Changes in myosin heavy chain mRNA and protein isoforms of rat muscle during forced contractile activity

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Changes in myosin heavy chain mRNA and protein isoforms of rat muscle during forced contractile activity. Am. J. Physiol. 274 (Cell Physiol. 43): C365–C370, 1998.—A quantitative reverse transcriptase-polymerase chain reaction was established to determine absolute amounts of mRNAs specific for four myosin heavy chain isoforms [MHCI\(\beta\), MHCII\(d(x)\), MHCII\(a\), and MHCII\(\beta\)] in rat extensor digitorum longus muscle during forced contractile activity by chronic (10 h/day) low-frequency stimulation (CLFS). The induced changes in absolute and relative mRNA amounts were similar. MHCI\(\beta\) mRNA decreased rapidly after 1 day, and MHCII\(a\), MHCII\(\beta\), and MHCII\(d(x)\) started to decrease at day 7. After 42 days, the MHCII\(b\), MHCII\(d(x)\), MHCII\(a\), and MHCII\(\beta\) mRNAs amounted to 2, 6, 90, and 2% of total MHC mRNAs, respectively. Changes at the protein level were studied in a second experimental series increasing CLFS (24 h/day, up to 100 days). Also under these conditions, MHCII\(a\) reached only a fraction of 12% (2-fold elevation). The changes at the protein level remained restricted to the MHCII\(b\) to MHCII\(a\) transition, which agrees with the notion that the induced changes in MHC isoform expression primarily resulted from altered pretranslational activities. Rat fast-twitch muscle thus exhibits a restricted capacity for fast-to-slow conversion.

chronic low-frequency stimulation; myosin heavy chain isoforms; messenger ribonucleic acid; quantitative reverse transcriptase-polymerase chain reaction

FAST-TWITCH MUSCLES can be transformed into slower-contracting muscles by chronic low-frequency stimulation (CLFS; for reviews see Refs. 16 and 17). This process encompasses fast-to-slow fiber-type transitions concomitant with sequential changes in myosin heavy chain (MHC) composition. In hindlimb muscles of rabbit in which MHCII\(d(x)\) is the predominant fast isoform (2), these transitions follow the order MHCII\(d(x)\) → MHCII\(a\) → MHCII\(\beta\). MHCII\(\beta\), the slowest isoform, is predominant in 60-day stimulated muscles (12). In rat fast-twitch muscle in which MHCII\(b\) represents the predominant fast isoform, the fast-to-slow conversion comprises the MHCII\(b\) → MHCII\(d(x)\) transition and the corresponding type II\(B\) → type II\(D(x)\) conversion (3, 7, 22).

The present study was undertaken to investigate, in rat fast-twitch muscle exposed to CLFS, transitions in MHC expression at the mRNA level. We were especially interested in the temporal patterns of induced transitions in various MHC mRNA isoforms. Because sequences of the three fast MHC isoforms (8) and MHCII\(\beta\) (15) are available, we have established a protocol for quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR). The quantification of absolute amounts of mRNAs was based on the use of external cRNA standards (18). This approach made it possible to assess changes in absolute amounts of MHC mRNA isoforms in extensor digitorum longus (EDL) muscle of rats exposed to CLFS for various durations. Muscles stimulated for up to 42 days exhibited changes in the expression pattern of the fast MHC isoforms, whereas an induction of the slow MHCII\(\beta\) was not observed. This was in agreement with previous studies at the protein level in which we showed that, contrary to the rabbit (4, 12, 20), only a minor upregulation of MHCII\(\beta\) occurred in low-frequency stimulated rat muscles (7, 10, 22).

Meanwhile, an upregulation of slow myosin has been reported in rat EDL muscle when stimulated for periods of up to 61 days (14). The difference between this study and our results may reflect a dose-response relationship, since CLFS lasted for 10 h/day in our studies (7, 22), whereas a 24-h protocol was used in the former investigation (14). In view of this discrepancy, we performed an additional experimental series in which we extended CLFS to periods of up to 100 days and also increased the total amount of activity imposed on the muscle by using a 24-h stimulation protocol. This experimental design should be suitable to study whether the MHCII\(a\) → MHCII\(\beta\) transition takes place in rat muscle. Moreover, the chosen conditions should allow testing of the hypothesis that species-specific differences exist between fast-twitch muscles of rat and rabbit with regard to their adaptive changes in response to forced contractile activity.

METHODS

Animals and muscles. Adult male Wistar rats (initial body weight 400–470 g) were used. CLFS (10 Hz, 10 h/day) of the EDL muscle of the left hindlimb was performed via electrodes implanted laterally to the peroneal nerve (19). After various periods of stimulation (1, 3, 7, 14, 28, 42, days), the animals (3–4 for each time point) were killed, and EDL muscles from both the stimulated (left) and contralateral hindlimb were excised, immersed in liquid nitrogen, and stored at −70°C until analyzed. In a second series, stimulation was performed continuously (24 h/day) for 1, 3, 6, 12, 24, 28, 36, 60, 80, and 100 days. Three animals were studied for each time point.

Preparation of total RNA. Frozen muscle tissue was pulverized under liquid N\(_2\) in a small steel mortar and homogenized (1:20, 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 three modifications. 1) After homogenization, proteins and insoluble material were removed by 10 min of centrifugation at 4°C at 12,000 g. 2) Phase separation was performed using 1-bromo-3-chloropropane (Fluka, Buchs, Switzerland). 3) Iso- propanol (0.25 ml) and 0.25 ml of a solution containing 1.2 M sodium citrate and 0.8 M NaCl were used for RNA precipitation. Pellets were resuspended in 40 μl of 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.0). RNA con-
Table 1. Primers for cDNA synthesis and polymerase chain reaction of sarcomeric MHC isoforms

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Product Length, nt</th>
<th>Primer Sequence</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHCIIId(x)</td>
<td>120</td>
<td>Antisense: TCCAAAGGTCAGTACAAAATGG</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sense: CCGAGGTTCCACACAAAA</td>
<td></td>
</tr>
<tr>
<td>MHCIIb</td>
<td>197</td>
<td>Antisense: CTGGGAGTTCCTTGCCCTCCT</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sense: CCGAGGAAACATCCAACGTC</td>
<td></td>
</tr>
<tr>
<td>MHCII 8</td>
<td>288</td>
<td>Antisense: GGGCTTCAAGGATCCTTAG</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sense: ACGAGGAAAGCAAGGAAAGAATCTAC</td>
<td></td>
</tr>
<tr>
<td>MHCIIa</td>
<td>310</td>
<td>Antisense: TAAAAGAACATCGGAGGACA</td>
<td>8, 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sense: TATCCTCAGGCTTCAAGATTTG</td>
<td></td>
</tr>
</tbody>
</table>

MHC, myosin heavy chain; nt, nucleotides.

centration was assessed spectrophotometrically. For quantification experiments, RNA stock solutions (0.1, 0.01, and 0.001 µg/µl) were prepared in 20 mM ribonuclease vanadyl complexes (Sigma). Dilution series were prepared as previously described (18).

Oligonucleotide primers. Oligonucleotide primers specific to MHCIIb, MHCIIId(x), MHCIIa, and MHCIIβ (Table 1) were derived from published cDNA sequences. The 5′-ends of sense primers were labeled with digoxigenin to allow chemiluminescence detection of the amplified products.

Preparation of external standards. cDNA standards were synthesized by in vitro transcription using the transcription vector pCRTCII (Innogenetix, San Diego, CA) into which the various PCR products had been cloned. For MHCIIβ [288-nucleotide (nt) PCR product], MHCIIa (310-nt PCR product), and MHCIIb (197-nt PCR product), EcorV was used for linearization, and SP6 polymerase was used for transcription. For MHCIIId(x) (120-nt PCR product), BamHI was used for linearization, and T7 polymerase was used for transcription. In vitro transcription was performed using the T7/SP6 MEGA-Script kit from Ambion (Austin, TX). Template digestion was performed in two steps, first by 30-min incubation with 1 U/µl ribonuclease (RNase)-free deoxyribonuclease (DNase), and after ethanol precipitation the transcripts were incubated for 60 min with 1 U/µl RNase-free DNase and 1 U/µl Alu I for MHCIIβ, MHCIIa, and MHCIIb, and 1 U/µl Bsp143I for MHCIIId(x), using the restriction enzyme buffer recommended by the supplier (MBI, Vilnius, Lithuania). The second step was performed to completely digest the template. Unincorporated nucleotides were removed by Chroma Spin 100 (Clontech, Palo Alto, CA) centrifugation columns. cRNA quantification and serial dilutions were performed as previously described (18).

cDNA synthesis, PCR, and product detection. cDNA synthesis for each sequence was performed from 2 µl of RNA stock solution in a 20-µl volume using the following assay mixture: 20 units avian myoblastosis virus RT (Pharmacia, Uppsala, Sweden), 25 units RNAguard (Pharmacia), 0.625 mM each deoxyribonucleoside triphosphate (dNTP), 1 µM specific antisense primer, 50 mM Tris·HCl (pH 8.3), 8 mM MgCl2, and 30 mM KCl. Incubation was performed for 60 min at 50°C. One microliter of the assay was transferred to 24 µl of the incorporation mixture for PCR [50 mM Tris·HCl, 15 mM (NH₄)₂SO₄, 0.2 µM antisense and sense primers, 0.25 mM dNTPs]. For MHCIIβ, MHCIIb, and MHCIIId(x), pH was 8.50, MgCl2 was 2.5 mM, and 0.75 units Taq polymerase (Pharmacia) were used. For MHCIIa, pH was 8.30, MgCl2 was 2 mM, and 1.4 units Expand High-Fidelity polymerase (Boehringer Mannheim) were used. The annealing temperatures were 59°C for MHCIIb and MHCIIa, 64°C for MHCIIβ, and 55°C for MHCIIId(x). Depending on the initial amount of template, the number of cycles was varied between 18 and 25 cycles to allow product detection in the exponential range of amplification.

Chemiluminescent product detection was performed after electrophoresis on a 6% polyacrylamide gel and electroblotting to a nylon membrane (Hybond N; Amersham). The digoxigenin-labeled DNA was visualized by an antibody-linked assay followed by a peroxidase-catalyzed chemiluminescence reaction (Boehringer Mannheim). Signals were photographically documented (Hyperfilm ECL; Amersham) and evaluated by integrating densitometry (18). At least two measurements were performed on each sample (animal and time point).

MHC protein electrophoresis. MHC protein isoforms were analyzed by gradient gel electrophoresis as previously described (22). The silver-stained gels were evaluated by integrating densitometry. At least two measurements were performed on each sample.

Statistical analyses. A t-test was used to determine if differences existed between values from stimulated and control muscles. The acceptable level of significance was set at P < 0.05. Values are presented as means ± SD.

RESULTS

Changes in MHC mRNA isoforms. To assess absolute amounts of the different MHC mRNA isoforms in total RNA extracts, a quantitative RT-PCR has been established. Quantification of the various MHC mRNA isoforms was based on external cRNA standards obtained by in vitro transcription and serial dilutions. As previously shown, the detection limit of this method is in the range of 50 mRNA molecules (18). Oligonucleotide primers for the different isoforms were selected to yield amplification products of different length, i.e., 310 nt for MHCIIa, 288 nt for MHCIIβ, 197 nt for MHCIIb, and 120 nt for MHCIIId(x). Their specificity was verified by restriction fragment analysis (Fig. 1).

The normal EDL muscle was characterized by a predominance of the MHCIIb mRNA amounting to 10.87 ± 0.27 × 10⁸ molecules/µg total RNA (Fig. 2A). MHCIIId(x) and MHCIIa mRNA amounted to 3.75 ± 0.70 × 10⁸ and 4.44 ± 0.90 × 10⁸ molecules/µg total RNA, respectively, and thus scaled 2.9- and 2.4-fold lower than MHCIIb, respectively. To assess absolute amounts of the different MHC mRNA isoforms in total RNA extracts, a quantitative RT-PCR has been established. Quantification of the various MHC mRNA isoforms was based on external cRNA standards obtained by in vitro transcription and serial dilutions. As previously shown, the detection limit of this method is in the range of 50 mRNA molecules (18). Oligonucleotide primers for the different isoforms were selected to yield amplification products of different length, i.e., 310 nt for MHCIIa, 288 nt for MHCIIβ, 197 nt for MHCIIb, and 120 nt for MHCIIId(x). Their specificity was verified by restriction fragment analysis (Fig. 1).

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CLFS elicited marked changes, such that the amount of MHCIIb mRNA was −50% reduced in 3-day stimulated muscle. Its amount continued to decay, reaching an almost 35-fold lower level in 42-day stimulated muscles. As shown in Fig. 3, the decrease of MHCIIb mRNA followed an exponential time course with an
apparent half-life of 62 h. Based on the assumption that transcription was completely suppressed in the stimulated muscle, the half-life of 62 h may be considered as a maximal value. Even though there was a steep decrease in MHCIIb mRNA, the sensitivity of the RT-PCR made it possible to detect small amounts even after 42 days (0.31 ± 0.29 x 10^8 molecules/µg RNA).

MHCIIa mRNA was unaltered in 3-day stimulated muscles, started to increase after 7 days, and was significantly elevated by 14 days. After 42 days, the amount of MHCIIa was 3.4-fold elevated over control (15.17 ± 2.09 x 10^8 molecules/µg RNA; Fig. 2A). MHCIIId(x) mRNA increased after 1 day but decayed with longer stimulation periods. Its decrease was significant by 14 days, and, compared with control, it was 4.5-fold reduced by 42 days (1.06 ± 0.51 x 10^8 molecules/µg RNA). MHCβ exhibited only small changes and was 1.8-fold elevated in muscles exposed to CLFS for 42 days (0.37 ± 0.09 x 10^8 molecules/µg RNA; Fig. 2A).

The determination of molecule numbers made it possible to verify that changes in absolute amounts of the individual mRNAs had little effect on the level of total MHC mRNAs. As can be derived from Fig. 2A, the total amount of MHC mRNAs remained rather stable during the time period of major changes in isoform expression.

A similar picture emerged when the changes in mRNA isoform distribution were expressed as percentage of total MHC mRNAs (Fig. 2B). Compared with Fig. 2A, the increase in MHCIIa mRNA was more pronounced, especially during 7 and 28 days. This difference may be explained by a transient (~1.6-fold) increase in total RNA during the same time period (data not shown).

Changes in MHC protein isoforms. Because our studies at the mRNA level did not indicate an appreciable induction of slow myosin, the possibility existed that the total amount of stimulation was not sufficient to elicit the ultimate step of the fast-to-slow conversion. Therefore, we performed a second time course study in which the daily stimulation was increased to 24 h and
the maximum stimulation period amounted to 100 days. To definitively clarify whether or not CLFS induces slow myosin in rat muscle, this part of our investigation focused on changes in MHC expression at the protein level (Fig. 4).

According to electrophoretic analyses, normal rat EDL muscle contained 51.1 ± 2.7% MHCIIb, 31.6 ± 2.5% MHCIIId(x), 12.5 ± 1.8% MHCIIa, and 4.8 ± 1.3% MHCIβ (Fig. 4). The first significant changes of the MHC pattern were detected in 6-day stimulated muscles when MHCIIb decayed steeply and the relative concentration of MHCIIa increased. These reciprocal changes progressed with continued stimulation such that MHCIIb reached a minimum of 6–7% by 36 days, when MHCIIa attained a maximum of ~80%. The decrease of MHCIIb followed an exponential time course with an apparent half-life of 11 days (Fig. 5). Significant decreases in the relative concentration of MHCIIId(x) were first detected in muscles stimulated for 12 days. Longer stimulation periods led to further reductions in MHCIIId(x). Due to the similar electrophoretic mobilities of MHCIId(x) and MHCIIa, the former could not be separated unambiguously from the latter in long-term stimulated muscles in which MHCIIa was predominant and MHCIId(x) had decreased to minute amounts. MHCIβ was unaltered up to 36 days, but a first significant increase was noted in 60-day stimulated muscles when it amounted to ~10%. In 100-day stimulated muscles, MHCIβ amounted to 12.0 ± 4.0%, corresponding to an approximately twofold increase over control. Compared with MHCIIa (81.7 ± 2.1%), however, its fraction remained low.

**DISCUSSION**

The present study investigates the time courses of adaptive changes in MHC isoform expression of rat fast-twitch muscle in response to CLFS. Previous studies focusing on contractile properties (11), histochemically assessed myofibrillar ATPase (7), and MHC isoform analysis (21, 22) have provided evidence that rat fast-twitch muscle displays a restricted capacity for fast-to-slow conversion in response to CLFS. The existence of species-specific differences regarding the readiness for fast-to-slow conversion is most obvious when comparing rats with larger mammals, such as rabbits (4, 12, 20), dogs (1, 9), goats (6), and sheep (5). Whereas muscles of these animals respond to CLFS with a pronounced upregulation of slow myosin, the stimulation-induced adaptation of the MHC complement in rat muscle seems to be restricted to an exchange of MHCIIb with MHCIIa. The present study shows that this restriction applies to muscles exposed to CLFS as long as 100 days. We therefore disagree with the notion of an unrestricted adaptive capacity of rat fast-twitch muscle (14). However, as previously demonstrated, slow myosin can be induced by CLFS in rat fast-twitch muscle if the animal is hypothyroid. Thus hypothyroidism lowers the threshold for inducing the fast-to-slow conversion (10). A barrier to turn slow has also been observed in chronically stimulated fast-twitch muscles of mouse (A. Termin, N. Hämäläinen, and D. Pette, unpublished results), which supports the notion of a restricted adaptive capacity of small rodents compared with large mammals (16).

Furthermore, differences between responses of rats and larger animals, e.g., the rabbit, to enhanced neuromuscular activity emerge from species-specific isoform transitions and their time courses. In rabbit EDL, the fast-to-slow transformation starts at the stage of MHCIIId(x), its predominant fast isoform (2, 12). In rat EDL, the fast-to-slow transition starts at the stage of MHCIIb, which represents the predominant isoform of this muscle. Moreover, in the rabbit, the transformation encompasses sequential transitions in the order MHCIIId(x) → MHCIIa → MHCIβ (12). In this sequence, MHCIIa is transiently upregulated but decreases when MHCIβ starts to rise (12). Conversely, one might get the impression that, in the rat, MHCIIb is directly exchanged with MHCIIa, as the steep decrease in MHCIIb coincides with a steep increase in MHCIIa while the level of MHCIIId(x) is stable (Figs. 2 and 4). However, the possibility exists that, in rat

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**Fig. 4.** Time course of changes in relative concentrations of MHC protein isoforms in extensor digitorum longus muscle of rat in response to chronic low-frequency stimulation. *First time points at which changes of the individual MHC isoforms exhibited significance (P < 0.05) compared with control (n = 9). Relative concentrations (means ± SD, n = 3) of MHCIIb (○), MHCIIId(x) (▲), MHCIIa (■), and MHCIβ (●).

**Fig. 5.** Time-dependent decay of MHCIIb protein in low-frequency stimulated rat extensor digitorum longus muscle. Values (means ± SD) were replotted from Fig. 4, and broken line was obtained by regression analysis. Inset: half-logarithmic plot for determining the apparent t₁/₂ [in days (d)].
muscle, the transition from MHCIIb to MHCIIId(x) occurs in synchrony with the transition from MHCIIId(x) to MHCIIId. This could explain the observation of relatively stable MHCIIId(x) mRNA and protein levels during the initial phase of the transformation process. However, the possibility also exists that the changes in MHC expression do not follow a strictly sequential time course but occur continuously. This would result in an expansion of various hybrid fiber types as previously shown in transforming muscles of rabbits (20) and rats (3, 22).

Our findings disclose remarkable similarities between mRNA (Fig. 2) and protein data (Fig. 4). The changes in MHC protein isoform levels follow and mirror the changes in MHC mRNA isoforms, although these results were obtained from two separate experimental series differing in the daily amount of stimulation. Thus the mRNA data are based on a 10 h/day stimulation protocol, whereas a 24-h protocol was used for the protein study. In spite of the lower amount of stimulation in the first series, the changes in mRNA isoforms occur earlier than the changes at the protein level in the second series. Our suggestion that changes at the mRNA level occur earlier than at the protein level is also in agreement with the steeper decay of MHCIIb at the mRNA level (apparent half-life of 62 h) compared with the protein level (apparent half-life of 11 days). Moreover, the increase in MHCIIa mRNA in the first series occurs at an earlier time point than that of the corresponding protein in the second series.

The changes in MHC protein isoforms recorded in the present study follow a time course similar to a previous study in which we used a 10 h/day protocol (22). In both studies, the sum of MHCIIa and MHCIIId(x) attained a plateau of ~80% relative concentration by the same time, i.e., 35 days (22) and 36 days (present study), respectively. This agreement suggests that the higher amount of daily stimulation applied in the second series of the present study did not enhance the alterations in MHC isoform expression. Similarly, as shown in an independent study, increasing the impulse frequency from 10 to 20 Hz did not enhance the fast-to-slow transition in the stimulated rat EDL muscle (14).

It appears justified, therefore, to compare the temporal patterns of the CLFS-induced changes in the two experimental series of the present study. The observation that absolute and relative amounts of the various mRNAs and protein isoforms exhibited changes in the same ranges suggests that altered transcriptional activities or changes in pretranslational regulation are primarily responsible for the induced alterations in MHC isoform expression. It is also evident that the minor increase in MHCIIb mRNA in 42-day stimulated muscles matches the findings at the protein level from the second series. The suggestion that changes at the pretranslational level indeed play a primary role in determining MHC isoform transitions is in agreement with our previous study, which demonstrated that the CLFS-induced upregulation of MHCIIb in hypothyroid rats is accompanied by pronounced increases in the level of the corresponding mRNA (10).

Taken together, we show that CLFS induces, in rat fast-twitch muscle, pronounced changes in MHC isoform expression, although, compared with larger mammals, the rearrangement of the isoform profile remains essentially restricted to the fast myosins. The failure to detect, in rats, appreciable increases in slow myosin points to the existence of an elevated threshold to undergo the fast-to-slow transition commonly induced by CLFS in fast-twitch muscles of larger mammals.

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