Profiles of connectin (titin) in atrophied soleus muscle induced by unloading of rats

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Goto, Katsumasa, Ryoko Okuyama, Masanori Honda, Hiroshi Uchida, Tatsuo Akema, Yoshinobu Ohira, and Toshitada Yoshioka. Profiles of connectin (titin) in atrophied soleus muscle induced by unloading of rats. J Appl Physiol 94: 897–902, 2003.—Responses of the properties of connectin molecules in the slow-twitch soleus (Sol) and fast-twitch extensor digitorum longus muscles of rats to 3 days of unloading with or without 3-day reloading were investigated. The wet weight (relative to body wt) of Sol, not of extensor digitorum longus, in the unloaded group was significantly less than in the age-matched control (P < 0.05). Immunoelectron microscopic analyses showed that a monoclonal antibody against connectin (SM1) bound to the I-band region close to the edge of the A band at resting length and moved reversibly away from the Z line as the muscle fibers were stretched. In Sol, the displacement of the SM1-bound dense spots in response to stretching decreased after hindlimb suspension. There were no changes in the molecular weights and the percent distributions of α- and β-connectin in both muscles after hindlimb suspension. A significant increment of percent β-connectin in Sol was observed after 3 days of reloading after hindlimb suspension (P < 0.05). It is suggested that the elasticity of connectin filaments in the I-band region of the atrophied Sol fibers was reduced relative to that of the control fibers. The lack of the elasticity in atrophied muscle fibers may cause a decrease in contractile function.

Fast and slow muscles of rat; hindlimb suspension

Tin molecules (11, 12, 40). However, it is not fully understood how the connectin molecule responds to transformation of fiber phenotype. It is reported that β-connectin (titin 2) is the proteolytic product of the mother molecule, α-connectin (titin 1) (15, 22, 27). The protease, which is responsible for splitting a pure connectin molecule (α-connectin) into β-connectin, is partially inhibited by 1 mM E64c (an inhibitor for a thiol protease) or leupeptin and is thought to be a thiol protease such as calpain (13, 21). However, the physiological role of β-connectin is still unclear.

Unloading causes a striking atrophy of skeletal muscle, especially of the antagonistic muscle such as the soleus (Sol) (1, 2). Such a morphological adaptation is generally associated with the slow-to-fast transformation of muscle fiber phenotype. Changes in the expression of contractile proteins, such as myosin heavy chain and troponin, in slow-twitch muscle has been reported (1, 30, 31, 34). It is generally considered that muscular atrophy is caused by both the activation of proteolysis and the inhibition of the synthesis of muscular proteins (3, 38). However, it is unclear how the changes in proteolysis caused by unloading and reloading affect the characteristics of connectin. It is also not known how the elasticity of muscle fibers, which may be closely associated with the altered characteristics of connectin, is influenced.

Protein breakdown is induced after only a few days of hindlimb unloading (2, 3). However, it is still unclear how the profiles of connectin are influenced when Sol atrophies at a higher rate. Thus the present study was carried out to examine our working hypothesis that the profiles of connectin molecules are altered drastically when the...
muscle proteins turnover at a higher rate. Three-day hindlimb suspension followed by 3-day reloading, which may cause muscle fiber damage and change in connectin profiles, was performed in rats. The response of muscle fiber elasticity to unloading and/or reloading was also investigated.

**MATERIALS AND METHODS**

**Animals and treatments.** This study was performed following the Guiding Principles for the Care and Use of Animals Approved by the Council of the Physiological Society of Japan. Male Wistar rats, 10 wk of age, were used. Rats were randomly divided into three groups: 1) control group (n = 5), 2) hindlimb-suspended group (n = 5) subjected to 3 days of hindlimb suspension, and 3) recovery group (n = 5) subjected to 3 days of hindlimb suspension followed by 3 days of ambulation recovery. Rats were anesthetized with pentobarbital sodium (5 mg/100 g body wt ip) and killed by decapitation. Immediately after decapitation, the Sol and extensor digitorum longus (EDL) muscles were dissected and weighed.

A portion of each muscle (cut cross sectionally) was solubilized in 3 ml of SDS-DTT buffer consisting of 5 mM ethylenediaminetetraacetic acid, 10% SDS, 50 mM DTT, and 0.1 M Tris·HCl (pH 8.8), boiled for 3 min, and clarified by centrifugation for 20 min at 13,000 g at 20°C (19, 28). Then, the supernatant was mixed with 10 μl of 50% glycerol solution (50% glycerol, 0.1% bromophenol blue) at a 1:1 ratio and used for SDS-PAGE. For immunoelectron microscopic study, single muscle fibers were dissected from each muscle in relaxing solution, which consisted of 10 mM EGTA, 3.5 mM MgATP, 15 mM phosphocreatine, 0.3 mM DTT, 1.5 mM Mg2+, and 20 mM piperezine-N,N′-bis(2-ethanesulfonic acid) (PIPES) with pH 7.0 at 20°C. The ionic strength was adjusted to 0.2 M with potassium methansulfonate.

**SDS-PAGE.** SDS-PAGE was carried out by using 3–6% polyacrylamide (bisacrylamide/acylamide, 1:20 (wt/wt)) slab gel (60 × 85 × 1 mm) containing 0.5% SDS at a constant current (10 mA) for 60 min, as was described by Itoh et al. (17). The proteins on the gels were visualized by silver staining with Bio-Rad Silver Stain Plus (Bio-Rad, Hercules, CA) and were analyzed by using NIH Image software (National Institutes of Health). The molecular weights of α- and β-connectin were estimated by the densitometric scanning of the bands.

**Immunoelectron microscopy.** A single muscle fiber, dissected from each muscle, was tied to a platinum bar and transected from each muscle, was tied to a platinum bar and subjected to a skinning solution consisted of 10 mM EGTA, 3.5 mM MgATP, 15 mM phosphocreatine, 0.3 mM DTT, 1.5 mM Mg2+, and 20 mM piperezine-N,N′-bis(2-ethanesulfonic acid) (PIPES) with pH 7.0 at 20°C. The ionic strength was adjusted to 0.2 M with potassium methansulfonate.

**RESULTS**

After the 3 days of hindlimb suspension, the wet weight of Sol relative to body weight was significantly less than that in the age-matched control (Table 1, P < 0.05). However, the weight of EDL was not different between two groups.

In both Sol (Fig. 1) and EDL (Fig. 2), the SM1 bound to the I-band region close to the edges of the A band at resting length. At resting fiber length, there was no change in the distance between the binding site of SM1 and the Z line (Z-S distance) for both muscles after 3 days of hindlimb suspension. As the muscle fibers were stretched, the Z-S distance was extended. In Sol, the magnitude of displacement of the SM1-bound dense spots from the Z line (Z-S distance) in response to stretching decreased after hindlimb suspension (Figs. 1 and 3). According to the analyses of the relationship between Z-S distance and the half sarcomere length, the extension of half sarcomere length by 100 nm caused the extension of Z-S distance by 47 nm in the control and by 35 nm in the hindlimb-suspended group, respectively. The Z-S-to-half sarcomere length ratio of the control Sol (0.34 ± 0.02, n = 37) was significantly greater than that of suspended Sol (0.28 ± 0.02, n = 53, P < 0.05). These results indicate that the region of Z-S in suspended Sol was less extensible than that in the control Sol. However, hindlimb suspension had no effects on Z-S distance in response to stretching of EDL fibers (Fig. 4).

Figure 5 illustrates the representative SDS-PAGE pattern of connectin molecules. Connectin molecules in both EDL and Sol displayed double connectin bands. It has been considered that the upper connectin band is α-connectin and the lower one is β-connectin. A subtle difference in the mobility of α- and β-connectin was observed between fast EDL and slow Sol. However, the molecular weights of α- and β-connectin, estimated

<table>
<thead>
<tr>
<th>Group</th>
<th>Sol</th>
<th>EDL</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.45 ± 0.03</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>Suspension</td>
<td>0.30 ± 0.04*</td>
<td>0.54 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SD of n = 5 in each group (in mg muscle wet wt/g body wt). Control, control group; suspension, hindlimb-suspended group (3-day hindlimb suspension). *Significantly different from the control value (P < 0.05).
from the mobility, in both muscles did not change after unloading and/or reloading.

Figure 6 showed the percent composition of connectin molecules in each group. There were no changes in the percent compositions of α- and β-connectin in both muscles after hindlimb suspension. However, a significant increment of β-connectin, associated with an insignificant decrease of α-connectin, was observed in Sol after 3 days of ambulation recovery from hindlimb suspension ($P < 0.05$). In EDL, there was no significant change in the composition of α- and β-connectin molecules after hindlimb suspension and ambulation recovery.

**DISCUSSION**

In a muscle sarcomere, connectin molecules span from the M line to the Z line and anchor the thick filament to the Z line. Connectin molecule in skeletal muscle contains three segments [tandem immunoglobulin (Ig), N2-A, and one that, which is rich in proline, glutamate, valine, and lysine residues (PEVK)] in the I-band region (25). It has been considered that the extensibility of the I-band region is mainly dependent on the PEVK segment and Ig-like domain (tandem Ig domain), which is serially linked to PEVK segment (12, 25, 26). The elastic nature of a connectin molecule is dependent on the I-band region in physiological condition. When the sarcomeres are stretched, tandem Ig segments are first straightened with little increase in tension. Further extension leads to unfolding of the PEVK segment, which is folded at the resting sarcomere length (12). In addition, the extensible region of the N2-A (4 Ig domains and a 106-residue unique sequence) or the N2-B element (3 Ig domains and a 572-residue unique sequence) is found in Z-line-linked tandem Ig domain in skeletal and cardiac muscles (25).

The connectin molecule in slow-twitch fibers is larger than that in fast-twitch fibers, and the difference in the
elastic properties between slow and fast muscle fibers is related to the difference in connectin molecules (11, 12, 40). Greater mobility of α- and β-connectin was observed in fast muscle compared with slow muscle, as was reported elsewhere (11, 39). It is suggested that the large differences in resting tension between Sol and EDL may be due to the different molecular size of each connectin (11, 12). It has been reported that muscles that express larger connectin isoforms tend to initiate passive tension at a longer sarcomere length, and therefore, the passive tension development at a certain sarcomere length is less than that in muscles with smaller connectin (40), suggesting that slow muscle is more elastic than fast muscle.

It has been reported that a monoclonal antibody for connectin, SM1, binds to the I-band region ~0.3 and 0.1 μm away from the Z line and the edges of the A band, respectively, at a resting sarcomere length of ~2.4 μm (12). In the present study, SM1 also bound to the I-band region ~0.3 μm away from the Z line at resting sarcomere length in the control and suspended Sol. However, the extensibility of the I-band region from the Z line to SM1 binding site (Z-S distance) in Sol, but not in EDL, decreased after hindlimb suspension.

Recently, it has been reported that hindlimb unloading caused a decrease in the content of connectin protein (37) and that the sizes of α- and β-connectin and connectin antibody localization were altered (18). These observations suggest that connectin in atrophied muscle may have some alterations in the molecular composition. However, none of the molecular weights, estimated from the mobility in the electrophoresis, and the percent distributions of α- and β-connectin in the unloaded group were different from those in the age-matched control. Kasper and Xun (18) and Toursel et al. (37) also observed that 14 days of hindlimb unloading had no effect on the expression of connectin molecules.

In atrophied Sol, the decrease in contractile protein contents (1–3), the depression of the isometric force generation (1, 41–43), Ca2+ sensitivity of myofilaments (41–43), and the increment of both maximal shortening velocities (41–43) are observed. The expressions of contractile proteins (1, 4, 30, 31, 34), regulatory proteins (4), sarcoplasmic reticulum Ca2+-pump protein (32), and metabolic proteins (5, 8, 44) are shifted from slow to fast type in slow-twitch muscles after unloading. In the present study, however, unloading by hindlimb suspension did not induce any change in the expression of connectin molecules. These observations suggest that the regulation system of the expression of muscular elastic protein might be different from that of other muscular proteins.

α-Connectin is considered to be easily degraded into β-connectin and a 1,200-kDa subfragment (20, 35). This degradation of α-connectin molecule is induced by the binding of Ca2+ to α-connectin (35) and/or calpain...
Increased intracellular Ca\(^{2+}\) concentration may activate calpain, and then \(\alpha\)-connectin may be degraded to \(\beta\)-connectin. In the present study, however, the percent composition of \(\alpha\)- and \(\beta\)-connectin did not change after hindlimb suspension. These observations suggest that the proteolysis by calpain may not be activated in the atrophied muscles. Muscular atrophy, in the present study, may be induced by a non-Ca\(^{2+}\)-activated proteolysis system, such as ubiquitin-proteasome and/or by the decreased synthesis of structural proteins.

Fiber damage is induced in response to reloading after unloading (6, 7, 23, 24, 36). Eccentric contraction-like lesions, such as an abnormal widening of sarcomeres with A-band disruption and excessively wavy and/or extracted Z line, are reported (23, 24). The reloading after unloading caused an increased percent-age of \(\beta\)-connectin in Sol but not in EDL. These observations suggest that the reloading on atrophied muscle may activate proteolysis, associated with calpain, for example, and then \(\alpha\)-connectin may be degraded to \(\beta\)-connectin. It has been reported that 1 day of reloading after 14 days of hindlimb suspension increased the resting intracellular Ca\(^{2+}\) concentration by 24\% (16).

Recently, Farges et al. (6) reported that increased cathepsin B activity and mRNA encoding cathepsin B, L, H, and C were observed in rat gastrocnemius muscle after unloading caused an increased percent-age of \(\beta\)-connectin in Sol but not in EDL. These observations suggest that the reloading on atrophied muscle may activate proteolysis, associated with calpain, for example, and then \(\alpha\)-connectin may be degraded to \(\beta\)-connectin. It has been reported that 1 day of reloading after 14 days of hindlimb suspension increased the resting intracellular Ca\(^{2+}\) concentration by 24\% (16).

In conclusion, the elasticity of connectin filaments in the I-band region of atrophied Sol fibers was reduced after 3 days of hindlimb suspension. But the molecular weights or percent distributions of \(\alpha\)- and \(\beta\)-connectin were not affected, although degradation of \(\alpha\)- to \(\beta\)-connectin was noted after 3 days of reloading. It is also unknown whether the properties of PEVK and/or tandem Ig segments were altered or not. Thus the mechanism responsible for the reduction of elasticity in atrophied muscle fibers is still unclear. The decreased elasticity in atrophied muscle fibers may, in part, play a role in the decrement of contractile function.

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