Specific isomyosin proportions in hyperexcitable and physiologically denervated mouse muscle

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Abstract We show here, by high resolution sodium dodecyl sulfate gel electrophoresis, that the proportions of myosin heavy chain (MyHC) isoforms of mouse muscles are specifically shifted by hereditary neuromuscular diseases. In wild-type and dystrophic MDX anterior tibial muscle (TA) about 60% of the MyHC is IIB, 30% IIX, at most 10% IIA and $<2\%$ type I (slow). In myotonic fast muscles, hyperexcitability leads to a drastic reduction of MyHC IIB which is compensated by IIA. Slow muscles, like soleus and diaphragm, were only marginally changed by myotonia. The MyHC pattern of TA of spinal muscular atrophy (SMA) 'wobbler' mice is shifted to a faster phenotype, with nearly 90% IIB. In the SMA mutant 'muscle deficient', all four adult isomyosins are expressed in the TA. These findings may be relevant for the future diagnosis of neurological disorders both in mouse disease models and in human patients.

Key words: Myosin heavy chain isoform; Muscular chloride channel ClC-1; High resolution polyacrylamide gel electrophoresis; Muscular dystrophy (*mdx*); Myotonia (*adr*, *mto*); Spinal muscular atrophy (*wr*, *mdf*)

1. Introduction

The approach to functional genomics by constitutional knockout of genes in the mouse has been criticized with the argument that loss of a single gene function, i.e. a single cut in the network of gene interactions, might alter the expression of a host of other genes [1]. The same argument would apply to spontaneous loss-of-function mutations. Yet, indirect changes of gene expression or secondary effects of mutations are instructive for the very reason that they highlight the network of gene interactions. These interactions may be of a more direct physical kind, by participation of different gene products in one protein complex, as in the case of the dystrophin glycoprotein complex that spans the sarcolemma. Alternatively, the relation may be more indirect in that a single gene determines a physiological property on which the expression of many other genes depends. An example of the latter case is the muscular chloride channel ClC-1 which regulates the excitability of skeletal muscle. Loss of function of the corresponding gene, *Clc1*, leads to hyperexcitability of muscle, the hallmark of the disease myotonia (muscle stiffness) [2–4]. Neurogenic muscle diseases like spinal muscular atrophy (SMA) in humans or mouse models of SMA like 'wobbler' (WR) [5,6] and 'muscle deficient' (MDF) [7] are due to motoneuron degeneration in the brainstem and spinal cord.

In the absence of pathological influences, muscle is able to adapt its fiber type profile to changing physiological and functional demands. This plasticity is reflected in the selective expression of the myosin heavy chain (MyHC) isoforms, the myofibrillar components that determine the speed of contraction. The four myosin isoforms expressed in adult mouse skeletal muscle (in order of decreasing contraction rate) are fast IIB, IIX (= IID), and IIA, and slow I. The IIB MyHC is expressed in the large diameter glycolytic fibers whereas the other isoforms are found in the more oxidative fibers. In muscles of small mammals, artificial chronic stimulation induces a fiber type shift from fast glycolytic in the direction of slow oxidative whereas disuse or decreased motor activity causes the opposite transformation (reviews [8–11]).

Hyperexcitable muscles of myotonic mice are transformed from a mixed glycolytic/oxidative to a pure oxidative phenotype [12,13]. This shift can be partially reversed by drugs that suppress the hyperexcitability [14,15]. Other consequences of myotonia in the mouse muscles are a downregulation of MyHC IIB [14,15] as well as of the calcium binding protein parvalbumin [16] and its mRNA [15,17]. The shift of isomyosin phenotype from fast glycolytic to slow oxidative in the myotonic muscle was confirmed at the level of mRNA [18,19] and was related to a differential expression of the myogenic factors: MyoD mRNA content was lowered, whereas myogenin mRNA was elevated [19].

An isomyosin shift in the direction fast to slow has also been observed in muscle biopsies from myotonia patients [20]. In Thomsen–Becker myotonia the loss of function of the muscle chloride channel causes milder symptoms than in the mouse and therefore there are less dramatic changes in muscle biochemistry than were observed in the myotonic mouse. Paradoxically, for the myotonic goat which also has a mutation in the muscle chloride channel a fiber type shift from slow to fast, i.e. in a direction opposite to that caused by

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Abbreviations: MyHC, myosin heavy chain; TA, anterior tibial muscle; SMA, spinal muscular atrophy; ALS, amyotrophic lateral sclerosis

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homologous mutations in mouse and man, has been reported [21].

Here we present, for the first time, a relation between the proportion of *all* major skeletal myosin isoforms to distinct hereditary diseases using mouse models of myotonia, muscular dystrophy and spinal muscular atrophy (SMA) or amyotrophic lateral sclerosis (ALS). Our results document the high specificity of isomyosin switches with respect to both the gene defect and the anatomical identity of the muscle. They could provide a tool to diagnose uncharacterized neurological diseases both in genetically manipulated mice and in patient biopsies.

2. Materials and methods

Adult dystrophic MDX, myotonic ADR, MTO and ADR*K, double mutant ADR.MDX, and atrophic WR and MDF mutant muscles (Table 1 [22]) were compared to muscles from unaffected littermates. The longissimus dorsi, tibialis anterior (TA), extensor digitorum longus, gastrocnemius, soleus, tongue, masseter and diaphragm muscles were dissected from freshly killed adult mice (70–150 days of age), immediately frozen in liquid nitrogen and stored at −80°C. At least three mice were used for each experimental point. Frozen muscles were extracted on ice for 60 min in four volumes of extracting buffer (pH 6.5) as previously described [23]. Following centrifugation, the supernatants were diluted 1:1 with glycerol and stored at −20°C. MyHCs were separated on 8% polyacrylamide gels which were made in the Bio-Rad mini-Protean II Dual slab gel cell system (0.75 mm thickness) as described previously [24]. Electrophoresis was carried out for 31 h at 72 V (constant voltage) at 4°C. Following electrophoresis, the gels were silver-stained according to [25]. The gels were scanned using a video acquisition system. The relative levels of MyHC isoforms were determined using a densitometric software (Scion Image, NIH, USA).

3. Results

Using high-resolution gel electrophoresis, the patterns of MyHC in TA muscle of adult myotonic mice (ADR, ADR*K, MTO, double mutant ADR.MDX) were compared to those of muscles affected by muscular dystrophy (MDX) and SMA/ALS-like atrophies WR and MDF (Table 1, Fig. 1). In normal adult mice the TA muscle contained predominantly fast IIB (62.4 ± 4.1% (S.D.) of the total MyHC content) and IIX (30.7 ± 2.8%) myosin isoforms and a small amount (6.6 ± 3.4%) of fast IIA isoform. In the TA muscle of adult MDX mutant mice no major change was observed as compared to wild-type (Fig. 1). The pattern of MyHC expression in myotonic mice was characterized by an increase of MyHC IIA at the expense of IIB which was reduced from about 60 to 5–15%. This shift was similar regardless of the alleles or the different genetic backgrounds of the myotonic mutants, but MTO muscle showed the strongest reduction in MyHC IIB. It is not clear whether this slight difference is due to the allele or

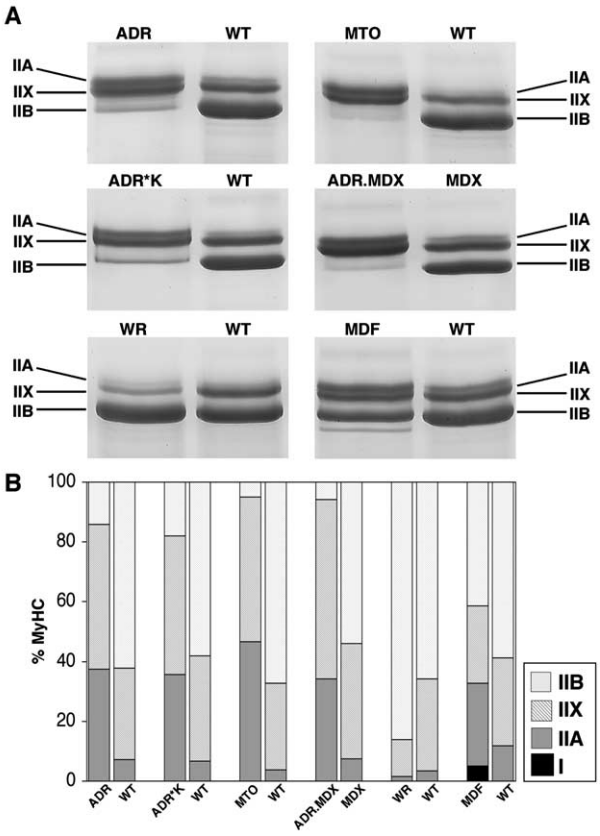

Fig. 1. MyHC isoforms in the TA muscles of mouse mutants affected by the neuromuscular disorders myotonia (ADR, ADR*K, MTO), muscular dystrophy (MDX), and spinal muscular atrophy (WR, MDF) in comparison to wild-type (WT) controls. A: Separation of four MyHC isoforms by high-resolution sodium dodecyl sulfate gel electrophoresis. Isoforms are fast IIB, IIX and IIA and slow type I. Migration from top to bottom (anode). B: Proportions of MyHC isoforms as determined by densitometric analysis of silver-stained bands. For details of mutants see Table 1.

to the genetic background, SWR/J×C57BL/6 hybrid. The mutant ADR*K is distinct from the two other alleles in that the ClC-1 antigen is retained in the sarcolemma (C. Wiegand and H. Jockusch, 2000, unpublished). The MyHC pattern in the TA of the double mutant ADR.MDX [26] was indistinguishable from that of the ADR mice (Fig. 1A).

The two mouse mutants with ALS/SMA-like diseases, WR and MDF, showed MyHC patterns distinct from myotonic muscles as well as from each other. In the TA muscle of WR mice a shift to a fast glycolytic phenotype was observed: the amount of IIA and particularly IIX MyHC was decreased and the amount of IIB MyHC was increased. In the TA muscle of the other SMA mutant, MDF, there was a moder-

Table 1
Mouse mutants used in this study

Disease/phenotype	Gene	Chromosome	Phenotype	Background	References
Arrested development of righting response, **ADR**	*Clc1*	6	myotonia	A2G	[2,13]
Arrested development of righting response kansas, **ADR*K**	*Clc1*	6	myotonia	C57BL/6	[31,32]
Myotonia, **MTO**	*Clc1*	6	myotonia	SWR/J, C57BL/6	[32,33]
Wobbler, **WR**	*wr*	11	atrophy	C57BL/6	[5,6]
Muscle deficient, **MDF**	*mdf*	19	severe atrophy	C57BL/6	[7]
X-linked muscular dystrophy, **MDX**	*Dmd*	X	dystrophy	C57BL/10	[34,35]
Double mutant, **ADR.MDX**	*Clc1*, *Dmd*	6, X	myotonia	A2G, C57BL/10	[26]

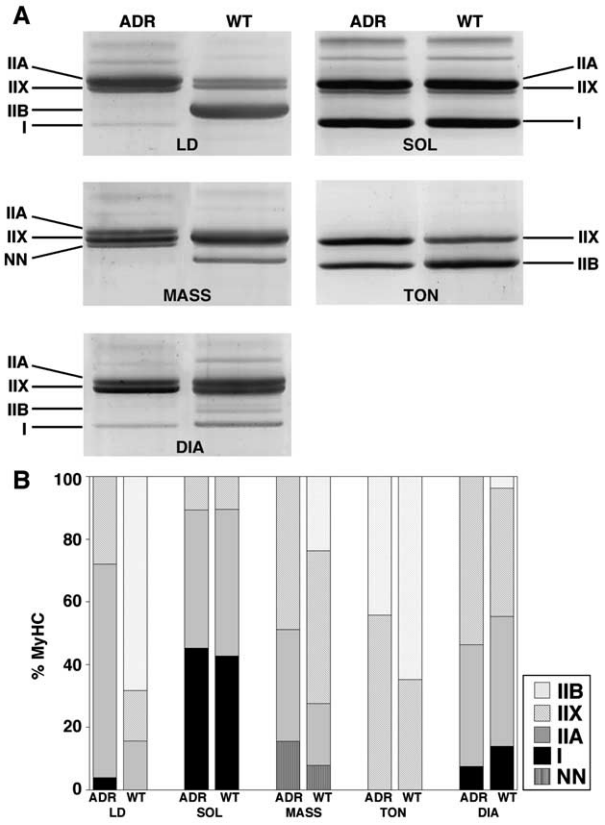

Fig. 2. MyHC isoforms in different adult myotonic (ADR) and wild-type muscles. A: Separation of five MyHC isoforms (four adult MyHC as in Fig. 1 and one neonatal, NN, which is restricted to the adult masseter). B: Proportions of MyHC isoforms. Abbreviations for muscles: LD, longissimus dorsi; SOL, soleus; MASS, masseter; TON, tongue; DIA, diaphragm. WT, wild-type control.

ate loss of MyHC IIB and slow MyHC I, normally absent from TA, was expressed (Fig. 1).

In order to determine if a shift to a slower MyHC pattern also occurred in functionally distinct skeletal muscles of myotonic mice, we analyzed the pattern of MyHC in the longissimus dorsi, soleus, tongue, diaphragm, and masseter muscles of ADR mutant mice in comparison to control littermates (Fig. 2). Significant changes were observed in all muscles of ADR mice except the slow contracting soleus. The longissimus dorsi is a fast, predominantly glycolytic muscle, and a more dramatic shift to a slower MyHC pattern than in the TA was observed: MyHC IIB, which represents 70% of the MyHC in wild-type longissimus dorsi, was totally eliminated. The soleus is fully oxidative and contains predominantly MyHCs IIA and I with about 10% MyHC IIX. Its contractility is severely affected by myotonia [14], however the MyHC pattern was identical to that of wild-type. In the tongue IIB MyHC expression was moderately downregulated with a compensatory upregulation of IIX. In the masseter and the diaphragm the wild-type pattern of MyHCs is different from that of limb muscles. In normal mice the masseter muscles, in addition to the three fast MyHC isoforms, contained a small amount of the neonatal myosin isoform, MyHC NN. In normal diaphragm all four adult MyHCs (IIB, IIX, IIA, I) are present. In myotonic (ADR) masseter and diaphragm the expression of IIB MyHC was totally eliminated. In the masseter the loss of IIB MyHC was compensated by increased levels of both IIA and MyHC NN isoforms, in the diaphragm by an increased expression of IIX MyHC.

4. Discussion

In mouse models in which the activity pattern of mammalian skeletal muscle was altered by chronic stimulation, disuse or surgical denervation, a sequence of transformations has repeatedly been found, with the MyHC isoforms being modified according to decreasing rate of contraction: IIB ↔ IIX (D) ↔ IIA ↔ I (Fig. 3). Chronic stimulation in a tonic pattern tends to shift the MyHC isoforms from left to right, i.e. fast glycolytic to slow oxidative [10,11], whereas disuse or denervation does so in the opposite direction. In the mouse, using chronic stimulation it is not possible to shift IIB fibers through the full range into type I fibers, which in wild-type limb muscles are largely confined to the soleus. In contrast to artificially stimulated adult animals, muscles in genetic mouse models of different neuromuscular diseases already develop under the influence of the physiological effects of the mutation. The muscular phenotype might be changed from the earliest time point the mutant becomes manifest. Despite this difference, all the evidence supports the notion that the MyHC shift observed in myotonic mice is caused by the hyperexcitability of muscle which leads to unscheduled episodes of trains of action potentials, equivalent to an artificial stimulation in a tonic pattern. Using physiological and histochemical criteria it was found that all muscles of myotonic mice that could be tested by contraction measurements of fiber type shifts were affected by myotonic hyperexcitability, indicating that these muscles do contain ClC-1 and that this is required for normal excitability. The muscles examined included the soleus (contraction measurements [12]), extraocular muscles (nystagmus and fiber type shift [27]), cross-striated muscle of the pharynx (fiber type shift [27]); however, no physiological or histochemical data were available for masseter and tongue, muscles which, according to reverse transcription polymerase chain reaction, contain levels of ClC-1 mRNA comparable to

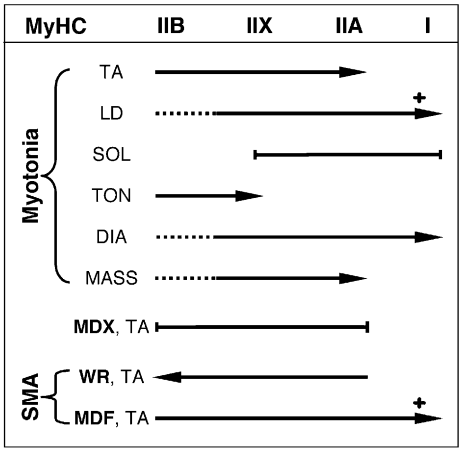

Fig. 3. Schematic representation of MyHC isotype transitions in mouse muscles affected by neuromuscular diseases. Bar, no significant change; arrow, isotype shift in the direction of the arrow; +, new appearance of a MyHC isoform in the mutant muscle; dashed line, disappearance of a MyHC isoform in a mutant.

those of limb and trunk muscles (C. Wiegand and H. Jockusch, unpublished results). We have not observed any obvious anomaly with food uptake, chewing and swallowing in the myotonic mice, but the masseter loses MyHC IIB and is thus clearly affected by the myotonia mutation. It is known that myotonic cramps (which in the masseter would have severe consequences for feeding) are suppressed by so-called warm-up, i.e. a temporary suppression of hyperexcitability by continuous use over several minutes. A similar argument may hold for the tongue and the diaphragm.

In contrast to myopathies in which the affected gene is expressed in muscle fibers, spinal muscular atrophies are characterized by indirect effects of a postnatal loss of motoneurons on muscle. Although the histological appearance of neurodegeneration in the ALS/SMA mouse models wobbler [5] and muscle deficient [7] are similar as are the elevated levels of mRNAs for acetylcholine receptor α subunit, myogenin, and MyoD and the reduced level of ClC-1 mRNA [22], we have observed very different MyHC phenotypes. After a few weeks, disuse or denervation tends to shift the isomyosin patterns from slow oxidative to fast glycolytic [28], in a direction opposite to that caused by chronic stimulation or myotonia. This is exactly what we have observed in the WR muscles in which biochemical analysis confirms physiological denervation and decreased activity [22]. Unexpectedly, MDF muscles show a MyHC pattern which is shifted towards a slower phenotype including slow MyHC I in muscles that normally do not express this isoform. The difference between WR and MDF may be due to the fact that in WR muscle denervation by motoneuron loss is partially compensated for by nerve sprouting and re-innervation [5] whereas in MDF muscle denervation may be complete and irreversible. Myographic analysis may aid in detecting the crucial physiological difference between these two muscle atrophies.

In dystrophic MDX mouse there is a massive degeneration of muscle fibers around weaning age so that the majority of muscle fibers in the adult have undergone degeneration/regeneration processes as confirmed by the centrally rather than peripherally located nuclei. The fact that adult MDX muscle shows a wild-type pattern of MyHCs rather than a pathological fiber type shift is additional evidence that ongoing physiological activity rather than the developmental history is the important determinant of isomyosin expression.

Future investigations of the secondary effects of myotonia and spinal muscular atrophies might include expression profiling of affected muscles, with the advantage that expression differences for a large number of genes are already known [16,17,29,30] and these might serve as positive controls. For the distinction between the secondary effects of the two mouse spinal atrophies, wobbler and MDF, to be mechanistically understood, the identification of the affected genes is probably a prerequisite.

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