Time-dependent changes in myosin heavy chain mRNA and protein isoforms in unloaded soleus muscle of rat

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Skeletal muscle has the capacity of changing its phenotype in response to altered functional demands. This “plasticity” has been documented in numerous studies, using various experimental protocols of increased and decreased mechanical loading, as well as enhanced or reduced contractile activity. Among these protocols, chronic low-frequency stimulation (CLFS) of fast-twitch muscle and unloading of slow-twitch muscle by hindlimb suspension represent extremes, leading to muscle fiber type transitions in opposite directions. CLFS induces fast-to-slow transitions, whereas unloading evokes slow-to-fast transitions (for reviews, see Refs. 1, 22, 30). The latter have become a challenging topic of muscle research in view of prolonged exposure to weightlessness under the conditions of spaceflight.

At the level of myofibrillar proteins, these adaptive fast-to-slow and slow-to-fast responses encompass exchanges of fast with slow and slow with fast protein isoforms, respectively. As shown by whole muscle and single fiber analyses, these changes are clearly reflected by sequential transitions in myosin heavy chain (MHC) isoform expression (17, 23). In low-frequency stimulated fast rat muscle, MHC isoform transitions generally follow the order of MHCIIb → MHCIId(x) → MHCIId → MHCIa (16). In rabbit, the MHCIa → MHCβ transition includes a transient upregulation of the MHCIα isoform (23, 24). In rat, however, the MHCIa → MHCβ transition is difficult to attain. It occurs only after very long stimulation periods (15, 35), and this may explain that, under our experimental conditions, an upregulation of MHCIα has as yet not been observed (26). The difficulty to attain in rat muscle the MHCIa → MHCβ transition, the ultimate step of the fast-to-slow transformation, appears to be in contrast to the facility to elicit the opposite change, namely the MHCβ → MHCIa transition by unloading rat slow muscle (3, 6, 19, 30). Studies at the mRNA and protein levels suggest that, under these conditions, the switch from MHCIβ to MHCIa is followed by further shifts toward other fast MHC isoforms, i.e., MHCIa → MHCIId(x) → MHCIb (12, 14). To our knowledge, the time course of transitions in the expression of MHC mRNA isoforms has been followed in unloaded rat soleus muscle only in a single study based on Northern blot hybridization (12). However, the question of MHCIα upregulation was not addressed.

The present study was undertaken to investigate in more detail the time course and complete MHC transition profile when a slow muscle turns fast. In this context, we were interested whether MHCIα is a member of the transition profile as in the rabbit. We quantitatively assessed changes in mRNA and protein levels of the various MHC isoforms in rat soleus muscle unloaded for 4, 7, 15, and 28 days by hindlimb suspension. Quantitative changes in the transcript levels of MHCIβ, MHCIα, MHCIa, MHCIId(x), and MHCIb were assessed by highly sensitive RT-PCR (16, 26) and correlated with quantitative changes in the pattern of MHC protein isoforms.

MATERIALS AND METHODS

Animals and muscles. Adult male Wistar rats (initial body wt 280 g) were randomly divided into two groups: control and hindlimb suspension unloading groups. The animal experiments, as well as the animal maintenance conditions, were approved by the French Ministries of Agriculture and Educ-
tion (veterinary service of health and animal protection, authorization no. 03805). Hindlimb suspension was performed as previously described (28). After various periods of hindlimb suspension (4, 7, 15, and 28 days), the animals (5–7 for each time point) were anesthetized by intraperitoneal injection of ethyl carbamate and killed by exsanguination, and soleus muscles from both control and hindlimb-suspended rats were removed. The muscles were weighed (Table 1), frozen in liquid N2, and stored at −70°C until analyzed. Frozen muscle tissue was pulverized under liquid N2 in a small steel mortar. One part of the muscle powder was used for MHC mRNA analysis, and the other was used for MHC protein analysis.

Preparation of total RNA. Muscle powder was homogenized (1:10, wt/vol) in cold TRI reagent (Molecular Research Center, Cincinnati, OH). Total RNA was isolated according to the producer’s instructions for RNA preparation using the following modifications: 1) after homogenization, proteins and insoluble material were removed by 10 min centrifugation at 12,000 g at 4°C; 2) phase separation was performed using 1-bromo-3-chloropropane (Fluka, Buchs, Switzerland); and 3) isopropanol (0.125 ml) and 0.125 ml of a solution containing 1.2 M sodium citrate and 0.8 M NaCl were used for RNA precipitation. Pellets were resuspended in 30 µl of diethyl pyrocarbonate-treated water. RNA concentration (µg/ml) was assessed spectrophotometrically.

Oligonucleotide primers. Oligonucleotide primers specific to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-skeletal actin, MHCIβ, MHCIId(x), MHCIId, MHCIIα, and MHCIIα (26) were derived from published cDNA sequences. The 5′-ends of sense primers were labeled with digoxigenin to allow chemiluminescence detection of the amplified products.

RT-PCR. Total RNA stock solution (2 µg) was reverse-transcribed (25) in a 20-µl volume using the following assay mixture: RT buffer (50 mM Tris·HCl, pH 8.5, 8 mM MgCl2, and 30 mM KCl), 2 units avian myoblastosis virus RT, 40 units RNase inhibitor (Roche), 2.5 µM primer p(dT)15 (Roche), and each 0.625 mM deoxynucleoside triphosphate (dNTP; Pharmacia). Incubation was performed for 60 min at 42°C. Two microliters of the 1:5 diluted RT assay were amplified separately for each of the seven sequences and transferred in 23 µl of the following PCR incubation mixture: PCR buffer (50 mM Tris·HCl and 15 mM (NH4)2SO4), specific antisense and sense primers (0.2 µM), dNTPs (0.25 mM), and 0.63 units Expand High-Fidelity polymerase (Roche). The optimized MgCl2 concentrations were 2 mM for GAPDH, α-skeletal actin, MHCIβ, MHCIId, and MHCIIα and 2.5 mM for MHCIβ, MHCIId(x), and MHCIIα. For amplification, the following conditions were chosen: denaturation at 94°C, annealing at 59°C, and synthesis at 72°C. The number of cycles was adjusted to allow product detection in the exponential range of amplification. Cycle numbers were 13 for α-skeletal actin, 18 for GAPDH, and varied according to their expression levels from 17 to 26 for the different MHC sequences. To allow quantitative analysis of gene expression, the amplification reactions were monitored using cDNA standards. For this purpose, PCR fragments were purified from primers, probe oligonucleotides, and nonspecific reaction products using the QIAEX DNA gel extraction procedure (QIAGEN) after electrophoretic separation on 1.5% agarose gels (25). The purified DNA was spectrophotometrically quantified, and known amounts of each sequence were amplified in parallel to the samples. The calculated first-strand copy numbers of standard cDNA fragments were 106, 4 × 105, and 105 molecules for GAPDH and α-skeletal actin; 103, 4 × 103, and 102 molecules for MHCIIα; 4 × 102, 102, and 101 molecules for MHCIIα; and 102, 102, and 101 molecules for MHCIIβ and MHCIIid(x).

Product analysis and quantitative evaluation. The digoxigenin-labeled DNAs were electrophoretically separated on 6% polyacrylamide gels and visualized after electroblotting (Hybond N; Amersham) by an antibody-linked assay followed by a peroxidase-catalyzed chemiluminescence reaction (Roche). The signals were photographically documented (Hyperfilm ECL; Amersham) and evaluated by integrating densitometry. At least two measurements were performed on each sample (animal and time points).

Immunohistochemistry. A monoclonal antibody directed against MHCIIα (clone F-88 12F8, 8.1) was from Biocytex (Marseille, France). The following monoclonal antibodies directed against adult MHC isoforms were also used: 7HCS-15 (specific to MHCII) and SC-71 (specific to MHCIα). Biotinylated horse anti-mouse IgG (rat-absorbed, affinity-purified) was from Vector Laboratories (Burlingame, CA). Freshly cut, 9-µm-thick, frozen sections were air-dried at room temperature for 2 h. Sections were washed in PBS containing 0.1% Tween 20 and in PBS alone. They were then incubated for 15 min in 3% H2O2 in methanol, washed, and incubated for 2 h in a blocking solution (1% BSA, 10% horse serum, and 0.1% Tween 20 in PBS, pH 7.4). The primary monoclonal antibody was applied overnight at 4°C. Primary mouse IgG monoclonal antibodies were used as undiluted culture supernatants (F-88) or were diluted in blocking solution (7HCS-15, 1:40; SC-71, 1:1,000). Control sections were processed in parallel incubations in which the primary antibody was substituted with nonspecific control mouse IgG. After 30 min of incubation with biotinylated horse anti-mouse IgG (diluted 1:200 in blocking solution), sections were washed and incubated for 30 min with biotin-avidin horseradish peroxidase complex (Vectastatin Elite; Vector Laboratories). Staining solution (0.07% diaminobenzidine, 0.05% H2O2, and 0.03% NiCl2 in 50 mM Tris·HCl, pH 7.5) was applied for 4 min.

MHC protein electrophoresis. MHC isoforms were analyzed as previously described (13). Gels were silver-stained, and relative concentrations of MHC isoforms were evaluated by integrating densitometry. At least two independent measurements were performed on each sample.

Statistical analyses. Data are presented as means ± SD. All data were analyzed using Student’s t-test to determine differences between values from control and unweighted muscles. The acceptable level of significance was set at P < 0.05.

RESULTS

Changes in MHC protein isoforms. Unloading of soleus muscle up to 28 days led to progressive loss of

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Table 1. Wet weights of soleus muscle from age-matched control rats and rats exposed to hindlimb suspension for various time periods

<table>
<thead>
<tr>
<th>Condition</th>
<th>Weight, mg</th>
</tr>
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<tbody>
<tr>
<td>Control 4 days</td>
<td>120 ± 6</td>
</tr>
<tr>
<td>4 Days unloaded</td>
<td>100 ± 7*</td>
</tr>
<tr>
<td>Control 7 days</td>
<td>120 ± 6</td>
</tr>
<tr>
<td>7 Days unloaded</td>
<td>91 ± 5*</td>
</tr>
<tr>
<td>Control 15 days</td>
<td>137 ± 5</td>
</tr>
<tr>
<td>15 Days unloaded</td>
<td>70 ± 4*</td>
</tr>
<tr>
<td>Control 28 days</td>
<td>151 ± 8</td>
</tr>
<tr>
<td>28 Days unloaded</td>
<td>75 ± 6*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5–7 for each time point. *Significant change from age-matched control (P < 0.05).
weight (Table 1) and changes in the pattern of the electrophoretically separated MHC isoforms (Figs. 1 and 2). MHCI, the predominant isoform in normal soleus muscle (~95%), decreased within the first week of unloading, reaching a relative concentration of ~75% at day 7. MHCIIa, which represented 5% of total MHC isoforms in control soleus, was elevated by day 4 and reached a maximum of ~25% after 1 wk. With longer periods of unloading, when MHCIId(x) and also MHCIIb started to rise, MHCIIa decayed. Thus 28-day unloaded soleus muscle exhibited a thoroughly rearranged MHC isoform pattern in which all fast isoforms were elevated at the expense of MHCI. Even though MHCI remained the predominant isoform (~70%) in 28-day unloaded soleus muscle, MHCIId(x) now represented the major fast isoform (~18%). MHCIIa and MHCIIb amounted to ~8 and 3%, respectively.

Under the applied conditions of electrophoresis, only a single MHCI band was separated, such that MHCIa and MHCIb could not be distinguished. Therefore, changes in their proportions due to unloading were not detected. An interesting observation was that MHCIa, an additional slow MHC isoform recently described and electrophoretically characterized by a mobility higher than MHCIb (8, 9), seemed to be present at elevated levels in the 15- and 28-day unloaded muscles (Fig. 1).

Changes in MHC mRNA isoforms. To assess mRNA concentrations by molecule number, RT-PCR were performed using specific primers for the various MHC isoforms, as well as for α-skeletal actin and GAPDH. Known amounts of purified PCR fragments were used as external DNA standards for quantitative evaluations of molecule numbers (25). α-Skeletal actin and GAPDH mRNAs served as controls to assess the validity of the method.

Quantitative evaluations of the data from several animals at different time points of hindlimb suspension are summarized in Figs. 3 and 4. Over the time period under study, the level of GAPDH mRNA remained stable (Fig. 3). Conversely, the amount of the α-skeletal actin mRNA decreased in muscles unloaded for longer periods of hindlimb suspension.
than 7 days. MHCI mRNA, the predominant isoform in control soleus (2.42 ± 0.26 × 10^6 molecules/µg total RNA), tended to decline in the unloading muscle, especially during the second week, although the decreases attained at 15 and 28 days were not significant. Very low concentrations of MHCIα mRNA (0.5 ± 0.2 × 10^6 molecules/µg total RNA) were detected in control soleus, but, after 4 days of unloading, MHCIα mRNA was markedly elevated (2.7 ± 1.1 × 10^6 molecules/µg total RNA). It thus attained a similar level as MHCIIb mRNA in the 28-day unloaded muscle (Fig. 4). However, the rise of MHCIα mRNA was transitory, and it started to decay after 15 days.

The three fast MHC mRNA isoforms were already elevated in the 4-day unloaded muscle. MHCIIa mRNA, present at 1.8 ± 0.2 × 10^6 molecules/µg total RNA in the control, had increased threefold by 15 days (Fig. 3). With prolonged unloading (28 days), however, MHCIIa mRNA decayed, attaining its basal level. Among all MHC isoforms, MHCIId(x) mRNA displayed the steepest rise within the first 4 days (Fig. 4). The low amount in control soleus (2.5 ± 0.26 × 10^5 molecules/µg total RNA) had increased ~40-fold after 4 days. Thereafter, it continued to rise, but at a lower rate, being ~60-fold elevated (1.5 ± 0.08 × 10^7 molecules/µg total RNA) over control in 28-day unloaded soleus muscle. Elevated transcript levels were also observed for MHCIIb (Fig. 4). Its basal level in control soleus (2.0 × 10^6 molecules/µg total RNA) was in the same range as that of MHCIId(x) mRNA. It was elevated after 4 days and continued to rise, reaching an ~13-fold increase in 28-day unloaded soleus muscle (2.7 ± 0.3 × 10^6 molecules/µg total RNA).

A summary of the major alterations in MHC isoform expression at the level of relative MHC mRNA concentrations is given in Fig. 5. The temporal pattern of the changes in relative magnitudes resembles that shown for the proteins in Fig. 2. The only exception is MHCIIa peaks by 15 days, at a time when its protein has started to decay.

Immunohistochemical findings. To investigate the histological distribution of MHCIα expression, we performed immunohistochemical stainings with an antibody previously proven to be highly specific to MHCIα (24). As expected, MHCIα expression was restricted in control soleus muscle to the muscle spindles (results not shown). However, 15-day unloaded soleus muscles displayed an additional weak reaction in extrafusal fibers. In comparison with MHCIIa and MHCIβ, however, the staining intensity for MHCIα was very low, even with the highly sensitive method of enhancement used. Nevertheless, not all fibers were equally reactive. The predominant MHC complement of those fibers displaying weak MHCIα reactivity was determined by immunohistochemical stainings of serial sections. As can be seen, MHCIα expression was observed in MHCIIa-positive fibers (Fig. 6, fiber 1) and in MHCIβ-positive fibers (Fig. 6, fiber 2).

**DISCUSSION**

As first shown by Vrbová (33) in 1963, unloading of rabbit soleus by tenotomy turns this slow muscle into a faster-contracting muscle. Numerous studies on rats have since confirmed that mechanical unloading of soleus muscle induces slow-to-fast transitions (22, 30). Besides alterations in contractile properties (28), these transitions are best illustrated by changes in the pattern of MHC isoforms (4, 6–8, 18–20, 27, 29). Collectively, these studies have demonstrated increases in the relative concentration of MHCIIa and an induction of MHCIId(x) that is normally not expressed in soleus muscle of adult rats. A few studies even reported the additional appearance of low amounts of MHCIIb under these conditions (2, 5, 8).

As judged from whole muscle analyses, the slow-to-fast conversion in response to unloading seems to encompass a similar sequential order in MHC isoform exchanges, although in the opposite direction, as observed during the fast-to-slow transition. However, the changes during unloading are less pronounced than those during enhanced neuromuscular activity (16). This may be due to the fact that two processes occur in parallel during hindlimb suspension: 1) altered gene expression and 2) muscle atrophy. Especially in the case of isoforms that are upregulated in the unloaded muscle, their increases at both the mRNA and protein levels are attenuated or obscured by the atrophy of the muscle. The pronounced atrophy (Table 1) could explain the observation that MHCIIa protein starts to decrease at a time when its mRNA is still rising. Another difference between the two models is that MHCIIa is persistently upregulated in muscles undergoing fast-to-slow transition, whereas it is only transiently elevated during slow-to-fast transition. Thus fast-to-slow conversion leads to an accumulation of MHCIIa after downregulation of MHCIIb and MHCIId(x) (16), whereas expression of MHCIIa in the
unloaded muscle represents an early stage of the transformation process. As judged from the time course of the increases in MHCIIa and MHCIId(x) mRNAs, it appears that the upregulation of these two mRNA isoforms occurs in synchrony rather than sequentially. A similar temporal relationship between the changes in MHCIId(x) and MHCIIa mRNAs has been observed during fast-to-slow conversion in rat muscle (16) and also in rabbit muscle, where single fiber analyses identified numerous fibers coexpressing up to four MHC mRNA isoforms. The observation that increases in MHC isoforms, which seem to occur in parallel at the mRNA level, differ at the protein level could point to posttranscriptional regulation. However, one also must consider that the changes at the protein level are given as relative concentrations. Thus the relative increase in MHCIId protein (Fig. 2) may not be due to enhanced translation but obviously results from the decrease in MHCI protein.

The parallel increases in MHCIId and MHCIId(x) mRNAs raise the question as to their upregulation within the same or in different fibers. The possibility exists that analyses of whole muscle RNA preparations reveal parallel MHCIId and MHCIId(x) mRNA increases because type I fibers start to express MHCIId at the same time when preexisting type IIA fibers start to upregulate MHCIId(x). Therefore, single fiber analyses are necessary to further investigate this question. Such studies would answer the question as to the homogeneity or heterogeneity of myonuclear expression patterns within individual fibers.

The increases in MHCIId(x) and MHCIa are the most conspicuous changes in the MHC mRNA pattern. MHCIId(x) mRNA exhibits the steepest increase, ultimately even exceeding the transcript level of MHCIIa in control soleus. Contrary to findings based on Northern blot hybridizations (12), the rise in MHCIId(x) is not transitory but persists under our experimental conditions. Similarly, MHCIId mRNA continues to rise during the whole time period under study.

The detection of the MHCIa transcript in total RNA preparations from unweighted soleus muscle raises the question as to the site of its enhanced expression. MHCIa is normally expressed only in intrafusal fibers of limb muscles, especially the bag, type (21). This is consistent with the very low amounts of MHCIa mRNA in normal soleus muscle and the finding that the immunohistochemical reaction for MHCIa is restricted to spindles. The immunohistochemical detection of low amounts of MHCIa in some type I and type IIA fibers of 15-day unloaded soleus muscle, however, shows that this isoform is expressed in some fibers of transforming rat muscle, although not as a major MHC isoform compared with the rabbit (24).

Different experimental models such as microgravity, hindlimb suspension, tenotomy, and denervation elicit in soleus muscle comparable slow-to-fast transitions (1, 22, 30). Their common denominator could be the reduction of the tonic impulse pattern normally delivered to the soleus muscle by its nerve (32). Contractile activity and specific neural impulse patterns are regarded as major regulatory factors in the control of MHC isoform expression and other phenotype properties in adult skeletal muscle (10, 11, 33, 34).

Taken together, the changes in expression levels of MHC mRNA and protein isoforms in unloaded rat soleus muscle reflect slow-to-fast transitions in the order MHCIβ → (MHCIα) → MHCIIda → MHCIId(x) → MHCIId. Contrary to the rabbit, the upregulation of MHCIα does not seem to be a major stage of the transformation.

Fig. 6. Serial cross sections of 15-day unloaded soleus muscle immunohistochemically stained with anti-MHCIa (F-88), anti-MHClIa (SC-71), anti-MHCIβ (7HCS-15), and control IgG. Two MHCIa-positive fibers coexpressing MHCIIda (1) or MHCI (2) are labeled. Bar, 50 µm.
transformation process in rats, and probably, the inter-
mediate expression of MHCIα does not occur in all
fibers.

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