Cytoskeletal protein contents before and after hindlimb suspension in a fast and slow rat skeletal muscle

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Chopard, Angele, Françoise Pons, and Jean-François Marini. Cytoskeletal protein contents before and after hindlimb suspension in a fast and slow rat skeletal muscle. Am J Physiol Regulatory Integrative Comp Physiol 280: R323–R330, 2001.—Transversal cytoskeletal organization of muscle fibers is well described, although very few data are available concerning protein content. Measurements of desmin, α-actinin, and actin contents in soleus and extensor digitorum longus (EDL) rat skeletal muscles, taken with the results previously reported for several dystrophin-glycoprotein complex (DGC) components, indicate that the contents of most cytoskeletal proteins are higher in slow-type fibers than in fast ones. The effects of hypokinesia and unloading on the cytoskeleton were also investigated, using hindlimb suspension. First, this resulted in a decrease in contractile protein contents, only after 6 wk, in the soleus. Dystrophin and associated proteins were shown to be reduced for soleus at 3 wk, whereas only the dystrophin-associated proteins were found to increase after 6 wk. On the other hand, the contents of DGC components were increased for EDL for the two durations. Desmin and α-actinin levels were unchanged in the same conditions. Consequently, it can be concluded that the cytoskeletal protein expression levels could largely contribute to muscle fiber adaptation induced by modified functional demands.

Cytoskeleton; fast and slow muscle fibers; quantitative analysis; atrophy

SKELETAL MUSCLE FIBERS, because of force generation and external loading, must withstand mechanical constraints exerted on their longitudinal and transversal axis (22, 37). The intensity, frequency, and duration of muscle loading depend on the muscle functions. These variable mechanical constraints cause structural differences in the myotendinous junction (MTJ) (39) and in Z lines (40) of the longitudinal axis of slow (type I) and fast (type II) muscle fibers. But little is known about functional consequences on the transversal cytoskeletal organization. Studies have revealed the role of specific structures, like dystrophin-glycoprotein complex (DGC) (35) or other protein complexes with a costameric organization (25, 26), in the transversal cohesion of muscle fibers and their relation with the extracellular matrix (ECM) (1, 6, 22). Genetic defects of the DGC in human congenital muscular dystrophies (14, 35) as well as in animal models (20) have highlighted the importance of this complex in the muscle fiber cohesion and in the protection of the sarcolemma from contraction-induced damage. Also, studies have revealed the relation between muscle function and myosin heavy chain (MHC) contents (30), myofibrillar protein diversity (31), and MTJ ultrastructure (38). However, although the proteins of specific compartments of the cytoskeleton are well described and localized, little is known about their respective contents in different muscle types. In a previous study, we developed a quantitative method that demonstrated that DGC relative content was twofold higher in a slow muscle (soleus) than in a fast one [extensor digitorum longus (EDL)] rat skeletal muscles (4). Considering the important differences between postural and phasic muscles, it seemed of a particular interest to determine the effects of changing functional demand on several muscle cytoskeletal proteins.

Muscle atrophy is a consequence of disuse or hypokinesia. It is accompanied by changes in MHC isoforms during hindlimb suspension (HS) in rats (17, 24) and during spaceflights in rats (21), humans (11), and rhesus monkeys (3) and by changes in MTJ during HS (42) and spaceflight (29). These changes indicate that, with respect to some characteristics (MHC or metabolic profiles for example), the soleus muscle was transformed into a faster muscle type. Concerning the cytoskeletal proteins, which play a crucial role in the resistance to imposed mechanical constraints (which specific role is still unclear), it can be postulated that a modified functional demand could also affect their expression. Thus the understanding of their physiological role, especially for DGC, could benefit from the evaluation of cytoskel-
etal protein modifications for both slow and fast muscle types submitted to unloading conditions.

In the present study, we first determined the relative contents of several proteins belonging to cytoskeletal compartments other than DGC, previously established (4), that is the intra- and perisarcromeric compartment (α-actinin and desmin) and the contractile compartment (actin and myosin) in slow (soleus) and fast (EDL) rat skeletal muscle. Second, we studied the changes in the cytoskeletal protein relative contents as a consequence of hypokinesia and hypotension, not only in the atrophied soleus muscle, but also in the fast EDL muscle during 3 and 6 wk of rat HS.

METHODS

Animal Care and Suspension

Female pathogen-free, 6-wk-old Sprague-Dawley rats weighing ~150 g were obtained from IFFA CREDO (L’arbresle, France). The rats were housed in a temperature-controlled room at 21°C with a 12:12-h light-dark cycle and were provided with rodent chow and water ad libitum. After 2 wk, they were randomly divided into HS and control groups. Nine rats were suspended by their tails for 3 wk and 9 for 6 wk according to the Morey procedure (23). The animals were suspended at about a 20° head-down tilt angle to minimize lordosis. They were allowed to move in their individual cages in a full range of motion, freely able to gain access to food and water by walking on their forelimbs. The other 18 rats were used as controls.

Muscle Preparation

At the end of different HS periods (3 and 6 wk), the experimental and control animals were weighed and killed with a solution of pentobarbital sodium (6%) administered intraperitoneally. The lethal dose of pentobarbital sodium was at least sixfold the one used for anesthetized animals, that is 500 mg/kg of body mass. The soleus and EDL muscles were removed from their right hindlimbs. All muscles were frozen in isopentane precooled by liquid nitrogen and were stored at −80°C.

Antibodies

Anti-α-actinin monoclonal antibody was purchased from Sigma (St. Quentin Fallavier, France). Anti-desmin, anti-β-dystroglycan, anti-γ-sarcoglycan, and anti-α-sarcoglycan monoclonal antibodies were purchased from Tebu (Le Perray en Yvelines, France). Anti-slow (I) MHC (8H8), anti-fast (IIa + IIb) MHC (15F4), and anti-dystrophin (5G5) monoclonal antibodies were previously described in Refs. 7, 19, 27, respectively. The anti-fast MHC 14E8 was prepared with psoas myosin as antigen and characterized by Western blotting and immunofluorescence, and it was specific to the IIb MHC (Pons and Marini, unpublished data). Secondary labeling was performed with the use of horseradish peroxidase-conjugated, anti-mouse Ig from Amersham Pharmacia Biotech (Les Ulis, France) or FITC-conjugated, anti-mouse Ig from Tebu.

Immunohistochemical Classification of Muscle Fibers

Serial cross sections (10 µm thick) at the middle part of the muscle were cut in a cryostat, maintained at −30°C, and mounted onto glass microscope slides. Muscle fibers were identified on the basis of their reactivity with anti-MHC antibodies. The percentage of the different muscle fiber types was evaluated, with ~200 muscle fibers on each section.

Cross-Sectional Areas

The areas of 50 slow-type fibers and 50 fast-type fibers in each muscle middle part were calculated with the use of an image-analysis software (Analysis, Olympus, France).

Tissue Sampling and Extraction

A whole section (20–30 mg) was cut in the middle of each right hindlimb frozen muscle, quickly weighed, and then immediately homogenized in 200 µl of buffer (pH 6.8) containing 5 mM Tris, 10% SDS, 0.2 M 1,4-dithiothreitol, 1 mM EDTA, and 2.5% protease inhibitor cocktail (Sigma). The samples were subsequently boiled for 2 min and centrifuged for 10 min at 10,000 g. The supernatant was then separated, with one part for total protein concentration determination and the other for electrophoresis analysis.

Total Protein Concentration

The total protein concentration of each sample was determined with the use of a BCA protein assay (Interchim, Montluçon, France) modified as described elsewhere (4). A fresh set of protein standards (bovine serum albumin) was prepared and used to calibrate the protein concentrations for each sample at the absorbance of 562 nm. We made each measurement in triplicate and used the mean values. The protein standards were read before and after all the other samples to evaluate the optical density (OD) variation during the measurements.

SDS Gel Electrophoresis

Proteins were separated onto a 4–12% slab gradient SDS polyacrylamide gel according to the method of Laemmli (18). All the samples used for each comparison were loaded at the same time on the same gel. The wells contained alternately 10 µl of control or HS extracts of EDL or soleus muscle to determine the effect of HS or 10 µl of control EDL or control soleus extracts to compare the two muscle types (Fig. 1A). The two edge wells were excluded from the comparison. We quantified and compared several protein relative contents of two groups of nine animals, both in the same working conditions as previously described (4). Myosin and actin, the major muscle components (respectively 200 and 42 kDa), were directly identified on the gels stained with Coomassie brilliant blue. The other cytoskeletal protein levels were determined by immunoblotting after the gel transfer.

Western Blot

The upper part of the gel containing polypeptides >300 kDa was transferred overnight onto a nitrocellulose sheet (30 V, 15°C) in a Tris, methanol, and glycine buffer. The lower part of the gel was submitted to a transfer procedure for 1 h at 80 V in the same buffer. After that, both parts of the gel were stained with Coomassie brilliant blue to check the uniformity of transfer. Nitrocellulose blots were blocked 12 h at 4°C in Tris-buffered saline (TBS) containing 100 mM Tris (pH 8) and 150 mM NaCl by the addition of 0.1% Tween 20 and 5% nonfat dry milk. Blots were then incubated for 30 min at 37°C with antisera diluted in TBS Tween as follows: anti-dystrophin, 1:15; anti-α-actinin, 1:5,000; anti-desmin,
1:4,000; anti-α-sarcoglycan, 1:40; anti-β-dystroglycan, 1:500; and anti-γ-sarcoglycan, 1:300.

Blots were subsequently washed 1 × 5, 1 × 20, and 2 × 5 min in TBS Tween and then incubated 1 h at room temperature with peroxidase-labeled, species-specific second antibodies (Amersham Pharmacia Biotech) diluted 1:5,000 for anti-mouse. After being washed 1 × 5, 1 × 20, and 2 × 5 min in TBS Tween, bound antibodies were detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech) with different times of exposure to obtain a signal in a linear set (Fig. 1B).

Scanning Densitometry

Gels and blots were digitized with the use of a scanner densitometer with a resolution of 1,200 pixel per inch. The digitized images were quantitatively analyzed with the use of a specific software, Phoretix 1D (Phoretix International, Newcastle Upon Tyne, UK). The OD of a protein band in a sample was expressed as relative OD (ODr) according to the amount of protein loaded into the corresponding well.

Each gel or blot was triplicated. To avoid errors due to differences in signal intensity among the three gels or blots, the ODr was normalized as follows. For each protein, the ODr of all samples was summed onto each gel or blot (Si), and the ODr of each band on the gel or blot was then normalized according to the mean of these sums: normODr = ODr × (S1 + S2 + S3)/3 × S1.

Statistics

Results were expressed as means ± SD of the normalized ODr in the three assays. The values obtained were used to compare means of protein relative content expressed in arbitrary units, that is absolute OD divided by the amount of protein loaded for each sample. Finally, for each protein relative content, the two groups were compared by Mann-Whitney’s rank sum test. P values <0.05 were considered statistically significant.

RESULTS

Comparison of Cytoskeletal and Contractile Protein Relative Contents in Slow-Type Muscle (Soleus) and Fast-Type Muscle (EDL)

Quantitative analysis showed that cytoskeletal and contractile protein relative contents were significantly different between the slow-type muscle (soleus) and the fast-type muscle (EDL) (Fig. 2). Unlike myosin, whose relative content was 10% lower (P < 0.01) in the soleus, the other proteins exhibited higher relative contents in the slow-type muscle. Actin exhibited only a 10% higher (P < 0.005) relative content, whereas α-actinin and desmin exhibited greater difference (50 and 90%, respectively) (P < 0.005).

Effect of HS

Body mass, muscle mass, cross-sectional area, total protein concentration, and MHC expression. Body mass (Table 1) was not significantly changed after 3 and 6 wk of HS. Soleus and EDL muscle weights decreased significantly after 3 and 6 wk of HS. On the other hand, at these two times, soleus showed a higher decrease (43.5 and 57%, respectively, P < 0.001) than EDL (17 and 30%, respectively, P < 0.001) in muscle mass. In both muscle types and durations, HS did not change the total protein concentration in the middle part of the muscles. The mean cross-sectional area (CSA) of both slow- and fast-twitch soleus fibers significantly de-
Table 1. Body mass, soleus EDL muscle mass, total protein concentration, CSA, and MHC expression after 3 and 6 wk of HS

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>HS</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3 wk</td>
<td>6 wk</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>249 ± 17</td>
<td>258 ± 15</td>
</tr>
<tr>
<td>Soleus mass, mg</td>
<td>124 ± 6</td>
<td>130 ± 7</td>
</tr>
<tr>
<td>EDL mass, mg</td>
<td>120 ± 7</td>
<td>135 ± 6</td>
</tr>
<tr>
<td>Soleus, protein g/muscle</td>
<td>145 ± 14</td>
<td>160 ± 16</td>
</tr>
<tr>
<td>EDL, protein g/muscle</td>
<td>135 ± 11</td>
<td>160 ± 10</td>
</tr>
<tr>
<td>Soleus, slow fiber CSA, ( \mu m^2 )</td>
<td>3,044 ± 324</td>
<td>2,949 ± 380</td>
</tr>
<tr>
<td>Soleus, fast fiber CSA, ( \mu m^2 )</td>
<td>2,736 ± 309</td>
<td>1,980 ± 285</td>
</tr>
<tr>
<td>EDL, slow fiber CSA, ( \mu m^2 )</td>
<td>1,185 ± 181</td>
<td>1,158 ± 105</td>
</tr>
<tr>
<td>EDL, fast fiber CSA, ( \mu m^2 )</td>
<td>2,271 ± 341</td>
<td>2,258 ± 256</td>
</tr>
<tr>
<td>Soleus, slow MHC, %</td>
<td>82 ± 2.8</td>
<td>83 ± 4.5</td>
</tr>
<tr>
<td>Soleus, fast MHC, %</td>
<td>18 ± 2.8</td>
<td>17 ± 4.5</td>
</tr>
<tr>
<td>Soleus, MHC coexpression, %</td>
<td>9 ± 2</td>
<td>6 ± 1.3</td>
</tr>
<tr>
<td>EDL, slow MHC, %</td>
<td>91 ± 2</td>
<td>94 ± 1.3</td>
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</tbody>
</table>

Values are means ± SD. Significant difference between hindlimb suspension (HS) and control values: *P < 0.001; †P < 0.005; ‡P < 0.01.

EDL, extensor digitorum longus; CSA, cross-sectional area; MHC, myosin heavy chain (expressed in percentage of total fibers).
titative differences in other cytoskeletal proteins is still unclear. With the use of quantitative analysis of gels and immunoblots, we compared the relative contents of cytoskeletal and contractile proteins in fast (EDL) and slow (soleus) rat skeletal muscle before and after HS.

Cytoskeletal and Contractile Protein Relative Contents in Slow and Fast Rat Skeletal Muscle

Myosin, whose isoforms' composition differ for the two muscle types (30), exhibited a 10% lower relative content in soleus in our comparative study. This slight difference is probably related to the main characteristics of the fiber types, i.e., the larger mitochondrial content and lower maximum force of the type I fibers. The higher desmin and α-actinin relative contents in soleus than in EDL muscle fit with the 100% higher relative content of DGC in soleus than in EDL muscles we previously showed (4). Moreover, Ho-Kim and Rogers (15) reported a twofold higher dystrophin relative content in the soleus compared with the vastus lateralis (a fast muscle). Actin relative content is slightly higher (10%) in soleus muscle, as are filamin and spectrin relative contents (32). In that context, and because soleus muscle is mainly composed of type I fibers and EDL of type II fibers, we suggest that the major part of cytoskeletal protein relative contents is higher in slow-type fibers than in fast ones.

The quantitative differences in cytoskeletal protein relative contents we observed may correspond to morphological differences between fiber types, reflecting their adaptation to functional demand. Functional requirements impose different mechanical stresses on the cytoskeleton and on the structure of the MTJ, which could, for example, correspond to the structural ability of the muscle fiber adaptation to the functional demand (39). In the same way, slow fibers exhibit a thicker Z band than do fast fibers; this thickness can even serve to discriminate among fiber types (12, 33). The higher relative content we observed for α-actinin and actin in slow soleus muscle may be related to Z-band morphological differences because these proteins are their major components (2, 41). The ability of slow muscle, mainly type I fibers, to maintain position and to generate forces during large periods would probably necessitate a cytoskeleton reinforcement. This reinforcement clearly appears in the longitudinal axis, at.

Fig. 3. Effect of 3 wk (A) and 6 wk (B) of hindlimb suspension (HS) on contractile protein (actin and myosin) (4A) and desmin and α-actinin (4B) relative contents of slow soleus and fast EDL muscles. Values are expressed in AU (means ± SD, n = 9 in each group). Significant difference between control (open bars) and HS (filled bars) protein relative contents: *P < 0.001; †P < 0.005.
the MTJ, for which it has been established that membrane folding is determined not only by the magnitude of loading but probably also by its duration (37). As in the longitudinal axis, muscle structure must also adapt to constraint in its transversal axis, as shown at the Z-band level. The thickness of this anchorage structure was demonstrated to increase when fast fibers, stimulated by low frequencies, are converted into slow fibers (12), which is supported by our results. Finally, it is now well established that the cytoskeleton plays a crucial role in muscle ability to resist internal and external constraints (6, 16, 22, 37). Cytoskeleton components appear to be involved in the transversal resistance and transmission of forces, and the different protein relative contents illustrate the adaptation of the muscle fibers to duration, magnitude, and frequency of the imposed mechanical loading.

**Effect of HS**

The decreases in soleus and EDL muscle mass of ~40 and 20% after 3 wk and ~60 and 30% after 6 wk, respectively, is in agreement with the classically reported muscle atrophy during HS (9). Moreover, muscle fiber CSA decreased for slow and fast soleus fibers by 24 and 20%, respectively, after 3 wk and ~55 and 34% after 6 wk; in EDL, it changed by 17 and 24% only after 6 wk. These results indicate that muscle atrophy is progressive and decreases in proportion with time (36), whereas the individual changes in the protein relative contents are probably more subtle.

The total protein concentrations remained unchanged after either 3 or 6 wk of HS, as reported for shorter durations of inactivity (9, 34). However, the quantitative analysis of protein relative contents from several compartments of the cytoskeleton indicated different changes according to muscle type, HS duration, and protein localization. The consequences of inactivity on the contractile apparatus are marked in soleus muscle where we observed the classically reported increase in muscle fibers that express type II MHC, resulting essentially from a coexpression of slow and fast MHC (5, 17, 21, 24). The present results indicating 17 and 24% decreases, respectively, of actin and myosin solely after 6 wk of HS, and only in the soleus and EDL muscles.
soleus, confirmed the greater susceptibility to inactivity and/or unloading of this muscle compared with a fast one such as EDL.

The potential functional or structural consequences of changes in cytoskeletal protein relative contents resulting from disuse atrophy are of great interest. The importance of cytoskeletal proteins is illustrated by the consequences of defects in one or several proteins, which result in severe disruption of muscle architecture and in muscle fiber degeneration and tissue necrosis, as demonstrated by different muscular dystrophies in human (35) or in animal models, for example, mdx mice (8), desmin nulle mice (20), or mice with \( \gamma \)- and \( \alpha \)-sarcoglycan deficiency (10, 13).

Contrary to desmin and \( \alpha \)-actinin relative contents, which did not change after 3 and 6 wk in EDL and soleus muscles, dystrophin and its associated protein relative contents are markedly reduced for the two durations of HS. These results suggest that proteins of DGC or from Z lines react differently to changes of stress. The decrease in dystrophin relative content in soleus after 3 wk of HS, which is amplified 3 wk later, goes along the same line as atrophy. On the other hand, it could indicate a shift of soleus muscle toward EDL muscle characteristics because the amount of cytoskeletal protein relative content is much lower in fast than in slow muscle. Inversely, the increased dystrophin relative content, 20 and 30\%, respectively, after 3 and 6 wk of HS in EDL muscle brings EDL closer to soleus with respect to dystrophin relative content. This evolution is supported, for EDL, by the changes in dystrophin-associated protein relative content, which increased by 20, 30, and 40\%, respectively, for \( \beta \)-dystroglycan, \( \alpha \)-, and \( \gamma \)-sarcoglycan after 3 wk, and by 40, 40, and 60\% after 6 wk of HS. Concerning the changes in fast muscle, Rezvani et al. (28) reported an increase in dystrophin, vinculin, and acinulin contents in tibialis anterior and biceps muscles, respectively, after 7 and 21 days of denervation and after 6 days of spaceflight.

The changes in soleus muscle observed during HS are at least biphasic complex, because after the first relative content decrease of \( \sim 20\% \) for \( \beta \)-dystroglycan, \( \alpha \)-, and \( \gamma \)-sarcoglycan during the first 3 wk of HS, as for dystrophin, an increase of 30, 60, and 60\% occurred, respectively, for \( \beta \)-dystroglycan, \( \alpha \)-, and \( \gamma \)-sarcoglycan after 3 more wk of HS could be due to transitory adaptations. The increase in \( \beta \)-dystroglycan and \( \alpha \)- and \( \gamma \)-sarcoglycan after 3 more wk of HS could be interpreted as such a transitory step in the reorganization of soleus muscle to a different functional demand. The relations between the different DGC proteins appear complex, and their functional interdependence is not clearly established. This is illustrated by the results of Hack et al. (13) indicating that the dystrophin-dystroglycan-laminin mechanical link was unaffected by a \( \gamma \)-sarcoglycan deficiency, although it resulted in a secondary reduction of \( \beta \)- and \( \delta \)-sarcoglycan. The relation between the changes in DGC relative content and membrane modifications during HS need to be clarified, because, for example, the length of the membrane interface between muscle fiber and tendon is largely increased after HS or spaceflight (29). Plasma membrane remodeling during muscle atrophy is probably one of the key points for interpreting the modifications in DGC relative content that result from different loading conditions.

Besides its functional role during force transmission, the cytoskeleton, managing the forces generated or supported in the transversal and longitudinal axis of muscle fiber, is able to protect the muscle fiber from contraction-induced damage. In the same way as the longitudinal relation with tendons through the MTJ (38) involves series of successive sets of myofibrillar proteins, the transversal relation of cytoskeleton with the ECM, via the sarcolemma, involves DGC and other protein complexes, which seem to be required in the fiber damage prevention (35). The differences in cytoskeletal protein relative contents between fast and slow muscles and the changes resulting from a reduction of the load imposed on muscles illustrate the importance of these proteins in a structure-function relation in muscles and their ability to contribute to muscle fiber adaptation induced by modified functional demands.

Perspectives

In terms of understanding the adaptation of cytoskeleton muscle to disuse, further investigation is required to specify the time course progression of the expression of the proteins studied to highlight the adaptation of muscle fibers to the duration, magnitude, and frequency of the imposed mechanical loading.

The application of such analysis to human tissue, for which little material is available, will help in understanding the physiological role of the cytoskeletal proteins and muscular dystrophy research or diagnosis.

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REFERENCES


EFFECT OF UNLOADING ON CYTOSKELETAL PROTEIN CONTENTS


