Non-weight bearing-induced muscle weakness: the role of myosin quantity and quality in MHC type II fibers

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Kim JH, Thompson LV. Non-weight bearing-induced muscle weakness: the role of myosin quantity and quality in MHC type II fibers. Am J Physiol Cell Physiol 307: C190–C194, 2014. First published May 14, 2014; doi:10.1152/ajpcell.00076.2014.—We tested the hypothesis that non-weight-bearing-induced muscle weakness (i.e., specific force) results from decreases in myosin protein quantity (i.e., myosin content per half-sarcomere and the ratio of myosin to actin) and quality (i.e., force per half-sarcomere and population of myosin heads in the strong-binding structural state during muscle contraction) in single myosin heavy chain (MHC) type II fibers. Fisher-344 rats were assigned to weight-bearing control (Con) or non-weight bearing (NWB). The NWB rats were hindlimb unloaded for 2 wk. Diameter, force, and MHC content were determined in permeabilized single fibers from the semimembranosus muscle. MHC isoform and the ratio of MHC to actin in each fiber were determined by gel electrophoresis and silver staining techniques. The structural distribution of myosin from spin-labeled fiber bundles during maximal isometric contraction was evaluated using electron paramagnetic resonance spectroscopy. Specific force (peak force per cross-sectional area) in MHC type IIB and IIXB fibers from NWB was significantly reduced by 38% and 50%, respectively. MHC content per half-sarcomere was significantly reduced by 21%. Two weeks of hindlimb unloading resulted in a reduced force per half-sarcomere of 52% and fraction of myosin strong-binding during contraction of 34%. The results suggest that reduced myosin and actin content (quantity) and myosin quality concomitantly contribute to non-weight bearing-related muscle weakness.

METHODS

Animals and hindlimb unloading protocol to mimic non-weight bearing. The animal care protocol was approved by the Institutional Animal Care and Use Committee in accordance with guidelines established by the American Physiological Society. Male Fisher-344 rats were obtained from the aging colony maintained by the Minneapolis Veterans Administration. Rats were randomly assigned to a control group (Con), weight-bearing group, or non-weight bearing (NWB) group (n = 6 and 5, respectively). We used a hindlimb unloading (HU) model as a mimic of the non-weight bearing condition. Animals were hindlimb suspended for 2-wk periods as described previously (11). Briefly, the hindlimbs were suspended by using a tail harness attached to the proximal two-thirds of the rat’s tail with orthopedic traction tape. The height of the suspension was vertically adjusted to a spinal orientation of 40–45° above horizontal so that the hindlimbs could not contact the cage floor.

Tissue preparation. After 2 wk of HU or normal cage activity for the Con group, rats were anesthetized with pentobarbital sodium (55 mg/kg ip). To avoid reloading-induced muscle injury, NWB rats were anesthetized while suspended. Semimembranosus (SM) muscles were dissected, trimmed free of fat and connective tissue, and weighed. SM muscles were then placed in cold relaxing solution [in mM: 0.016 CaCl2, 5.6 MgCl2, 80 KCl, 20 imidazole (pH 7.0), 14.5 phosphate, 4.8 ATP]. The SM muscles were divided into small bundles of fibers ~8 mm long and ~1 mm in diameter for single-fiber
contractile analyses. These bundles were tied to pieces of capillary tubes and stored in skinning solution (50% glycerol:50% relaxing solution) for up to 5 wk at −20°C. Additional bundles of fibers, ~25 mm long and ~2 mm in diameter, were permeabilized in fresh glycerol solution containing 0.1 mM DTT for EPR experiments and stored at −20°C.

**Single muscle fiber force determination.** To determine maximal force-generating capacity, individual fiber segments (~2 mm long) were randomly isolated from the permeabilized bundles and studied at 15°C as described in detail previously (22). Next, the fiber segments were mounted to an experimental bath filled with relaxing solution, the segment length was adjusted to 2.5 μm of sarcomere spacing by moving the microscope stage with a micrometer. Fiber length (L0) was determined directly from the micrometer displacement, and diameter was determined as the average of three measurements made along the length of the fiber. Isometric maximal force (P0) was determined by transferring the fiber from relaxing solution to activation solution [in mM: 7 EGTA, 5.6 MgCl2, 80 KCl, 20 imidazole (pH 7.0), 14.5 creatine phosphate, 4.8 ATP, and CaCl2 to achieve pCa 4.5] and measured at the point where the force output reached a maximum. Six to ten fibers from each muscle were studied. After force measurements were made, each fiber segment was solubilized in 20 μL of sample buffer (24 mM EDTA, 60 mM Tris; pH 6.8, 1% SDS, 5% β-mercaptoethanol, 15% glycerol, 2 mg/ml bromophenol blue) and stored at −80°C.

**MHC content, MHC isoform, and MHC-to-actin ratio determination.** MHC content was determined following the procedures described by Geiger et al. (5). Briefly, to determine the MHC content, myosin standard as protein reference was purchased (Sigma M7659) and diluted (0.1–1.5 μg/μL) in 1× SDS sample buffer. The diluted myosin standards and the single-fiber segments (tested for force determination) were loaded on a gel (3.5% acrylamide stacking/12% acrylamide separating). Silver-stained gels were scanned on a flatbed scanner, and image analysis software (Sigma-Scan, Jandel Scientific) was used to quantify the content of all MHC isoforms in each fiber segment.

Specifically, a linear relationship between densitometric measurement of electrophoretic bands and the myosin standard concentration was used to determine the MHC content. The MHC content in the entire single fiber was determined by dividing the MHC concentration calculated from the myosin standard curve by the fiber volume. To calculate MHC content per half-sarcomere, the MHC content was then multiplied by the half-sarcomere volume of the fiber, normalized to L0, of 2.5 μm/sarcomere. Force per half-sarcomere MHC content (N/μg) was obtained by dividing peak force by the value of MHC content per half-sarcomere as previously described (5). About 1 nl of fiber volume was calculated and then loaded into a second gel (3.5% acrylamide stacking/12% acrylamide separating) to determine the ratio of MHC to actin content. The relative content of MHC to actin in each fiber was determined from silver-stained gels using a flatbed scanner and image analysis system (Sigma-Scan) (25). MHC isoform expression was also determined.

**EPR spectroscopy.** To determine the role of the fraction of strong-binding cross bridge in the force-generating state, the permeabilized fiber bundles were further dissected into 0.25- to 0.5-mm-diameter bundles and the bundles were spin labeled at Cys-707 (sulfhydryl group 1; SH1) in the catalytic domain of the myosin head with iodoacetamide spin label (IASL, Sigma) (12, 16). Each end of the bundle was tied to a glass capillary tube with 7.0 silk suture and then placed in a TE102 cavity (4102ST/8838; Bruker Instruments, Billerica, MA) perpendicular to the magnetic field. The capillary tube was along the lines of tubing and a peristaltic pump, such that fresh buffers were supplied over the fibers at a rate of 2 ml/min at 22°C. One end of the fiber bundle was attached to a force transducer (SensoNor Ackers, model 801 strain gauge, Aksjeskapet, Norway); the other end of the fiber bundle was stabilized to hold the bundle isometrically. Force was measured throughout EPR spectra. Low-field EPR spectra were measured under rigor, relaxation, and contraction conditions with an E500

**RESULTS**

Two weeks of non-weight bearing significantly reduces specific force in MHC type II single muscle fibers. Table 1 summarizes the effect of hindlimb unloading on diameter and specific force (force/CSA) in single fibers. With hindlimb unloading, diameter and specific force were significantly reduced in MHC type IIB fibers by 24% and 38% and in MHC type IIXB fibers by 29% and 18%, respectively. The data indicate that the force-generating capacity per unit CSA in MHC type II fibers is impaired with hindlimb unloading.

Non-weight bearing significantly reduces MHC content per half-sarcomere and force per half-sarcomere in MHC type II single muscle fibers (myosin quantity and quality). MHC content per half-sarcomere, determined from the MHC concentration and half-sarcomere volume of each fiber, was 21% lower in fibers from NWB rats (6.59 ± 10−4 μg/half-sarcomere) compared with fibers from Con rats (8.38 ± 10−4 μg/half-sarcomere) (Fig. 2). To determine the effect of myosin content

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Diameter, μm</th>
<th>Specific Force, kN/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC type IIB fibers</td>
<td>Con (n = 32)</td>
<td>97 ± 1</td>
<td>100 ± 3</td>
</tr>
<tr>
<td></td>
<td>NWB (n = 13)</td>
<td>74 ± 2*</td>
<td>62 ± 6*</td>
</tr>
<tr>
<td>MHC type IIXB fibers</td>
<td>Con (n = 8)</td>
<td>98 ± 4</td>
<td>93 ± 7</td>
</tr>
<tr>
<td></td>
<td>NWB (n = 18)</td>
<td>70 ± 2*</td>
<td>76 ± 5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of fibers. Myosin heavy chain (MHC) isoform expression was determined by sensitive gel electrophoresis and silver staining techniques. We evaluated a total of 87 fibers and report 71 fibers with MHC type IIB or IIXB. *Values of non-weight bearing group (NWB) are significantly different from values of weight bearing control (Con) group (P < 0.05).
on specific force, peak force values of the individual muscle fibers were normalized to the amount of MHC per half-sarcomere volume (N/µg). A significant reduction in force per half-sarcomere MHC content was found in fibers from NWB rats (0.54 ± 0.03 N/µg) compared with fibers from Con rats (1.13 ± 0.12 N/µg) (Fig. 3).

The ratio of myosin to actin, however, was not different between Con and NWB groups (1.00 ± 0.02 vs. 0.97 ± 0.04, data not shown). These data indicate that non-weight-bearing-induced decline in specific force can, in part, be explained by reduced myosin content and average force per unit area.

DISCUSSION

The overall goal of the present study was to evaluate myosin quantity and quality in type II single muscle fibers following a period of non-weight bearing. Force per half-sarcomere MHC content and fraction of myosin strong-binding structural state were identified as the parameters to evaluate myosin quality whereas MHC content/half-sarcomere and MHC-to-actin ratio were selected to characterize myosin quantity. Using a model of hindlimb unloading to mimic the non-weight bearing condition we found that the quantity and quality of myosin were reduced.

Myosin quantity. MHC content per half-sarcomere fiber volume is an indirect estimate of the number of cross bridges per unit area (4). This measure is a valuable outcome for determining the quantity of the contractile machinery because confounding effects, such as changes in extracellular components of the muscle CSA, are avoided (4). The myosin content in the single fibers from the semimembranosus muscle from normal weight-bearing rats of 8.38 × 10^{-4} µg determined in the present study is in good agreement with previous reports (−6.0 × 10^{-4} µg) in fibers from the diaphragm muscle (4, 5).

Because there are many steps in the determination of MHC content per half-sarcomere in single skeletal muscle fibers, the comparable value to previously published work indicates that our experimental techniques (extraction of MHC from the single-fiber samples, determination of MHC concentration by comparison to calibration curve, electrophoresis, and obtaining measures of fiber volume) are sound.

Muscle atrophy is associated with a decrease in total protein content following denervation (7). For instance, 12 days of denervation results in soleus and plantaris muscle atrophy of 30–33% and this atrophy is associated with a reduction in total protein content of 30–34% (7). Atrophy and reductions in total protein content are also present at the single skeletal muscle fiber level. Two weeks of denervation results in significant single-fiber atrophy of 50% and a 50% reduction in MHC content per half-sarcomere in MHC type IIX fibers (4). Denervation is a model of muscle atrophy by which the atrophy is rapid and very severe.

In contrast to the denervation model, hindlimb unloading is a model of muscle atrophy that is relatively mild. Previous studies show that hindlimb unloading (14 days) induced muscle atrophy and a decrease in total protein content, but the extent of atrophy and protein content reductions is ~20% (15, 17). Indeed, there are many studies demonstrating single-fiber atrophy with 2 wk of hindlimb unloading (3, 11, 13) and the current study supports this atrophy (24–29%). The results of the present study demonstrate, for the first time, a reduction in MHC content per half-sarcomere of 21%. Because myosin and actin are closely associated within the sarcomere in a well-

Fig. 1. Fraction of the strong-binding myosin during an isometric maximal contraction. The values were determined by electron paramagnetic resonance spectroscopy of permeabilized fiber bundles from control (Con) and non-weight bearing (NWB) rats. Values are means ± SE. *Significantly different from Con group (P < 0.001).

Fig. 2. Myosin heavy chain (MHC) content per half-sarcomere of permeabilized semimembranosus (SM) fibers from Con and NWB rats. MHC content was determined by dividing the MHC concentration calculated from the myosin standard curve by the fiber volume. MHC content per half-sarcomere was determined by multiplying the MHC content and the half-sarcomere volume of the fiber, normalized to fiber length (L, of 2.5 µm/sarcomere). Twenty fibers for Con and 21 fibers for NWB were used for data analysis. Values are means ± SE. *Significantly different from Con group (P = 0.048).

Fig. 3. Force per half-sarcomere MHC content of permeabilized SM fibers from Con and NWB rats. Force per half-sarcomere MHC content was determined by dividing peak force by the MHC content per half-sarcomere (defined in Fig. 1). Twenty fibers for Con and 21 fibers for NWB were used for data analysis. Values are means ± SE. *Significantly different from Con group (P < 0.001).
ordered manner, there is likely a decrease in actin also. The decrease in actin is supported by the constant relative myosin and actin ratio at 14 days of hindlimb unloading.

It is well established that the degradation of myofibrillar proteins, myosin and actin, is a complex process and appears to occur via a timeline depending on the experimental model to induce atrophy. Collectively, studies evaluating both myosin and actin during various durations or periods of hindlimb unloading provide some insight into this time-dependent process. There is a significant reduction in MHC content compared with actin in the early phase of unloading (2 and 7 days) (6), which is switched to a selective loss of actin relative to myosin between 17 and 21 days (18, 19, 25). The current findings, hindlimb unloading for 14 days, suggest that both actin and myosin are contributing to single-fiber atrophy in this time period.

Overall, 2 wk of hindlimb unloading results in reduced myosin and actin content and these findings support a role for alterations in myofibrillar protein quantity in the observed reduction in single-fiber force-generating capacity.

Quality of muscle contraction (myosin). The quality of muscle contraction is often described by the physiological parameter of specific force or the force-generating capacity normalized to the size of the muscle. In the current study, the reduction of average 28% in single-fiber specific force (MHC type IIB and with MHC type IIXB from the semimembranosus muscles) is consistent with previously published studies investigating specific force in MHC type II fibers using an immobilization experimental model for humans to induce muscle weakness (8). In contrast to the 2 wk of hindlimb unloading, denervation for 2 wk results in a greater decline in specific force (40–50%) in MHC type IIB fibers (4), which is consistent with a model that induces muscle adaptations with a faster timeline.

Single-fiber specific force is dependent on three parameters: 1) the number of cross bridges per half-sarcomere, 2) the average force per cross bridge, and 3) the fraction of cross bridges in the force-generating state (5). The number of cross bridges per half-sarcomere or MHC content per half-sarcomere is a measure of myosin quantity. In the discussion above focused on myosin quantity, 2 wk of hindlimb unloading results in a decrease in the number of cross bridges per half-sarcomere (MHC content/half-sarcomere) that is consistent with the degree of atrophy. In contrast, the average force per cross bridge or peak force normalized for MHC content per half-sarcomere and the fraction of cross bridges in the force-generating state are measures of myosin quality. Based on these two outcome measures it is clear that myosin quality is reduced with 2 wk of hindlimb unloading and that changes in myosin structural states during contraction and force per cross bridge are involved.

In the current study we determined the ratio of myosin heads in the strong-binding structural state during an isometric maximal contraction using site-specific EPR spectroscopy (myosin quality). This method assumes that myosin affinity for actin is high and all myosin heads are in the strong-binding structural state with actin in the rigor state. On the other hand, in relaxation state all myosin heads are in the weak-binding structural state. In maximal isometric contraction state, myosin heads are intermediate to the relaxation and rigor state. Results from the present study show a significant reduction in the ratio of myosin heads in the strong-binding state during an isometric maximal contraction in rat SM muscle fibers after 2 wk of hindlimb unloading, suggesting that the quality of myosin is impaired. The decrement of population of myosin heads in strong-binding state during muscle contraction by 34% is similar to the range in hindlimb unloading-related decrement in specific force (28%) found in SM MHC type II fibers. Values reported here are also in agreement with results previously published with aging (12) and 3 wk of hindlimb unloading (25) studies. Our finding that fibers from control rats have 31% of myosin heads in the strong-binding structural state during maximal isometric contraction is similar to 32–34% found in fibers from control rats in other studies (12, 25). The underlying mechanism responsible for the altered structural states of myosin with hindlimb unloading is not clear; however, it likely involves posttranslational modifications (1).

The impaired quality of myosin was confirmed with a reduction in the average force per cross bridge (force/MHC content per half-sarcomere). Geiger and colleagues (4, 5) support this measure for myosin quality because the average force per cross bridge gives direct information of the contractile protein machinery [per half-sarcomere (contractile unit of a myofibril)]; 2) eliminates any confounding effects derived from noncontractile proteins such as connective tissue or interstitial fluid volume in CSA; and 3) minimizes the error of compliance effect caused by fiber segment length changes during contraction.

One interesting outcome of the current study is the difference observed in myosin quality between the published denervation study by Geiger et al. (4) and our hindlimb unloading study. Hindlimb unloading, a model of mild muscle atrophy, induces single-fiber weakness that is associated with a reduction in myosin quality involving changes in both the structural states of myosin and the force per cross bridge. In contrast, denervation, a model of severe muscle wasting, induces single-fiber weakness that is associated with a reduction in force per cross bridge only (4). Notably, Geiger et al. (4) did not find any change in myosin structural states with 2 wk of denervation when the myosin structural states were evaluated by fiber stiffness. It is possible the rapid degradation of myofibrillar proteins with denervation plays a role in this observed difference (1, 7). Geiger et al. (4) suggest that the decline in force per cross bridge was linked to lattice spacing or titin elasticity.

In conclusion, we have shown that the decline in force-generating capacity with hindlimb unloading is due to a decrease in myosin and actin content (quantity) and myosin quality. The quality of myosin is likely impaired due to damage or site-specific posttranslational modification of myosin structure (1).

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS


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