The roles of myosin ATPase activity and myosin light chain relative content in the slowing of type IIB fibers with hindlimb unweighting in rats

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Zhong S, Thompson LV. The roles of myosin ATPase activity and myosin light chain relative content in the slowing of type IIB fibers with hindlimb unweighting in rats. Am J Physiol Cell Physiol 293: C723–C728, 2007. First published May 9, 2007; doi:10.1152/ajpcell.00009.2007.—We tested the hypothesis that slowing of shortening velocity generated by type IIB fibers from hindlimb-unweighted (HU) rats resulted from a reduced ATPase activity and/or a reduction in the relative content of myosin light chain 3f isoform content (MLC3f). After 2, 3, and 4 wk of HU, maximal unloaded shortening velocity (V_o) of single permeabilized semimembranosus muscle fibers was determined by the slack test. Subsequently, the myosin heavy chain and the relative content of MLC were determined by SDS-PAGE. The ratio of MLC2f to MLC2f was determined by densitometric analysis. In addition, myofibrils were prepared from permeabilized fibers (soleus and semimembranosus muscles) and assayed for resting myosin ATPase and Ca2+ -activated myosin ATPase. After HU, V_o declined by 28–40% and the MLC3f/MLC2f ratio decreased by 32 to 48%. A significant correlation between the relative amount of MLC3f and V_o was found (r = 0.48, P < 0.05). Resting myosin ATPase rates were not different between myofibrils prepared from corresponding muscles of control and HU rats (P = 0.86). Ca2+ -activated myosin ATPase activities also were not different between myofibrils prepared from corresponding muscles of control and HU rats (P = 0.13). These data suggest that the slowing of maximal unloaded shortening velocity in type IIB fibers with HU is, at least in part, due to a relative change in the essential light chain composition, a decrease in the relative amount of MLC3f and most likely a concomitant increase in MLC1f. However, this reduction in V_o is independent of myosin ATPase activity.

Indeed, the unloading-induced increase in velocity is correlated with an elevation in ATPase activity in MHC type I fibers (17). Currently, the relationship between changes in ATPase and velocity in MHC type IIB fibers following unloading is unknown.

The alkali myosin light chains (MLC; essential) play a role in determining unloaded shortening velocity in fibers with MHC type II isoforms. Sweeney et al. (25) and Bottinelli et al. (4) report that velocity is elevated in type II fibers that contain larger relative amounts of MLC 3f isoform (MLC3f). Moreover, velocity is proportional to the relative content of MLC3f in a group of single fast fibers (MHC type II) (4, 11). These results suggest that the proportion of MLC3f is an important determinant of unloaded shortening velocity among fast fibers. Thus it is possible that the decline in shortening velocity in MHC type IIB fibers following unloading is due to changes in the relative content of MLC3f. To date, the effect of unloading on the relative content of MLC3f in skeletal muscle fibers that contain MHC type IIB has not been thoroughly investigated.

Consequently, the purpose of this study is to define the roles of myosin ATPase activity and the relative content of MLC3f in the muscle unloading-induced decline in shortening velocity in MHC type IIB and MHC type IIB-IIX fibers. We hypothesize that the decrease in velocity following muscle unloading is related to reduced ATPase activity and/or a reduction in the relative content of MLC3f. To study the effect of unloading on contractile properties the single-permeabilized fiber preparation is used. This cellular preparation allows investigation of contractile protein function in a cell with an intact filament lattice. Next, a sensitive SDS-PAGE is used to separate and quantify the relative content of the MLC isoforms and determine the MHC isoforms.

METHODS

Animals, unloading protocol, and tissue preparation. An animal care protocol was approved by the Institutional Animal Care and Use Committee and was in accordance with guidelines established by the American Physiological Society. Twenty-four Fisher 344 male rats were randomly assigned to a control (weight bearing) group or an unloading (non weight bearing) group (6 rats/group). The non-weight-bearing rats were suspended for either 2, 3, or 4 wk by a tail harness attached to the proximal two-thirds of the tail (1, 9, 22, 30). All rats were obtained from the colony maintained by the Minneapolis Veterans Administration. After 2, 3, or 4 wk of non-weight-bearing or normal cage activity for the control group, rats were anesthetized with pentobarbital sodium (55 mg/kg ip) and weighed. Next, the semimembranosus muscles were dissected and weighed. The semimembranosus muscle was selected because it is predominantly composed of MHC

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type IIB fibers and MHC type IIB-IIX fibers and shows hindlimb
unweighting-induced changes (36). Although the study focused on the
semimembranosus muscle, the soleus muscles from control rats,
comprised predominantly of MHC type I fibers, were dissected and
weighed, too. The soleus and semimembranosus muscles were used in
the ATPase experiments to demonstrate the difference in ATPase
activities between muscles composed of different fiber types in
non-weight-bearing rats.

Permeabilized single fiber preparation. The semimembranosus
muscles were placed in relaxing buffer composed of (in mM) 7
EGTA, 0.016 CaCl$_2$, 5.6 MgCl$_2$, 80 KCl, 20 imidazole, pH 7.0, 14.5
creatine phosphate, and 4.8 ATP on ice. Bundles of fibers (~8 mm
long and ~1 mm in diameter) were formed for single fiber contract-
tility analyses. These bundles were tied to pieces of capillary tubes
and stored in 50% glycerol: 50% relaxing buffer composed of (in mM) 60 KPr, 25 MOPS, 2 MgCl$_2$, 1 EGTA, and 1 NaN$_3$, containing 0.1 DTT for ATPase analyses.

ATPase activities. Glycerinated muscle bundles (from the semi-
membranosus muscles and soleus muscles) were homogenized in 20
mM Tris-HCl and protein concentration was determined using spe-
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muscles of control and hindlimb unloading rats \( (P = 0.86; \text{Fig. 2A}) \). \( \text{Ca}^{2+} \)-activated myosin ATPase activities also were not different between myofibrils prepared from corresponding muscles of control and hindlimb unloading rats \( (P = 0.13; \text{Fig. 2A}) \). In Fig. 2B, resting myosin ATPases rate for soleus samples (muscles composed of predominately MHC type I fibers) was 0.131 and increased to 0.302 \( \text{mol} \text{Pi} \cdot \text{min}^{-1} \cdot \text{mg} \text{protein}^{-1} \) with \( \text{Ca}^{2+} \) activation. In contrast, resting myosin ATPases rate for semimembranosus samples (muscles composed of predominately MHC type II fibers) was 0.110 and increased to 0.630 \( \text{mol} \text{Pi} \cdot \text{min}^{-1} \cdot \text{mg} \text{protein}^{-1} \) with \( \text{Ca}^{2+} \) activation. The \( \text{Ca}^{2+} \)-activated myosin ATPase of the semimembranosus muscles was predominantly fast MHC isoforms, 56\% type IIX/IIA, and 11\% type I. The MHC composition of the semimembranosus muscle (bundles) did not change with unloading.

Unloading-induced alterations in individual MHC type II fibers. The \( V_o \) of type II fibers decreased by 28\% with 2 wk of unloading, 40\% with 3 wk of unloading, and 36\% with 4 wk of unloading (Table 1). The decrease in \( V_o \) with unloading is also observed when the individual fibers are analyzed by specific fiber type group, fibers expressing only MHC type IIB (Table 2) or fibers co-expressing MHC type IIB-IIX only (Table 3).

Effect of unloading on \( V_o \) and MLC content of individual fibers expressing MHC type IIB and coexpressing type IIB-IIX fibers. To evaluate the role of MLC and \( V_o \) we focused our analysis on the individual fibers expressing MHC type IIB and MHC type IIB-IIX (grouped together). Table 1 shows the analysis of MLC compositions in the same fibers that were analyzed for unloaded shortening velocity \( [\text{changes of } V_o \text{ and the ratios of } \text{MLC}_3/(\text{MLC}_{2f}+\text{MLC}_{3f}), \text{MLC}_3/(\text{MLC}_{1f}+\text{MLC}_{3f}), \text{and } \text{MLC}_{1f}/(\text{MLC}_{1f}+\text{MLC}_{3f})] \). Two, three, and four weeks of unloading resulted in single fiber \( V_o \) decreasing from control values (Table 1). The ratio of the \( \text{MLC}_{3f} \) and \( \text{MLC}_{2f} \) of single fibers decreased by 37, 32, and 48\% with unloading, respectively (Table 1). The ratio of the \( \text{MLC}_3/(\text{MLC}_{1f}+\text{MLC}_{3f}) \) of the same single fibers followed the same hindlimb unweighting-induced pattern (decrease). Moreover, there was an increase in the ratio of \( \text{MLC}_3/(\text{MLC}_{1f}+\text{MLC}_{3f}) \) (Table 1).

In contrast to Table 1, Tables 2 and 3 summarize \( V_o \) and MLC isoform compositions by specific fiber type (fibers with only MHC type IIB or hybrid fibers that express both MHC type IIB/IIX). The hindlimb unweighting-induced decreases in \( V_o \) and in the ratios \( \text{MLC}_{3f}/(\text{MLC}_{2f}+\text{MLC}_{3f}) \) and \( \text{MLC}_{3f}/(\text{MLC}_{1f}+\text{MLC}_{3f}) \) are observed when the fibers are grouped by specific fiber type.

Relationship between \( V_o \) and MLC composition. Figure 3 shows the relationship between the \( \text{MLC}_3/(\text{MLC}_{2f}+\text{MLC}_{3f}) \) ratio and \( V_o \) for each individual fiber containing MHC type IIB and MHC type IIB-IIX. A significant correlation between the relative amount of \( \text{MLC}_3 \) and \( V_o \) was found. The slope of the linear regression was 5.18 for all fibers \( (r = 0.48, P = 0.05) \). In other words, as the relative amount of \( \text{MLC}_3 \) decreased among these fibers, so did \( V_o \). Moreover, the relationship between \( \text{MLC}_3/(\text{MLC}_{2f}+\text{MLC}_{3f}) \) and \( V_o \) is significant when the data was analyzed by specific fiber type. The slope of the linear regression was 5.43 for the MHC type IIB fibers \( (r = 0.52, P = 0.05) \) and 5.11 for the MHC type IIB-IIX fibers \( (r = 0.50, P = 0.05) \).

DISCUSSION

The purpose of the present study was to compare individual skeletal muscle fibers that contain identical MHC but express different ratios of myosin light chain (MLC\(_{3f}\)). Thus addressing the question of whether or not alterations in MLC\(_{3f}\) relative content can explain the non-weight-bearing-induced decline in contractile velocity. The hindlimb unweighting model was used to promote the non-weight-bearing condition, which alters muscle contractility \( (30, 31) \). The strength of the current study was using the same skeletal muscle fiber for velocity measurements and subsequent MHC and MLC analyses, which permitted an unambiguous analysis of the correlation between contractile function and relative MLC\(_{3f}\) isoform content.

The primary results of the present study are (1) muscle unloading induces a decline in maximal unloaded shortening...
non-weight-bearing-induced decline in activated myosin ATPase activity of freely shortening myofibrils. Animals.

IIB or coexpressed MHC type IIB-type IIX. HU2, HU3, and HU4 indicate unloading for 2, 3, and 4 wk, respectively. *Significantly different from control animals.

Table 1. $V_o$ and ratio of MLC3f/MLC2f, MLC3f/(MLC1f + MLC3f) of fast MHC type IIB fibers from SM of control and HU animals

<table>
<thead>
<tr>
<th>Animal Condition</th>
<th>$n$</th>
<th>$V_o$, fl/s</th>
<th>MLC3f/MLC2f</th>
<th>MLC3f/(MLC1f + MLC3f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27</td>
<td>9.1±0.3</td>
<td>0.63±0.04</td>
<td>0.37±0.02</td>
</tr>
<tr>
<td>HU2</td>
<td>4</td>
<td>7.4±0.9*</td>
<td>0.28±0.05*</td>
<td>0.16±0.02*</td>
</tr>
<tr>
<td>HU3</td>
<td>21</td>
<td>5.3±0.1*</td>
<td>0.38±0.02*</td>
<td>0.26±0.01*</td>
</tr>
<tr>
<td>HU4</td>
<td>10</td>
<td>5.0±0.3*</td>
<td>0.35±0.02*</td>
<td>0.24±0.01*</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, no. of individual fibers. The individual fibers expressed MHC type IIB. *Significantly different from control animals.

to a loss in MLC3f relative content (and a subsequent increase in MLC1f relative content).

Effects of unloading on ATPase. The shortening velocity of an individual muscle is thought to be primarily related to the ATPase activity of myosin within the muscle. Myosin ATPase activity is, in turn, related to distribution of MHC isoforms expressed in the fiber. For example, fast-twitch muscles (MHC type II) have faster ATPase activity compared with slow-twitch muscle (MHC type I) (26). So a logical explanation for the decrease of $V_o$ in the semimembranous fibers with unloading would be slower myosin ATPase activity in samples from non-weight-bearing rats. However, our Ca$^{2+}$-activated myosin ATPase measurements on myofibrils from the semimembranosus muscle do not substantiate this explanation. Although there is a significant difference in the ATPase activity between slow-twitch and fast-twitch muscles, fourfold (33), the difference between the fast myosins (MHC IIA, IIX, IIB) is not as great (33). Thus, it is not surprising that we did not detect a change in ATPase activity in the semimembranosus muscles following unloading, since the fiber-type composition of the muscle is predominantly MHC type IIB and MHC type IIB-IIX. Furthermore, this general fiber type composition of the muscle does not change with hindlimb unweighting.

In a previous study, we showed a shift in MHC isoform expression in individual fibers with 3 wk of unloading, such that the percentage of fibers that coexpressed MHC type IIB/IIX in non-weight-bearing rats was twofold greater than that in control rats, yet this shift did not explain the decrement in contractile velocity (36). It is important to point out, in the current study, the analysis by specific fiber type (MHC type IIB, MHC type IIB-IIX) and analysis by pooled fiber types (MHC type IIB and MHC type IIB-IIX) report the same conclusions. Therefore we conclude that other factors, such as changes in the essential MLC composition, must contribute to unloading-related reductions in contractile velocities.

Fig. 2. Resting myosin ATPase activity (i.e., low Ca$^{2+}$ condition) and Ca$^{2+}$-activated myosin ATPase activity of freely shortening myofibrils. A: ATPase activity values (means ± SE) of the semimembranosus muscles from rats that were cage control or hindlimb unloaded (HU) for 2 wk (HU2), 3 wk (HU3), and 4 wk (HU4). B: ATPase activity values (means ± SE) of the soleus muscles (composed of MHC type I fibers) compared with the semimembranosus muscles (composed of MHC type II fibers) from control rats. *Significantly different from soleus muscles.

Velocities among individual rat semimembranosus fibers (MHC type IIB and MHC type IIB-IIX), (2) variations in the MLC3f relative content of single fibers from rat semimembranosus were quantitatively correlated with $V_o$, suggesting the non-weight-bearing-induced decline in $V_o$ may be, in part, due

Table 2. $V_o$ and ratio of MLC3f/MLC2f and MLC3f/(MLC1f + MLC3f) of fast MHC type IIB fibers from SM of control and HU animals

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<td>31</td>
<td>8.5±0.3</td>
<td>0.61±0.04</td>
<td>0.37±0.02</td>
</tr>
<tr>
<td>HU2</td>
<td>14</td>
<td>6.3±0.5*</td>
<td>0.40±0.04*</td>
<td>0.26±0.02*</td>
</tr>
<tr>
<td>HU3</td>
<td>31</td>
<td>5.3±0.2*</td>
<td>0.41±0.02*</td>
<td>0.28±0.01*</td>
</tr>
<tr>
<td>HU4</td>
<td>15</td>
<td>5.5±0.4*</td>
<td>0.32±0.02*</td>
<td>0.23±0.01*</td>
</tr>
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</table>

Values are means ± SE; $n$, no. of individual fibers. The individual fibers expressed MHC type IIB or coexpressed MHC type IIB-type IIX. HU2, HU3, and HU4 indicate unloading for 2, 3, and 4 wk, respectively. *Significantly different from control animals.
Interact directly with actin through a NH₂-terminal extension that is lacking in MLC₃f (32). This extension is rich in proline and has four lysines at the distal end. The size and the positively charged residues enable this extension to interact with the negatively charged COOH-terminus of actin (2). It appears that the binding of the NH₂-terminal extension of MLC₁f to actin slows the speed of filament sliding but not the cross-bridge kinetics underlying the force transients and the rate of the whole cross-bridge cycle (ATPase activity) (19, 21). These contractile characteristics can be explained by assuming that MLC₁f binding to actin reduces the distance of filament displacement during one cross-bridge cycle, but does not affect the kinetics. The results of the present study are consistent with this theory that a decrease in velocity can occur without a change in ATPase (kinetics). Furthermore, a decrease in the relative content of MLC₃f would result in an increase in the relative content of MLC₁f. Although this study reports a decrease in the relative content of MLC₃f, most likely there is a concomitant increase in the relative content of MLC₁f, which is observed in the increase in the ratio MLC₁f/(MLC₁f + MLC₃f). The change in essential MLC isoforms (from MLC₃f to MLC₁f) is based on the assumption that one alkali light chain and one regulatory light chain wrap around the α-helix of the myosin molecule described in the proceeding paragraph.

Summary. In conclusion, the non-weight-bearing-induced changes in V₀ in the ratio of MLC₃f/MLC₂f and in the ratio of MLC₃f/(MLC₁f + MLC₃f) of single semimembranos fibers have been characterized. Non-weight-bearing changes induced a decline in fiber V₀ that was likely caused, at least in part, by a decrease in the relative MLC₃f content (and subsequent increase in MLC₁f content). However, this decline in V₀ is independent of ATPase activity. Future studies are needed to identify the cellular processes responsible for the preferential decline in MLC₃f content, such as changes in gene expression and upregulation of proteins involved in degradation processes.

### Table 3. V₀ and Ratio of MLC₃f/MLC₂f and MLC₃f/(MLC₁f + MLC₃f) of Fast MHC Type IIB-IIIX Fibers from SM of Control and HU Animals

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<th>V₀ (fl/s)</th>
<th>MLC₃f/MLC₂f</th>
<th>MLC₃f/(MLC₁f + MLC₃f)</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
<td>4</td>
<td>9.0 ± 0.5</td>
<td>0.80 ± 0.08</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td>HU2</td>
<td>10</td>
<td>5.2 ± 0.3*</td>
<td>0.47 ± 0.04*</td>
<td>0.30 ± 0.01*</td>
</tr>
<tr>
<td>HU3</td>
<td>10</td>
<td>4.9 ± 0.3*</td>
<td>0.50 ± 0.04*</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>HU4</td>
<td>5</td>
<td>6.3 ± 1.3*</td>
<td>0.28 ± 0.03*</td>
<td>0.22 ± 0.02*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of individual fibers. The individual fibers coexpressed MHC type IIX. HU2, HU3, and HU4 indicate unloading for 2, 3, and 4 wk, respectively. *Significantly different from control animals.

V₀ and MLCs. Although the MHC isoform composition of an individual muscle fiber is the primary determinant of its maximal shortening velocity, the MLC isoform complement has a modulatory influence on regulating this property, especially in fast fibers. Variations in the essential MLC isoform relative composition of single fibers, whether naturally occurring (8, 24), in transgenic animals (5, 18), or in experiments involving myofilament protein substitutions (18), are associated with differences in fiber contractile properties. The results of an in vitro motility assay utilizing myosin reconstituted with different MLC subunits (14, 15) further contribute to this theory. Collectively, these studies show that the unloaded velocity of shortening in fast fibers increases with greater relative levels of MLC₃f.

In the present study, the decrease in MLC₃f relative content may be the molecular mechanism for the decreases in contractile velocities with unloading because V₀ was proportional to MLC₃f relative content in fast fibers of same MHC composition. A change of 32–48% in MLC₃f/MLC₂f [25–38% in MLC₃f/(MLC₁f + MLC₃f)] or MLC₃f proportion induces a change in V₀ of 28–40%. The slope of the relationship of V₀ to MLC₃f/MLC₂f ratio (5:18) is similar to a previous study investigating MLC₃f/MLC₂f ratio and contractile velocities (8).

Muscle contraction and the role of MLCs. Muscle contraction is due to cyclic interactions of myosin and actin. Myosin is composed of two MHCs and two pairs of MLCs. MHCs are described to have a rod part that forms the backbone whereas the myosin heads form the globular ends. An α-helix (8.5 nm long) connects the globular end with the rod part of each MHC. One “alkali” or “essential” MLC and one “regulatory” MLC wrap around this helix, and form the myosin neck (20). The myosin neck is thought to act as a lever arm, which amplifies small conformational changes occurring in the catalytic domain of the MHC during ATP hydrolysis cycles into larger movements (20). The MLCs appear to be necessary for increasing the rigidity of the lever arm while transmitting force during the myosin head power stroke.

It is possible that any modulatory role of MLC may vary with its size, as well as with differences in primary sequence, which may, in turn, contribute to differences in contractile properties of fast fibers. For instance, a higher proportion of MLC₁f is associated with slower unloaded shortening velocity (8, 25). This slowing of velocity by MLC₁f is likely due to interaction of its NH₂-terminal extension with actin (2, 8, 14, 25, 32). MLC₁f appears to interact directly with actin through a ~40 amino acid sequence, which may, in turn, contribute to differences in contractile properties of fast fibers.
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