isoforms

The muscle thin filament regulatory system consists of troponin and Tm (18). We examined the isoform expression of Tm and two subunits of troponin, TnI and TnT, for changes during skeletal muscle unloading. Consistent with the unchanged MHC isoform expressions in EDL (Fig. 4A), no significant change in the thin filament regulatory protein isoforms was detected

in EDL by Western blotting and densitometry analysis during the 4 weeks of hind limb suspension (Fig. 5).

The unloading-induced myosin isoform changes in soleus muscle were accompanied by multiple changes in thin filament regulatory protein isoform expression. Fig. 6 shows a switching of Tm isoforms in the soleus after four weeks of hind limb suspension. The CH1 Western blot in Fig. 6A detected comparable levels of α - and β -Tm isoforms in the control soleus muscle. Consistent with a recent report that β -Tm decreased in unloaded soleus (44), progressive decrease of β -Tm was found during hind limb unloading and accompanied by a complementary increase of α -Tm. The time course of the switching between β and α Tm isoforms was analyzed by densitometry of the Western blots (Fig. 6B). The increase in α -Tm/ β -Tm ratio in the unloaded soleus muscle changed the expression pattern to mimic that in the EDL (Fig. 5A). The expression of γ -Tm is considered as a feature of the slow skeletal muscle fibers (41). The CG3 Western blot in Fig. 6A shows that γ -Tm began to decrease in rat soleus after 14 days of hind limb unloading and became barely detectable after 28 days of unloading, supporting a slow to fast fiber type switching.

The expression of TnI isoforms in soleus muscle was also affected by hind limb suspension. The Western blots in Fig. 7A show that the control rat soleus expressed both slow and fast skeletal muscle TnI (ssTnI and fsTnI, respectively) with ssTnI as the dominant isoform. A decrease in ssTnI and an increase in fsTnI were seen in the soleus muscle after 14 days of unloading. The ratio of ssTnI to fsTnI was reversed with fsTnI becoming dominant after 28 days of unloading. The time course of the TnI isoform switching is demonstrated by densitometry analysis (Fig. 7B). These results further support the observation that unloading induced a slow to fast fiber type switching in the soleus, a normally weight bearing slow fiber muscle.

The most complex change in myofilament protein isoform contents found in the unloaded soleus muscle was the regulation of TnT isoforms (Fig. 8). The level of slow skeletal muscle TnT (ssTnT) in soleus underwent a down-regulation during the 4 weeks of hind limb suspension (Fig. 8A). Densitometry analysis showed that the ssTnT level began to decrease after 7 days of unloading. This trend continued to 28 days of unloading when ssTnT became less than 20% of the control. It is worth noting that the ratios of the alternatively spliced high and low molecular weight ssTnT isoforms that differ by inclusion or exclusion of an 11 amino acid N-terminal segment encoded by exon 5 (24) remained stable while the expression of ssTnT gene was down regulated. In comparison to the later changes in MHC, Tm and TnI isoform expression, the time course of the decrease in the amounts of ssTnT in the soleus (Fig. 8B) demonstrated an earlier response to hind limb unloading.

While the level of ssTnT was decreasing, the level of fast skeletal muscle TnT (fsTnT) was increasing in soleus muscle during the four weeks of unloading (Fig. 8A). The increase in fsTnT expression was detectable after 7 days of unloading, became obvious after 14 days of hind limb suspension, and remained at the predominant level after 28 days of treatment. Densitometry analysis of the Western blots (Fig. 8B) showed that the up-regulation of fsTnT was a complementary change to the decrease in ssTnT expression. The switch of TnT isoforms also agrees with the slow to fast fiber type switching observed in the soleus muscle in hind limb unloading (17).

Decreased Fatigue Resistance of Unloaded Soleus Muscle

Fig. 9A and 9B show representative high frequency fatigue (12) recordings of tetanic contraction of soleus and EDL muscles from control and 4-week hind limb suspended rats. As typically seen in slow fiber muscles, the tetanic tension raised to peak and declined slowly in the control soleus. In contrast, the tetanic tension in the unloaded soleus declined much faster

(Fig. 9A), showing a pattern similar to that of EDL. The rapid high frequency fatigue pattern of EDL was not much affected by hind limb unloading (Fig. 9B). We used the normalized force at the thirtieth second of continuously tetanic contraction vs. the peak tetanic force (P_{30}/P_0) as an index for the high frequency fatigability of soleus muscle. The results showed decreases by 28%, 32% and 66% after 7, 14, and 28 days of hind limb unloading, respectively (Fig. 9C).

Since the high frequency fatigue in continuous tetani may involve the functionality of the T-tubular system (1), we further assessed the fatigue resistance of the unloaded soleus muscle in intermittent tetanic contraction that reflects more closely the function of myofilament, especially thin filament Ca^{2+} sensitivity (53). Fig. 9D shows the representative recordings of intermittent tetanic contraction of four-week unloaded soleus and control muscles. The results demonstrate a decreased resistance of the unloaded soleus to intermittent tetanic contraction. Like that seen in the high frequency fatigue experiments, the pattern also became similar to that of the EDL muscle. Fig. 9E and Fig. 9F summarize the course of tension declining during intermittent tetanic contraction of soleus and EDL muscles from control and 1-, 2- and 4-week hind limb suspended rats. The faster decline of the unloaded soleus versus the control demonstrates that unloading produced a decrease in fatigue resistance in the soleus muscle under intermittent tetani. The decrease was significant as early as after 7 days of hind limb suspension. It is interesting to note that there was no further decrease in the next 3 weeks of hind limb unloading (Fig. 9E). The fatigue patterns of EDL muscle in intermittent tetani showed rapid declines of force reflecting a low resistance to fatigue, which was not affected by hind limb suspension (Fig. 9F).

Alternative Splicing Regulation of Fast TnT Isoforms in Unloaded Soleus Muscle

Accompanying the increased level of fsTnT gene expression in the unloaded soleus muscle, there was a switching of alternatively spliced isoforms to produce more of the higher molecular weight isoforms (Fig. 8A). We have previously demonstrated that the size differences among chicken and mouse fast skeletal muscle TnT isoforms are solely due to alternative splicing of the NH_2 -terminal variable region (39,51). The relative amounts of the five fast TnT bands resolved by SDS-PAGE and identified by mAb T12 Western blots were quantified by densitometry. The results in Fig. 10 show that the switching of alternatively spliced fsTnT isoforms was detectable in the soleus muscle after 14 days of unloading and became more clear after 28 days of treatment. It is known that the size of the acidic NH_2 -terminal variable region corresponds well with the overall charge and isoelectric point of the fsTnT isoforms and normal adult skeletal muscle expresses mostly the basic isoforms (39,51). Therefore, the increase in the amounts of high molecular weight fsTnT isoforms in the unloaded soleus muscle indicated a switch of fsTnT towards the production of more acidic isoforms that are normally seen in embryonic skeletal muscles (51).

DISCUSSION

Skeletal muscle undergoes rapid and significant morphological and functional changes during physiological and pathological adaptations. Mechanical unloading produces complex phenotypic changes in weight bearing muscles, in which the regulation of myofilament proteins needs to be systematically investigated in order to understand the molecular basis of the functional alterations. Using the rat hind limb suspension model, the present study simultaneously investigated the regulation of both thick and thin filament protein isoforms in correlation with analyses of contractility.

Differential regulation of troponin isoforms

The expression of thick and thin filament protein isoforms in rat soleus all showed progressive changes during the four weeks of continuous unloading. At matching time points, the decrease

in MHC I and increases in MHC IIx and IIb in the unloaded soleus muscle were accompanied by changes in Tm, TnI and TnT isoforms. A previous study has detected a slow to fast isoform switch of TnC in soleus muscle after 15 days of unloading (29). The time courses of the myofilament protein isoform switches were coordinated but not completely synchronized at the protein level. Slow to fast MHC and Tm isoform switches in the unloaded soleus were not significant until 28 days of hind limb suspension. The decrease in slow and increase in fast TnI isoforms were detectable at 14 days of unloading but the change in isoform ratio was not apparent until 28 days of unloading. In contrast, the slow to fast switch of TnT isoforms in the unloaded soleus occurred earlier and was seen after 7 days of hind limb suspension. In normal adult muscle fibers, TnI and TnT, two subunits of the troponin complex, have a coupled expression of the fast and slow isoforms correlating to the fast and slow muscle fiber types (10). The earlier switching of TnT isoforms than that of TnI isoforms in soleus muscle during mechanical unloading, which was also seen in a previous study (45), is an interesting observation. Another previous report demonstrated a decrease in slow TnI mRNA and an increase in fast TnI mRNA in mouse soleus muscle after 7 days of hind limb suspension (15), demonstrating that the regulation of TnI gene expression is initiated in the unloaded soleus muscle as early as the changes in TnT isoform proteins. We previously reported that the equilibrium of TnT protein in muscle cells is effectively regulated by proteolysis (52). Therefore, while coordinated thick and thin filament protein isoform switches in the unloaded soleus muscle represent a slow to fast switch of fiber types, the more rapid response of TnT isoform switching than that of other myofilament protein isoforms may have occurred due to a more effective regulation at the protein turnover rate. Considering the rapid atrophic effects unexceptionally observed during the unloading of weight bearing muscles (9), the sensitivity of TnT to proteolytic regulation may lead the reduction of myofibrils and underlies the early atrophy seen in the soleus after 7 days of hind limb suspension (Table 1).

Potential contribution of thin filament regulatory proteins to the decreased contractility

Although atrophy-related decreases in the amount of myofibrils and increase in connective tissue contribute to the reduction of contractile force (33), the large decrease in maximum twitch and tetanic contractions normalized by cross-sectional area indicate a reduction in force output per contractile unit. Single fiber studies have demonstrated that myosin isoforms determine the maximum force and troponin isoforms affect the Ca^{2+} sensitivity of myofilament (10,23). It is known that muscle fibers containing mainly MHC II develop greater tension than that contain mainly MHC I (19). Therefore, the decrease in MHC I and increases in MHC IIx and IIb in the unloaded rat soleus could not be the cause but a compensation for the reduction of contractile force during this adaptation. The primary decrease in contractile force production per cross-sectional area must result from non-myosin changes. Similarly, although the increased expression of MHC IIx or IIb may explain the increase the velocity of contraction (18), the unloading-induced faster rate of relaxation requires further explanation. The significant changes in the thin filament regulatory protein isoforms in the unloaded soleus muscle provide new insights into the molecular basis of muscle adaptation to unloading.

The intermittent tetanic contractile force of unloaded soleus muscle was not changed until four weeks of hind limb suspension (Fig. 3B). This change corresponds to the slow to fast switch of in MHC, Tm and TnI isoforms. Previous studies have shown that troponin has potentiation effects on actomyosin ATPase activity and the contractile force development (49). Therefore, the switching of TnI and TnT from slow to fast isoforms in the unloaded soleus muscle may contribute to the decrease in contractile forces and requires further investigation.

Corresponding to the early switch of TnT isoforms, the shortened time to develop peak tension (TPT) in isometric twitch contraction occurred in soleus at 7 days of unloading but the shortening of time to 50% peak tension (TP_{50}) was less proportional. TP_{50} is an indicator of

sarcoplasmic reticulum Ca^{2+} release velocity (2). Therefore, sarcoplasmic reticulum Ca^{2+} release might have limited contribution to the contractility changes and the shortened TPT may be largely attributed to a shortened phase of force development, suggesting a faster response of myofibrils to Ca^{2+} . With the early slow to fast TnT to isoform switching in the unloaded soleus muscle (Fig. 8), this notion is supported by the previous finding that fast TnT confers a higher cooperativity of Ca^{2+} activation of the myofibrils (21).

The TR_{75} is determined by a combination of the rate of sarcoplasmic reticulum Ca^{2+} uptake and the release of Ca^{2+} from troponin in the myofilaments (3), which was also shortened in the unloaded soleus muscle after 7 days of hind limb suspension. No significant change in soleus sarcoplasmic reticulum Ca^{2+} uptake was detected at 7 days of unloading (46,56). Therefore, the shortened TR_{75} may suggest a decreased affinity of troponin to Ca^{2+} due to the slow to fast isoform switches. This observation is supported by the data that fast troponin is known to corresponds to lower sensitivity to Ca^{2+} in skinned muscle preparations (10,23) and in agreement with the report that the sensitivity of myofibrils to Ca^{2+} decreased in skinned hind limb muscle fibers after 7 days of space flight (20).

Role of slow TnT in the decreased fatigue resistance of unloaded soleus muscle

In contrast to the control, the tetanic force of the unloaded soleus declined very rapidly during continuous high frequency stimulation, demonstrating a decreased fatigue resistance mimicking that of the fast (EDL) muscle (Fig. 9A and 9B). This phenomenon is in agreement with the slow to fast fiber type switch induced by hind limb unloading. This decrease in fatigue resistance was also seen in intermittent tetani (Fig. 9D–F). Inhibition of sarcoplasmic reticulum Ca^{2+} release in the unloaded muscle has been correlated to the decreased fatigue resistance in intermittent tetanic contractions (54). However, studies have showed that the sarcoplasmic reticulum Ca^{2+} release actually increased in soleus after 14 days of unloading (46, 56). Therefore, the decreased resistance to intermittent tetanic contraction of unloaded soleus muscle needs further explanation. Interestingly, the unloaded rat soleus muscle began to show significantly increased fatigability during intermittent tetanic contraction after 7 days of unloading without further change in the next 3 weeks during the treatment (Fig. 9B). This early functional response to unloading corresponds to the slow to fast switching of TnT isoforms (Fig. 8) that was the only early myofilament protein change detected. This result suggests that TnT isoforms may be a determinant for the fatigability of skeletal muscle. TnT is the Tm-binding subunit of the troponin complex and interacts with TnC, TnI, Tm, and F-actin as an organizer in the muscle thin filament regulatory system (40). The central position of TnT in the regulation of muscle contraction and the unique role of TnT isoform switch in fatigue resistance during muscle adaptation to unloading suggest a direction for future studies.

Dual regulation of TnT isoform expression by transcriptional control and RNA splicing

Slow skeletal muscle TnT and adult fast skeletal muscle TnT are acidic and basic isoforms, respectively (23). Previous studies have shown that acidic TnT isoforms confer a higher sensitivity to Ca^{2+} activation than that of basic TnT isoforms (38). Slow and fast TnT are encoded by different genes (*TNNT1* and *TNNT3*). The complementary increase in fast TnT and decrease in slow TnT expression in the unloaded soleus indicate a coordinated gene regulation that maintains the total TnT contents stable. It is known that slow TnT is indispensable in skeletal muscle function and its absence causes a lethal form of nemaline myopathy (23). Therefore, the decrease in slow TnT may have negative effect on the function of weight-bearing muscles such as the soleus. TnT structure and function is further regulated by alternative RNA splicing that generates acidic and basic protein isoforms (39,51). When the expression of fast TnT was up-regulated in the unloaded soleus muscle, the ratio of alternatively spliced isoforms shifted to encode more acidic isoforms. The alternatively spliced acidic and basic TnT isoforms are known to convey conformational and functional differences (8,25,27,50). We previous

demonstrated that while the presence of adult fast TnT (basic) cannot compensate for the loss of slow TnT (acidic) in an inherited nemaline myopathy, the transient expression of cardiac TnT and embryonic fast TnT, both are acidic TnTs, in the neonatal skeletal muscle of these patients might be compensatory (23). Therefore, for the biochemical similarity between acidic fast TnT and slow TnT, the up-regulation of acidic fast TnT may compensate for the decrease in slow TnT to sustain the function of slow muscle fibers.

The differential regulation of TnT isoforms by gene regulation, proteolytic control and alternative RNA splicing in muscle unloading suggests a direction for the development of countermeasures to prevent weightless-induced muscle dysfunction in astronauts that has been a challenging task during long space flights (16,36). The present study demonstrates that the regulation of myofilament protein isoform expression, especially the TnT isoforms, is worth further study for the role in countering the unloading effects on skeletal muscle function. This line of investigation may also lead to a better understanding of the prevention and treatment of muscle dysfunction in various pathological disuse conditions.

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Fig. 1. Quantitative Western blotting using specific mAbs against thin filament protein isoforms (A) Mouse soleus (predominantly slow fiber) and EDL (fast fiber) muscle protein extracts were analyzed By Western blotting to show the specificity of the anti-slow TnT mAb CT3 and the anti-fast TnT mAb T12. The high molecular weight mouse slow skeletal muscle TnT isoform (ssTnT) expressed in *E. coli* from cloned cDNA (24) was used as control. Although mAb T12 is known to cross-react with cardiac TnT (22), it did not show any cross-reaction in Western blots with slow TnT at the working dilution and under the high stringent washing conditions used in the present study. (B) Serial dilutions of total protein extracts from rat muscles were used to verify the quantitative conditions for Western blots using CH1, TnI-1, CT3 and T12 mAbs. The densitometry plots against the relative amounts of protein loading showed an excellent positive correlation in a wide range, justifying the use of these mAbs to quantitatively detect Tm, TnI, slow TnT and fast TnT. (C) Serial dilutions of total protein extracts from rat muscles were resolved by SDS-PAGE and the actin bands were analyzed by gel densitometry. The plot also showed an excellent positive correlation in a wide range of protein loadings, justifying the use of the actin level in this study for normalization of the Western blots. The Values are means $\pm$ SEM. $n = 3$ muscles in each group.

Fig. 2. Altered twitch contractility of soleus muscle during hind limb unloading

The values are means $\pm$ SEM, * $P < 0.05$ and ** $P < 0.01$ vs. control. (A) Decreases in maximum twitch tension (P_1) measured on muscles from 1-, 2- and 4-week hind limb suspension and control rats normalized by cross-sectional area, $n = 6$ muscles in each group. (B) Increases in the maximal rate of twitch tension development ($+dT/dt_{max}$), $n = 5$ muscles in each group. (C) Increases in the maximal rate of relaxation ($-dT/dt_{max}$), $n = 5$ muscles in each group. (D) Shortened time to peak tension (TPT). $n = 6$ muscles in each group. (E) Unchanged time to 50% peak tension (TP_{50}). $n = 6$ muscles in each group. (F) Shortened time for 75% relaxation (TR_{75}). $n = 6$ muscles in each group.

Fig. 3. Decreased tetanic contraction of soleus muscles during hind limb unloading

The tension was normalized by cross-sectional area of the muscle. The values shown are means $\pm$ SEM. ** $P < 0.01$ vs. control. (A) Maximum tetanic tension under continuous high frequency stimulation. $n = 6$ muscles in each group. (B) Maximum tetanic tension under intermittent stimulation. $n = 6$ muscles in each group.

Fig. 4. MHC isoform switching in unloaded rat soleus but not EDL muscle

(A) Total protein extracts from soleus and EDL muscles of control and hind limb suspended rats were resolved by glycerol-SDS-PAGE to examine the MHC isoform contents. The representative SDS-gel show significant decreases in MHC I in the soleus muscle after 14 days of hind limb unloading. After 28 days of unloading, MHC IIa was also significantly decreased in the soleus muscle. In the meaning time, MHC IIx and IIb were significantly increased in the unloaded soleus muscle. No change in MHC isoform contents was seen in EDL muscle after 28 days of hind limb suspension. Rat diaphragm muscle protein extract was used as a control for the gel mobility differences among the four MHC isoforms. (B) Shown by the Western blots using mAb FA2 against MHC I and densitometry analysis of multiple samples, the

expression of MHC I in soleus muscle decreased significantly after 28 days of hind limb suspension as compared with the control. The Values are means $\pm$ SEM. n = 3 muscles in each group. $**P < 0.01$ vs. control.

Fig. 5. No change in Tm, TnI and TnT isoform expression in the EDL muscle during four weeks of hind limb suspension

Representative Western blots using mAbs against Tm, TnI, slow TnT, and fast TnT (A) and densitometry analysis (B) showed no change in the thin filament regulatory protein isoforms in rat EDL muscle after four weeks of hind limb suspension. The Values are means $\pm$ SEM. n = 3 muscles in each group.

Fig. 6. Switching of Tm isoforms in rat soleus muscle during hind limb unloading

(A) Total protein extracts of soleus muscle from control and 5-, 7-, 14-, or 28-day hind limb suspended rats were resolved by SDS-PAGE and analyzed by Western blotting for the expression of Tm isoforms using mAb CH1 against α - and β -Tm and mAb CG3 against γ -Tm. The representative blots detected decreases in the slow fiber-specific γ -Tm and the β -Tm/ α -Tm ratio in the unloaded muscles. (B) The Western blots were quantified by densitometry for the relative amounts of β - and α -Tm isoforms, demonstrating the significant decrease in β/α ratio after 4 weeks of unloading. The Values are means $\pm$ SEM. $n = 3$ muscles in each group. * $P < 0.05$ and ** $P < 0.01$ vs. control.

Fig. 7. Switching of TnI isoforms in rat soleus muscle during hind limb suspension

(A) Total protein extracts of soleus muscle from control and 5-, 7-, 14-, or 28-day hind limb suspended rats were resolved by SDS-PAGE and analyzed by Western blotting for the change in TnI isoforms using mAb TnI-1 recognizing both fast and slow skeletal muscle TnI isoforms. The representative blots showed decrease in slow and increase in fast TnI isoform expression during the 4 weeks of unloading. (B) Normalized by the actin bands in parallel SDS-gel, the Western blots were quantified by densitometry for the changes in slow (ssTnI) and fast (fsTnI) skeletal muscle TnI isoforms. The plots in the upper panel shows the significant changes in the soleus muscle TnI isoform contents after 14 and 28 days of unloading. The lower panel further

shows an inverted ratio of slow TnI and fast TnI in the unloaded soleus muscle. The values are means $\pm$ SEM. n = 3 muscles in each group. * P <0.05 and ** P <0.01 vs. control.

Fig. 8. Switching of TnT isoforms in rat soleus muscle during hind limb unloading

(A) Total protein extracts of soleus muscle from control and 5-, 7-, 14-, or 28-day hind limb suspended rats were resolved by SDS-PAGE and analyzed by Western blotting using the anti-slow TnT mAb CT3 and anti-fast TnT mAb T12. The results showed decreases of slow TnT (ssTnT) and increases of fast TnT (fsTnT). A switching of fast TnT to more high molecular weight isoforms was also noticeable. (B) The Western blots were analyzed by densitometry with normalization by the actin bands in accompanying SDS-gel to demonstrate the changes in slow and fast TnT isoforms in soleus muscle during 28 days of unloading relative to the original level. The values are means $\pm$ SEM. $n = 3$ muscles in each group. * $P < 0.05$ and ** $P < 0.01$ vs. control.

Fig. 9. Changes in the fatigability of soleus muscle during hind limb unloading

(A) Representative high frequency fatigue recordings of soleus muscles from control and 4-week hind limb unloaded rats. In contrast to the fatigue resistance of normal soleus muscle, the unloaded rat soleus showed a rapid decline of contractile force. (B) Representative high frequency fatigue recordings of EDL muscle from control and 4-week hind limb unloaded rat. The fast fiber EDL muscle showed a rapid decline of tetanic force, which was not significantly affected by hind limb unloading. (C) The decreases in fatigue tolerance of rat soleus muscle during 4 weeks of hind limb unloading were quantified by the ratio of tetanic tension at the thirtieth second (marked by the dash line in panel A) vs. the maximum tension in continuous high frequency fatigue protocol (P_{30}/P_0). $n = 6$ muscles in each group. * $P < 0.05$ and ** $P < 0.01$.

(D) Representative intermittent tetanic fatigue recordings of 4-week unloaded and control soleus muscles are shown together with a normal EDL control. The accelerated decline of tetanic force production indicates decreased fatigue resistance of the unloaded soleus, which mimics that of fast fiber muscles. (E) Fatigue resistance of soleus muscle from control and 1-, 2- or 4-week hind limb suspended rats under intermittent tetanic contraction was quantified to show a significant decrease after 1-week of unloading without further change in the next 3 weeks. The values are means $\pm$ SEM. $n = 6$ muscles in each group. $**P < 0.01$ vs. control. (F) Fatigue resistance of EDL muscle from control and 1-, 2- or 4-week hind limb suspended rats under intermittent tetanic contraction was quantified to show the significant decrease in force production, which was not significantly affected by hind limb unloading. The values are means $\pm$ SEM. $n = 5$ muscles in each group.

Fig. 10. Switching of alternatively spliced fast TnT isoforms in rat soleus muscle during hind limb unloading

Multiple copies of mAb T12 Western blots of unloaded soleus muscle protein extracts were quantified by densitometry for the relative amounts of five detectable alternatively spliced high and low molecular weight fast skeletal muscle TnT (fsTnT) isoforms. The results demonstrate a progressive down regulation of lower molecular weight (T3 and T5, Fig. 8A) and up-regulation of higher molecular weight isoforms (T1, Fig. 8A) during the 4 weeks of unloading treatment. The values are means $\pm$ SEM. $n = 3$ muscles in each group. * $P < 0.05$ and ** $P < 0.01$ vs. control.

Table 1
Changes in rat soleus and EDL muscles induced by hind limb suspension

		Soleus		EDL	
		CONTROL	SUSPENSION	CONTROL	SUSPENSION
7 days	Weight(mg/g)	0.38 ± 0.02	0.29 ± 0.02 [*]	0.45 ± 0.01	0.44 ± 0.01
	Pt(g/mm ²)	1.76 ± 0.18	1.46 ± 0.26	1.23 ± 0.13	1.24 ± 0.16
	TP ₅₀ (ms)	16.67 ± 3.33	13.33 ± 2.11	n.a.	n.a.
	TPT(ms)	45.00 ± 2.24	30.00 ± 2.58	20.00 ± 1.89	18.33 ± 3.07
	TR ₇₅ (ms)	78.33 ± 9.46	56.67 ± 4.22 [*]	21.25 ± 2.95	16.67 ± 2.11
	C-Po(g/mm ²)	8.56 ± 1.53	5.75 ± 1.04	n.a.	n.a.
	I-Po(g/mm ²)	8.77 ± 0.54	8.64 ± 0.61	4.21 ± 0.21	4.18 ± 0.29
14 days	Weight(mg/g)	0.40 ± 0.02	0.16 ± 0.01 ^{**}	0.47 ± 0.02	0.40 ± 0.02
	Pt(g/mm ²)	1.62 ± 0.10	1.25 ± 0.09 [*]	1.46 ± 0.26	1.16 ± 0.15
	TP ₅₀ (ms)	15.00 ± 2.89	12.00 ± 2.00	n.a.	n.a.
	TPT(ms)	50.00 ± 3.16	28.00 ± 7.35 [*]	20.00 ± 3.16	23.98 ± 2.45
	TR ₇₅ (ms)	82.00 ± 11.58	48.00 ± 6.63 [*]	18.00 ± 3.74	16.00 ± 4.00
	C-Po(g/mm ²)	8.61 ± 0.70	3.44 ± 0.47 [*]	n.a.	n.a.
	I-Po(g/mm ²)	8.84 ± 0.5	8.69 ± 0.35	4.01 ± 0.18	3.77 ± 0.74
28 days	Weight(mg/g)	0.35 ± 0.02	0.15 ± 0.01 ^{**}	0.41 ± 0.01	0.40 ± 0.01
	Pt(g/mm ²)	1.73 ± 0.16	0.90 ± 0.06 ^{**}	1.20 ± 0.10	0.80 ± 0.10
	TP ₅₀ (ms)	11.67 ± 1.67	11.25 ± 1.25	13.80 ± 1.80	10.80 ± 2.00
	TPT(ms)	43.75 ± 2.63	26.67 ± 2.11 ^{**}	32.50 ± 3.70	25.00 ± 3.40
	TR ₇₅ (ms)	72.50 ± 4.91	46.67 ± 6.15 ^{**}	27.10 ± 2.90	23.30 ± 2.10
	C-Po(g/mm ²)	9.34 ± 1.08	2.09 ± 0.15 ^{**}	2.48 ± 0.27	3.24 ± 0.52
	I-Po(g/mm ²)	9.02 ± 0.45	5.95 ± 0.64 ^{**}	4.43 ± 0.23	3.95 ± 0.30

Values are means ± SEM; n.a., not analyzed; H-Po, maximal tension during continuous high-frequency tetanic contraction; I-Po, maximal tension during intermittent tetanic contraction.

^{*} $P < 0.05$ and

^{**} $P < 0.01$ vs. control.