Changes in myosin heavy chain mRNA and protein isoforms in single fibers of unloaded rat soleus muscle

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Abstract Changes in myosin heavy chain (MHC) mRNA and protein isoforms were investigated in single fibers from rat soleus muscle unloaded by hindlimb suspension for 4 and 7 days. Dramatic changes were seen after 4 days, when all fibers co-expressed slow and fast MHC mRNAs. Most fibers contained mRNAs for MHCIβ, MHCIIa, MHCIId(x), and MHCIIb. The up-regulation of the fast isoforms was only partially transmitted to the protein level. Atypical combinations of MHC mRNA isoforms, which deviated from the 'next-neighbor rule', were frequent in fibers from unloaded soleus. These atypical combinations increased with time and were not observed in the controls. The results suggest that hindlimb suspension elicits in soleus muscle pronounced perturbations in the expression of MHC isoforms by disrupting transcriptional and translational activities.

Key words: Hindlimb suspension; mRNA; Myosin heavy chain isoform; Reverse transcriptase polymerase chain reaction; Single fiber study; Slow-to-fast transition

1. Introduction

The soleus muscle of the adult rat is predominantly composed of slow-twitch type I fibers characterized by their slow myosin heavy chain (MHC) MHCIβ complement. As shown by several studies, rat soleus muscle changes its phenotype when exposed to conditions which unload the muscle (for reviews, see [1–3]). Unloading of this normally weight-bearing muscle elicits orchestrated slow-to-fast transitions in functional and molecular properties. These transitions have been impressively illustrated by the induction of fast isoforms MHCIId(x) and MHCIIb which are normally not expressed in soleus muscle.

As recently shown for rat soleus muscle unloaded by hindlimb suspension, the slow-to-fast transitions in MHC mRNA and protein isoforms occur in the order MHCI → MHCIIa → MHCIId(x) → MHCIIb [4], i.e. in the reverse direction of experimentally induced fast-to-slow transitions [2]. Although for the most part the changes in MHC mRNA isoforms resemble those of the corresponding proteins in unloaded soleus muscle, some inconsistencies exist. For example, MHCIIa and MHCIId(x) mRNAs were found to increase in parallel, whereas the rise of MHCIIa protein preceded that of MHCIId(x). This and other observations suggested posttranscriptional regulation. However, the synchronous increases of MHCIIa and MHCIId(x) mRNAs may be related to their simultaneous expression in different fiber types. Thus, MHCIIa might be up-regulated in transforming type I fibers in parallel with MHCIId(x) in transforming type IIA fibers [4].

Single fiber analyses were performed to address this question in the present study. Rat soleus muscles were unloaded by hindlimb suspension for 4 and 7 days. Changes in mRNA and protein composition of the MHCIβ, MHCIIa, MHCIId(x), MHCIIb isoforms were assessed in single fiber fragments from control and unloaded soleus muscles by highly sensitive reverse transcriptase polymerase chain reactions (RT-PCR) and by MHC electrophoresis [5–7]. Expression patterns of MHC mRNA isoforms could thus be assigned to pure and hybrid fiber types classified according to their MHC protein complement.

2. Materials and methods

2.1. Animals and muscles

Control and unloaded soleus muscles were obtained from adult Wistar rats (initial body weight 280 g) exposed to hindlimb suspension for 0 (controls), 4, and 7 days (two rats for each time point). The muscles were from the same animals used in our previous study [4].

2.2. Dissection and classification of single fibers

Single fibers (~5 mm long) were isolated at random from freeze-dried fiber bundles by free-hand dissection under a stereomicroscope. Two small pieces were cut from each fiber, weighed on a quartz fiber balance, and analyzed for their MHC mRNA and protein compositions. The fragments for direct RT-PCR ranged from 80 to 100 ng, and those for protein analysis from 150 to 200 ng dry weight. MHC protein isoforms were separated by SDS-PAGE [8] and relative concentrations of MHC isoforms evaluated densitometrically. Because of similar mobilities of MHCIα and MHCIβ, these two isoforms co-migrated in the fastest band designated MHCI.

2.3. Direct RT-PCR on muscle fiber fragments

mRNA analysis by RT-PCR was performed by the oil well technique [5]. When necessary and possible, two pieces from each fiber were investigated. For total RNA extraction, the fiber fragment was transferred under visual control into 0.28 μl of high-salt extraction medium under mineral oil. The medium consisted of 50 mM Tris–HCl (pH 9.0), 250 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 U/μl human placenta RNase inhibitor (Roche), complemented with 5 mM ribonucleoside vanadyl complexes (Sigma). After 60 min incubation at 4°C, the sample was incubated for 5 min at 65°C and cooled on ice. Subsequently, reverse transcription was started by adding 0.86 μl of the following solution to the assay mixture: 50 mM Tris–HCl (pH 9.0), 10 mM $MgCl_2$, 10 mM DTT, 1.3 mM dNTP, 1 μM

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Abbreviations: DTT, dithiothreitol; MHC, myosin heavy chain; RT-PCR, reverse transcriptase polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

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oligo(dT)$_{15}$, 1 U/μl AMV reverse transcriptase, 1 U/μl human placenta RNase inhibitor. After 30 min incubation at 42°C, the mixture was heated for 10 min at 65°C. These small volumes were pipetted using a previously described automatic micropipette [9]. The assay was transferred into 30 μl PCR buffer (1× buffer, Expand High-Fidelity PCR system; Roche). Two microliters of the diluted RT assays were amplified separately for each of the four sequences and transferred into 23 μl of the following PCR incubation mixture (final concentrations): 1×Expand buffer, 0.25 mM of each dNTP, 0.2 μM of each oligonucleotide primer, 0.63 units of Expand polymerase (Roche). The oligonucleotide primers for the sequences were the same as previously described [4]. $MgCl_2$ concentrations were 2 mM for MHCIIa and 2.5 mM for MHCIβ, MHCIId(x) and MHCIIb. To ascertain that amplification from contaminating DNA did not occur, control PCR assays were run in the absence of RT. The PCR products of the different MHC isoforms were detected after 28 cycles.

After the amplification, 4 μl of each of the four assays was subjected to 6% polyacrylamide gel electrophoresis 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 and evaluated by integrating densitometry. At least two measurements were performed on each sample (animal and time point).

For evaluation of MHC mRNA expression levels, the signal intensities of MHC-specific mRNAs were referred to the signal intensities of purified PCR products of known concentrations run in parallel assays (10^3 molecules for MHCIβ and MHCIIa, and 10^2 molecules for MHCIId(x) and MHCIIb) as external standards.

3. Results

3.1. Changes in MHC protein isoforms of single fibers

Unloading the soleus for up to 7 days resulted in pronounced changes in the pattern of MHC protein isoforms in single fibers with decreases in pure and increases in hybrid fibers (Fig. 1). Pure type I fibers predominated in control soleus (∼68%), but decreased to 40% in 7-day unloaded muscles. The remaining portion of control soleus (∼32%) consisted of hybrid fibers. In these fibers, MHC isoforms co-existed in combinatorial patterns according to the 'next-neighbor rule', previously established from sequential transitions in the order of MHCI↔MHCIIa↔MHCIId(x)↔MHCIIb [2]. Most of the (∼68%) hybrid fibers from control soleus displayed the co-existence of MHCI and MHCIIa in varying ratios. A few fibers (∼4% of the total) contained MHCI, MHCIIa, and small amounts of MHCIId(x).

Unloading resulted in a decrease of hybrid fibers with the MHCI+MHCIIa complement and produced increases in hybrid fibers with MHCI+MHCIIa+MHCIId(x) and MHCI+MHCIIa+MHCIId(x)+MHCIIb combinations. These two

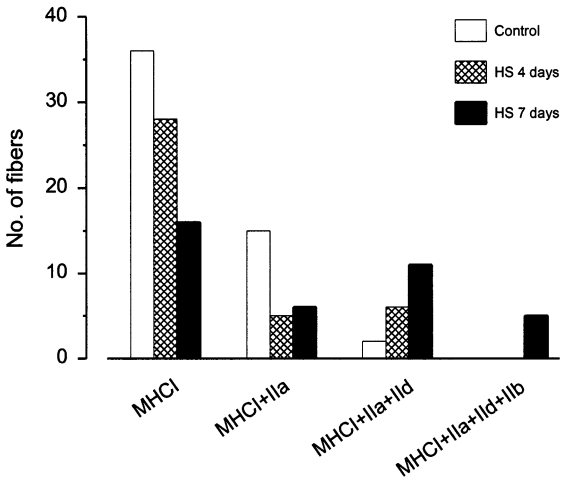

Fig. 1. Distribution of pure and hybrid fiber types in control, 4-day, and 7-day unloaded rat soleus muscles. Single muscle fibers ($n=130$) were classified according to their electrophoretically assessed MHC composition.

populations amounted to 23% and 13%, respectively, in the 7-day unloaded soleus muscles.

3.2. Changes in MHC mRNA composition of single fibers

A small fraction of the fibers electrophoretically classified as pure type I in control soleus contained only MHCIβ mRNA (Fig. 2). Most type I fibers in control soleus (∼80%) contained two (MHCIβ+MHCIIa), three (MHCIβ+MHCIIa+MHCIId), or four (MHCIβ+MHCIIa+MHCIId+MHCIIb) mRNA isoforms (Fig. 2, Table 1). However, MHCIβ mRNA was the predominant isoform in all these type I fibers. These results are in line with our previous findings on fast-twitch rabbit muscles in which a large percentage of fibers, classified according to their MHC protein complement as pure fiber types, proved to be hybrid at the level of MHC mRNA isoforms [5].

Fibers expressing only MHCIβ mRNA were no longer detected in the samples from 4-day unloaded muscles in which ∼80% of the fibers contained all four MHC mRNA isoforms. In 7-day unloaded muscles, such fibers were less frequent (∼35%). At this time, the percentage of fibers co-expressing two or three MHC mRNA isoforms increased. In view of the 'next-neighbor rule', most combinatorial patterns were atypical, e.g. MHCIβ+MHCIIa+MHCIIb (11%), MHCIβ+

Table 1
MHC mRNA complement of pure type I fibers from control, 4-day and 7-day unloaded soleus muscles

MHC mRNA complement	Control number of fibers	4-day unloaded number of fibers	7-day unloaded number of fibers
MHCIβ	7	–	–
MHCIβ+MHCIIa	10	1	–
MHCIβ+MHCIIa+MHCIId(x)	6	–	2
MHCIβ+MHCIIa+MHCIId(x)+MHCIIb	6	24	7
MHCIβ+MHCIId	–	2	4
MHCIβ+MHCIId(x)+MHCIIb	–	–	1
MHCIβ+MHCIIb	–	–	1
MHCIIa+MHCIId(x)+MHCIIb	1	–	–
MHCIIa+MHCIIb	–	1	–
MHCIId(x)+MHCIIb	–	–	1

Fiber typing was based on their electrophoretically determined MHC isoform composition. Atypical combinations of MHC mRNA isoforms are underlined. For explanation, see text.

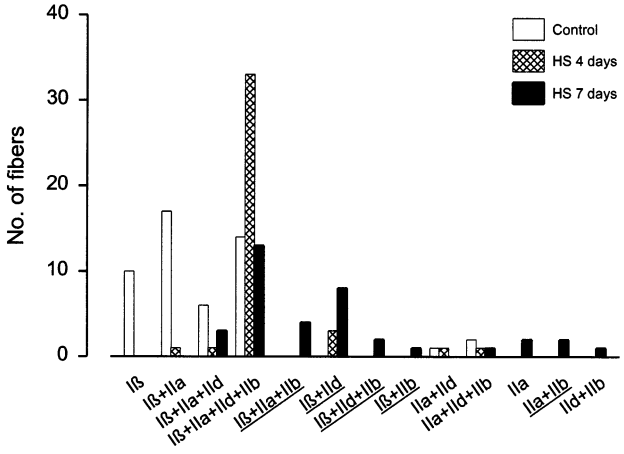

Fig. 2. Distribution of MHC mRNA isoform patterns in single fibers ($n = 127$) from control, 4-day, and 7-day unloaded rat soleus muscles. MHC mRNA isoforms were determined by RT-PCR in fragments of the same fibers studied in Fig. 1. Atypical MHC mRNA isoform combinations are underlined (see text for explanation).

MHCIId(x) (22%), MHCIβ+MHCIId(x)+MHCIIb (5%), and others (Fig. 2). A few fibers with the atypical MHCIβ+MHCIId combination were detected in 4-day unloaded muscles.

As judged by comparative evaluations of the amplified cDNA signals, MHCIβ mRNA was the predominant isoform in fibers with more than one mRNA isoform. In fibers with the MHCIβ+MHCIIa mRNA complement, MHCIIa mRNA amounted to ~70% of the MHCIβ mRNA content. These two isoforms existed in a similar ratio in those type I fibers additionally containing MHCIId(x) mRNA or MHCIId(x)+MHCIIb mRNAs. MHCIId(x) and MHCIIb mRNAs amounted to ~10% and ~5%, respectively, of the MHCIβ mRNA content in the latter fibers.

3.3. Correlations between MHC mRNA and protein patterns in single fibers

Discrepancies between mRNA and protein patterns were observed in control fibers, but more frequently in fibers from unloaded muscles in which all type I fibers contained more than one MHC mRNA isoform. Thus, pure and hybrid fibers existed which contained the MHCI protein without detectable amounts of MHCIβ mRNA (Tables 1 and 2). In addition, fibers from unloaded muscles displayed atypical mRNA combinations, especially after 7 days (Fig. 2, Table 2). As judged by the 'next-neighbor rule', mRNA combinations were considered atypical when an isoform was missing which normally bridges the gap between distant mRNA isoforms, e.g. MHIβ+MHCIId(x), MHCIIa+MHCIIb, MHCIβ+MHCIIa+MHCIIb, or MHCIβ+MHCIId(x)+MHCIIb. Atypical mRNA combinations were never detected in pure or hybrid fibers from control muscles (Tables 1 and 2).

4. Discussion

The availability of reliable micromethods [10] has made it possible to compare expression patterns of MHC mRNA and protein isoforms at the single fiber level. This type of analysis has provided insights regarding correlations between the levels of transcription and translation. In the present study, mRNA and protein patterns of MHC isoforms were not entirely coordinated in single fibers of normal and transforming muscles. For example, most pure type I fibers from normal soleus muscle (expressing only MHCI protein) contained almost equal amounts of MHCIβ and MHCIIa mRNAs. As such, either the MHCIIa transcript is not translated or its product remains below the limit of detection. This observation, as well as the detection of additional untranslated fast MHC mRNA isoforms in type I fibers, supports the notion that MHC protein isoform patterns of muscle fibers are under transcriptional *and* translational control.

As judged by the 'next-neighbor rule' [2], fibers from transforming muscles appear to contain 'atypical' combinations of MHC mRNA isoforms, e.g. MHCIβ+MHCIId or MHCIβ+MHCIIa+MHCIIb. However, failure to detect some mRNA isoforms may be due to methodology. Because reverse transcription was performed in a common assay for all four isoforms under study, the failure to detect a specific cDNA could only originate from the PCR, e.g. due to insufficient amplification of a low signal. This possibility cannot be excluded because the present PCR protocol was designed for semiquantitative evaluation and amplification, and, therefore, was restricted to 28 cycles. Because atypical combinations appeared only in transforming fibers where their frequency increased with the duration of hindlimb suspension, we suggest that such fibers represent incomplete stages of phenotype transition. However, atypical combinations or failure to detect

Table 2
MHC mRNA complement of hybrid fibers from control, 4-day and 7-day unloaded soleus muscles characterized by coexistence of MHCI and MHCII protein isoforms

MHC mRNA complement	Control number of fibers	4-day unloaded number of fibers	7-day unloaded number of fibers
MHCIβ+MHCIIa	9	–	–
MHCIβ+MHCIIa+MHCIId(x)	2	2	1
MHCIβ+MHCIIa+MHCIId(x)+MHCIIb	4	7	8
MHCIβ+MHCIIa+MHCIIb	–	–	4
MHCIβ+MHCIId(x)	–	1	3
MHCIβ+MHCIId(x)+MHCIIb	–	–	1
MHCIIa	–	–	2
MHCIIa+MHCIId(x)	1	–	–
MHCIIa+MHCIId(x)+MHCIIb	1	1	1
MHCIIa+MHCIIb	–	–	2

Fiber typing was based on electrophoretically determined MHC isoform composition. Atypical combinations of MHC mRNA isoforms are underlined. For explanation, see text.

a specific MHC mRNA may also have resulted from non-uniform distribution along the fibers, a phenomenon previously documented in fibers of transforming rabbit muscle [11]. Fibers with mismatches between MHC mRNA and protein isoform distribution have been detected by immunocytochemistry and in situ hybridization in normal and transforming human muscles and were interpreted as transitional fibers [12,13]. Their detection in control muscles in the present study suggests that fiber type transitions occur in soleus also under steady-state conditions.

Interestingly, fibers with atypical mRNA combinations did not exhibit similar atypical MHC protein patterns. The failure to detect fibers with atypical MHC protein patterns may, however, be due to the relatively short duration of unloading in the present study. Taking into account the differences between half-lives of MHC mRNA and protein isoforms [2], the 7-day period may not have been sufficient to translate short-term changes in mRNA composition into protein. In fact, single fibers with atypical combinations of the MHC protein isoforms have previously been observed in 14-day unloaded soleus muscle of the hyperthyroid rat [14].

The appearance of atypical MHC mRNA patterns is difficult to explain, but may relate to the fact that two opposite processes are overlapping in the unloaded muscle, namely fiber type transitions and fiber atrophy. Obviously, unloading has an immediate effect on the normally ordered expression of MHC isoforms. In the present investigation, most of the fibers in 4-day unloaded soleus muscle contained all four MHC mRNA isoforms. Moreover, our results on discrepancies between MHC isoform patterns at the transcript and protein levels suggest that unloading disrupts the processes of transcription and translation. It is noteworthy that these changes coincide with pronounced alterations in motoneuron activity. Electromyographic recordings on rat soleus muscle have previously shown that unloading leads to an almost immediate and drastic suppression ($\sim$90%) of its electrical activity [15]. Although reduced electrical activity was shown to be transitory, these changes may relate to the uncoordinated up-regulation of fast MHC isoforms. The impact of neural activity on gene expression in skeletal muscle, especially the inhibitory effect of tonic, slow motoneuron-like impulse patterns on the expression of fast myofibrillar protein isoforms, has been amply documented in studies using chronic low-frequency stimulation [2,16]. It is not unexpected, therefore, that silencing of slow motoneuron activity normally delivered to the slow soleus muscle up-regulates fast myofibrillar protein isoforms, similar to the effect of denervation [17] or tenotomy [18,19].

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