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Neuro-muscular function in the wobbler murine model of primary motor neuronopathy

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ABSTRACT

The wobbler mouse represents a model for neurodegenerative disease affecting motor neurons. This study explored the importance of fiber type specific changes for the contractile dysfunction of soleus and extensor digitorum longus (EDL) muscles from wobbler mice using a specific inhibitor of force generation by the type II myosin protein. Generally, wobbler condition was associated with ~50% reductions in muscle mass and contractile capacity in both muscles. In soleus, an increase in the relative abundance of type I myosin protein was observed. Since, however, only ~40% of the fibers containing type I myosin had functional innervation whereas almost all fibers containing type II myosin were innervated, the shift toward type I myosin was without significance for the *in vivo* contractile phenotype. Soleus muscles from wobbler mice were further characterized by a 2-fold increase in the width of the twitches, which was associated with a reduction in the excitation frequency necessary to elicit tetanic contractions. Since the SR Ca^{2+} ATPase in wobbler soleus was reduced from 22 ± 5 to 10 ± 2 nmol/g muscle tissue ($P = 0.0006$), the increase in twitch width was most likely caused by delayed recovery of cytosolic Ca^{2+}. Such changes were not observed in EDL. It is concluded that the shift in myosin protein from type II to type I previously reported in both innervated and denervated wobbler muscles primarily takes place in the population of denervated muscle fibers. Since these muscles do not contribute to force generation, the transition is, therefore, of limited relevance for the contractile phenotype of the muscles. Instead, the slow contractile phenotype of wobbler soleus muscles seemed to be a consequence of reduced SR content of Ca^{2+} ATPase.

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Introduction

Denervation of skeletal muscles or severe inactivity typically leads to atrophy and either no change in (Agbulut et al., 2009) or a decrease in the relative abundance of type I myosin heavy chain protein (for review, see Midrio, 2006). In contrast, muscle atrophy in association with several progressive neurodegenerative diseases and their related animal models is often paralleled by a transition of the affected muscles toward a slow-twitch and more fatigue resistant phenotype (Frey et al., 2000; Hegedus et al., 2008). Studies on the $SOD1^{G93A}$ transgenic mouse model of ALS indicate that this is the result of preferential loss of motoneurons that innervate type IIB muscle fibers and a better retained sprouting competence of motoneurons innervating type I and IIA/IIX muscle fibers (Frey et al., 2000; Hegedus et al., 2008; Pun et al., 2006). Other studies suggest that with disease progression, the changes in contractile phenotype of the muscles are related to the development of large motor units with mixed fiber type compositions (Baloh et al., 2007). Similarly to $SOD1^{G93A}$, the extensive loss of force generating capacity, atrophy and a myosin heavy chain transition of preferentially fast-twitch fibers toward a slow fiber type or to fibers with a mixed fiber type composition has been reported in the wobbler mouse model of primary motor neuronopathy (Agbulut et al., 2004; Duchen and Strich, 1968; Toursel et al., 2000). In this model, the mice suffer from an A to T transversion in exon 23 of the Vps 54 (vesicular protein sorting that plays a role in vesicular trafficking) gene present on chromosome 11 that results in the amino acid substitution L967Q (Schmitt-John et al., 2005). The primary effect of the mutation is compromised axonal transport (Mitsumoto and Gambetti, 1986) and degeneration of spinal and lower motoneurons (Mitsumoto and Bradley, 1982; Papapetropoulos and Bradley, 1972; Pollin et al., 1990).

Generally, studies of changes in muscle phenotype in relation to progressive neurodegenerative illnesses have focussed on histochemical and immuno-based determinations of fiber type transitions and on electrophysiological examinations of individual motor units. Such examinations have led to a comprehensive understanding of the importance of motor axon sprouting and muscle fiber re-innervation in fiber type transitions and, in addition, pointed to new diagnostic methods. The

Abbreviations: BTS, N-benzyl-p-toluenesulfonamide; EDL, extensor digitorum longus; WT, Wild type; SOD1, superoxide dismutase 1; Hz_{50}, Excitation frequency necessary to elicit 50% of the increase in muscle force with tetanic stimulation; SR, sarcoplasmic reticulum; ALS, amyotrophic lateral sclerosis.

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relation between the fiber type transitions and disease-induced contractile dysfunction in patients is, however, unclear. One particular problem is the inability of most studies to specifically determine the contractile function of the various fiber types in diseased muscles. Another problem is that often it cannot be determined whether the muscle fibers identified using histochemical and immuno-based methods are actually innervated. Their relative contribution to the total force generation in the muscles cannot, therefore, readily be evaluated. This problem is further exacerbated by contradictory reports on fiber type transitions in the wobbler mouse. Thus, a shift from myosin heavy chain type II toward type I, indicative of a transition from fast-twitch toward slow-twitch fibers, was reported by Toursel et al. (2000) in both the soleus and sternocleidomastoid muscles whereas a IIA to IIB transition was reported by Agbulut et al. (2004) in the tibialis muscle. The later finding was supported by a proteonomic study in which the most prominent finding was an increase in glycolytic enzymes (Staunton et al., 2011). In addition to these contradictory findings, other studies have identified numerous effects of neurodegenerative diseases that may interfere with the contractility and fatigability of individual fibers independent of their myosin heavy chain composition. These include changes in the abundance of acetylcholine receptors and ClC-1 chloride channels (Sedehizade et al., 1997), fiber segments with defective mitochondria (Yi et al., 2011) and profound reductions in parvalbumin levels (Sedehizade et al., 1997; Staunton et al., 2011). Furthermore, investigations of the contractile profile of single chemically skinned muscle fibers from the wobbler mouse have shown an increased Ca^{2+} sensitivity of the contractile filaments (Toursel et al., 2000). Based on this, we here investigated the relation between contractile dysfunction and fiber type transitions between type I and type II heavy myosin in response to neurodegenerative diseases. Specifically, we examined for fiber type specific changes in contractile function in intact muscles from wobbler mice. The specific contribution of type I and type II myosin to force generation was evaluated using the specific inhibitor of force generation by the type II myosin protein, N-benzyl-p-toluenesulfonamide (BTS) (Cheung et al., 2002; Shaw et al., 2003). To further differentiate fiber type specific effects of neurodegenerative conditions on muscle function, the examination included both a muscle with a mixed fiber type composition (soleus) and a muscle containing almost exclusively fast-twitch fibers (EDL).

Materials and methods

Animal handling and muscle preparation

Experiments were conducted in accordance with institutional, national, and EU guidelines (Directive 2010/63/EU) for the care and use of laboratory animals, and were approved by the Faculty of Health Sciences, Aarhus University. Wobbler mice were bred on a C57BL/6J background and kept with a 12:12 h light:dark cycle with unrestricted access to food and water. Offspring were genotyped postnatal as previously described (Schmitt-John et al., 2005). Wobbler (wr/wr, $N = 24$) and wild type (WT) mice (+/+, +/wr, $N = 20$) of both sexes and 6 ± 1 and 6 ± 1 weeks of age, respectively, were anesthetized with isoflurane and decapitated. Hereafter, the mixed fiber type soleus muscle and the predominantly fast-twitch EDL muscle (Agbulut et al., 2003) from the hind legs were dissected out with 0.5 to 1 mm of the distal part of the motor nerve still attached. The dissection was done as previously described (Kohn and Clausen, 1971). In short, muscles were dissected with as much tendon as possible. In soleus also a small part of the tibia was kept attached to the origin of the muscle. All handling of the muscles took place *via* the tendons. After dissection, the muscle–nerve preparations were in some experiments blotted, weighed and immediately frozen for later determination of Ca^{2+} ATPase. In experiments where force was measured *in vitro*, the muscles were incubated in standard Krebs-Ringer bicarbonate solution containing (in mM); 122 NaCl, 25 $NaHCO_3$, 2.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$ and 5.0 D-glucose. Throughout experiments, the solution was maintained at 30 °C and equilibrated with 5% CO_2 in O_2 leading to a pH of 7.4.

Contractile force

Muscle–nerve preparations were mounted isometrically on force transducers *via* their tendons or, in soleus, by fixing the tibia segment in a metal fork on the experimental set-up. Contractions were elicited by use of field stimulation across the central part of the muscle through platinum wire electrodes. Before experiments, muscles were stretched to the optimal length for an active twitch force production. If not otherwise stated, isometric tetani were elicited throughout experiments every 10 min using 2 s 120 Hz trains of pulses in soleus muscle and every 20 min using 1 s 200 Hz trains of pulses in EDL muscle, which ensured a smooth tetanic force development in all muscles (see Fig. 5, later). In standard experiments, supramaximal pulses of 30 V cm^{-1} and 0.2 ms duration were used, which led to the initiation of action potentials directly on the muscle fibers (direct stimulation) (see Fig. 4 later and Overgaard and Nielsen, 2001). To exclusively initiate contractions *via* stimulation of the motor nerve (nerve stimulation), pulse duration was reduced to 0.02 ms. As shown in Fig. 4 (see later), such contractions were in soleus completely abolished by the presence of 1 μM tubocurarine. This demonstrates that no direct excitation of the muscle fibers took place (Overgaard and Nielsen, 2001). In EDL stimulated with 0.02 ms pulses, however, 1 μM tubocurarine could only depress force by $92 \pm 1\%$ ($N = 4$), indicating that a small amount of direct stimulation of muscle fibers still took place. Isometric force was measured with force displacement transducers (Grass FTO3, W. Warwick, RI) and was digitalized at 1 kHz using a PowerLab data acquisition system (ADInstruments, Australia).

In some experiments, muscles were pre-incubated for 80 min in the presence of 50 μM BTS, a specific inhibitor of the type II myosin subfragment 1 actin-stimulated ATPase activity (Cheung et al., 2002). The effect of BTS-binding to myosin is a reduction in the affinity of myosin for actin and the prevention of sliding of the contractile filaments. Thus, in rat EDL muscles that primarily contain fast twitch fibers, 50 μM BTS inhibits tetanic force by more than 93% (Macdonald et al., 2005). Likewise, control experiments in the present study showed that in EDL muscles of the mice, 80 min pre-incubation was sufficient to reduce force by more than 95%.

Measurements of SR Ca^{2+} ATPase

The concentration of Ca^{2+} ATPase in the SR (SERCA) was determined by measurement of the Ca^{2+}-dependent steady state phosphorylation from [γ-P-32]ATP in crude muscle homogenates using an assay developed by Everts et al. (1989) on the basis of a phosphorylation assay for Ca^{2+} ATPase (Martonosi et al., 1977). For the assay, which effectively forces SERCA into a state in its pumping cycle where it binds one ATP molecule, muscles were in groups of 2 to 3 (10–12 mg muscle tissue) homogenized at 0 °C in 2 ml buffer containing 5 mM Hepes and 300 mM sucrose (pH 7.4). From this, 4 aliquots of 400 μl were incubated in duplicates for 30 s at 0 °C in a reaction-mixture containing, in mM: imidazole 100, KCl 100, MgCl 5, EGTA 0.5, ATP 0.05, and [γ-P-32]ATP (10^5 cpm/ml, pH 7.4) in the absence or presence of $CaCl_2$, (0.55 mM). The reaction was quenched with trichloroacetic acid (final concentration 300 mM). After repeated washings of the pellet with trichloroacetic acid (60 mM) at 0 °C, it was dissolved in NaOH and neutralized with H_2SO_4. P-32 activity in the suspension was measured by liquid scintillation counting. In this assay, the total Ca^{2+}-ATPase concentration was quantified from the difference in P-32 incorporation in the presence and absence of $CaCl_2$ in each sample assuming a stoichiometry of 1 to 1 for the Ca^{2+} dependent incorporation of P-32 (Everts et al., 1989). Analysis of the P-32 incorporation in the absence of Ca^{2+} showed that the unspecific P-32 incorporation in the samples was similar in muscles from WT and wobbler mice (10.4 ± 1.2 *versus* 9.6 ± 0.4 nmol/g tissue, respectively, in soleus $P = 0.39$).

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