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

Zhi Bin Yu,1,2 Fang Gao,2 Han Zhong Feng,1,2 and Jian-Ping Jin1

1Section of Molecular Cardiology, Evanston Northwestern Healthcare, Northwestern University Feinberg School of Medicine, Evanston, Illinois; and 2Department of Aerospace Physiology, Fourth Military Medical University, Xi’an, China

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Yu ZB, Gao F, Feng HZ, Jin J-P. Differential regulation of myofilament protein isoforms underlying the contractility changes in skeletal muscle unloading. Am J Physiol Cell Physiol 292: C1192–C1203, 2007.—Weight-bearing skeletal muscles change phenotype in response to unloading. Using the hindlimb suspension rat model, we investigated the regulation of myofilament protein isoforms in correlation to contractility. Four weeks of continuous hindlimb unloading produced progressive atrophy and contractility changes in soleus but not extensor digitorum longus muscle. The unloaded soleus muscle also had decreased fatigue resistance. Along with the decrease of myosin heavy chain isoform I and IIa and increase of 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 upregulated 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.

troponin T; fatigue resistance; troponin I; tropomyosin; myosin; hindlimb-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 spaceflight (17) and in bedridden conditions (6, 30, 42). Muscle unloading due to long exposure to weightlessness or simulated weightlessness causes atrophy, loss of functional capacity, impaired locomotor coordination, and decreased resistance to fatigue in the antigap 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 [Ca2+]i. Relevant changes in the Ca2+ handling system in unloaded muscle cells include the increased expression of Ca2+ channel in the sarcolemma (7) and the increase in the maximal velocity of shortening due to changes in the Ca2+ uptake and release by sarcoplasmic reticulum (43).

Downstream of the Ca2+ signaling pathway, the responsiveness of myofilaments is another determinant in skeletal muscle contractility. Ca2+ 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 with 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 hindlimb suspension rat model (55). Four weeks of continuous hindlimb unloading produced progressive atrophy and contractility changes in soleus but not extensor digitorum longus muscle.
longus (EDL) muscle. In addition to the decreases of MHC I and Ila 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 upregulated 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 mal-adaptation to mechanical unloading.

**Materials and Methods**

**Hindlimb suspension rat model of continuous skeletal muscle unloading.** Male Sprague-Dawley rats weighing 180–210 g were randomly divided into control and hindlimb suspension groups. The rats were housed in a 22 ± 2°C environment, subjected to 12:12-h light/dark cycles, and fed with water and rat chow ad libitum. After being individually caged for 1 wk, 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 hindlimb suspension and examined at the same schedule as controls. Muscle weight and body weight of the hindlimb-suspended and control rats were recorded at the time of functional measurements.

**Measurements of muscle contractility.** Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (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 MgSO4, 20.0 NaHCO3, 1.2 NaH2PO4, 2.5 CaCl2, and 10.0 glucose, pH 7.4, maintained at 37°C, and bubbled with 95% O2/5% CO2. The distal tendon of 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-PAGE of MHC isoforms.** As described previously (10), total protein was extracted by homogenizing the rat skeletal muscle tissues in 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 Bio-Rad mini-Protein II system and run at 70 V in an icebox for 24 h. 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 Bio-Rad mini-Protein II system. The resolved protein bands were electrically transferred to nitrocellulose membrane (0.45 μm pore size) using a Bio-Rad semi-dry transfer apparatus at 5 mA/cm² for 15 min. The membrane was blocked in Tris-buffered saline (TBS) composed of (in mM) 137 NaCl, 5 KCl, and 25 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 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 normalized with that of the actin band in parallel SDS gel. The Western blotting conditions for each antibody used were similar to that in normal fast skeletal muscle (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.
RESULTS

Muscle atrophy and changes in contractility during 4 wk of hindlimb unloading. The rat hindlimb 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 hindlimb suspension. Correspondingly, continuous hindlimb unloading rapidly produced contractility changes in soleus but not EDL during the 4 wk 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. Figure 2A demonstrates the changes in maximum isometric twitch tension ($P_t$) during 4 wk of hindlimb suspension. 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 hindlimb 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 of soleus muscle increased after 1 wk of unloading and progressed after 2 and 4 wk of unloading compared with the control values ($P < 0.05$ and $P < 0.01$; Fig.
The normal rat soleus expresses two MHC isoforms, MHC I and IIa, with MHC I being dominant isoform after 28 days when MHC IIa ceased expression and MHC IIb became significant. The results indicate that there was a slow transition of the fiber types in the soleus muscle during unloading that occurred significantly after 2 wk of hindlimb 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 blot and densitometry analysis during the 4 wk of hindlimb suspension (Fig. 5).

The unloading-induced myosin isoform changes in soleus muscle were accompanied by multiple changes in thin filament regulatory protein isoform expression. Figure 6 shows a switching of Tm isoforms in the soleus after 4 wk of hindlimb suspension. AJP-Cell Physiol • VOL 292 • MARCH 2007 • www.ajpcell.org

### Table 1. Changes in rat soleus and EDL muscles induced by hindlimb suspension

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<td>88.40±4.41</td>
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Values are means ± SE. EDL, extensor digitorum longus; muscle weight was normalized by per gram of body weight; P₀, peak tension per unit cross-sectional area; TP₅₀, time to 50% Peak tension; TPT, time to peak tension; TR₇₅, time from peak tension to 75% relaxation; NA, 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.

The changes in maximum tetanic tension (P₀) of the unloaded soleus muscle were illustrated in Fig. 3. Figure 3A shows that after 7 days of hindlimb suspension, the P₀ of continuous tetani decreased ~33%, although no statistical significance was established during shortening phenomena among individual animals. The decrease of continuous tetanic P₀ 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 P₀ in continuous tetani, Fig. 3B shows that the soleus P₀ 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 compared with the synchronous controls.

Unloading-induced MHC isoform switching. Four isoforms of MHC (I, Ia, 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 wk of hindlimb 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 progressive 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 upregulation of MHC IIX that was detectable after 14 days of unloading and became the dominant isoform after 28 days when MHC Ila 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 wk of hindlimb suspension.
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 hindlimb 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 hindlimb 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 downregulation during the 4 wk of hindlimb 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 <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 NH2-terminal segment encoded by exon 5 (24) remained stable while the expression of ssTnT gene was
downregulated. Compared with 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 hindlimb unloading.

While the level of ssTnT was decreasing, the level of fast skeletal muscle TnT (fsTnT) was increasing in soleus muscle during the 4 wk of unloading (Fig. 8A). The increase in fsTnT expression was detectable after 7 days of unloading, became obvious after 14 days of hindlimb 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 hindlimb unloading (17).

**Decreased fatigue resistance of unloaded soleus muscle.**

Figure 9, A and B, shows representative high-frequency fatigue (12) recordings of tetanic contraction of soleus and EDL muscles from control and 4-wk hindlimb-suspended rats. As is 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 hindlimb unloading (Fig. 9B). We used the normalized force at the thirtieth second of continuously tetanic contraction vs. the peak tetanic force (P30/Po) 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 hindlimb 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). Figure 9D shows the representative recordings of intermittent tetanic contraction of 4-wk 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. Figure 9, E and F, summarize the course of tension declining during intermittent tetanic contraction of soleus and EDL muscles from control and 1-, 2-, and 4-wk hindlimb suspended rats. The faster decline of the unloaded soleus vs. 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 hindlimb suspension. It is interesting to note that there was no further decrease in the next 3 wks of hindlimb unloading (Fig. 9E). The fatigue patterns of EDL muscle in intermittent tetani

Fig. 5. No change in Tm, TnI, and TnT isoform expression in the EDL muscle during 4 wk of hindlimb suspension. Representative Western blots using mAbs against Tm, Tnl, 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 4 wk of hindlimb suspension. Values are means ± SE; n = 3 muscles in each group.

Fig. 6. Switching of Tm isoforms in rat soleus muscle during hindlimb unloading. A: total protein extracts of soleus muscle from control and 5-, 7-, 14-, or 28-day hindlimb 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: 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 wk of unloading. Values are means ± SE; 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 hindlimb suspension. A: total protein extracts of soleus muscle from control and 5-, 7-, 14-, or 28-day hindlimb 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 TnI isoform and increase in fast TnI isoform expression during the 4 wk 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. Top, plots show the significant changes in the soleus muscle TnI isoform contents after 14 and 28 days of unloading. Bottom, an inverted ratio of slow TnI and fast TnI in the unloaded soleus muscle. Values are means ± SE; n = 3 muscles in each group. *P < 0.05 and **P < 0.01 vs. control.
more of the higher molecular weight isoforms (Fig. 8). We showed rapid declines of force reflecting a low resistance to fatigue, which was not affected by hindlimb 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 NH2-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 NH2-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 toward 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 to understand the molecular basis of the functional alterations. With the use of the rat hindlimb 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 4 wk 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 (29) has detected a slow to fast isoform switch of TnC in soleus muscle after 15 days of unloading. 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 hindlimb 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 hindlimb 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 Tnl mRNA and an increase in fast Tnl mRNA in mouse soleus muscle after 7 days of hindlimb suspension (15), demonstrating that the regulation of Tnl gene expression is initiated in the unloaded soleus muscle as early as the changes in TnI isoform proteins. We previously reported (52) that the equilibrium of TnT protein in the unloaded soleus occurred earlier and was seen after 7 days of hindlimb 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 Tnl 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 Tnl mRNA and an increase in fast Tnl mRNA in mouse soleus muscle after 7 days of hindlimb suspension (15), demonstrating that the regulation of Tnl gene expression is initiated in the unloaded soleus muscle as early as the changes in TnI isoform proteins. We previously reported (52) that the equilibrium of TnT protein in muscle cells is effectively regulated by proteolysis. 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 hindlimb 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 4 wk of hindlimb suspension (Fig. 3). This change corresponds to the slow to fast switch of 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.
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 wk 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 indicates a coordinated gene regulation that maintains the total TnT content 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 effects 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 upregulated 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 previously 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 upregulation 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 weightlessness-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 understand-
ing of the prevention and treatment of muscle dysfunction in various pathological disuse conditions.

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