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Muscle weakness in respiratory and peripheral skeletal muscles in a mouse model for nebulin-based nemaline myopathy

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Abstract

Nemaline myopathy is among the most common non-dystrophic congenital myopathies, and is characterized by the presence of nemaline rods in skeletal muscles fibers, general muscle weakness, and hypotonia. Although respiratory failure is the main cause of death in nemaline myopathy, only little is known regarding the contractile strength of the diaphragm, the main muscle of inspiration. To investigate diaphragm contractility, in the present study we took advantage of a mouse model for nebulin-based nemaline myopathy that we recently developed. In this mouse model, exon 55 of *Neb* is deleted ($Neb^{\Delta Exon55}$), a mutation frequently found in patients. Diaphragm contractility was determined in permeabilized muscle fibers and was compared to the contractility of permeabilized fibers from three peripheral skeletal muscles: soleus, extensor digitorum longus, and gastrocnemius. The force generating capacity of diaphragm muscle fibers of $Neb^{\Delta Exon55}$ mice was reduced to 25% of wildtype levels, indicating severe contractile weakness. The contractile weakness of diaphragm fibers was more pronounced than that observed in soleus muscle, but not more pronounced than that observed in extensor digitorum longus and gastrocnemius muscles. The reduced muscle contractility was at least partly caused by changes in cross-bridge cycling kinetics which reduced the number of bound cross-bridges. The severe diaphragm weakness likely contributes to the development of respiratory failure in $Neb^{\Delta Exon55}$ mice and might explain their early, postnatal death.

Keywords: Diaphragm; Nebulin; Respiratory failure; Nemaline myopathy

1. Introduction

Nemaline myopathy (NM) is a non-dystrophic congenital myopathy described for the first time in the 1960s [1]. Eleven genes have been implicated in NM. Six of these genes code for components of the skeletal muscle thin filament: alpha actin 1 (*ACTA1*) [2], alpha- and beta-tropomyosin (*TPM3* and *TPM2*) [3,4], nebulin (*NEB*) [5], leiomodin-3 (*LMOD3*) [6] and troponin T (*TNNT1*) [7]. Four genes code for proteins which play a role in the regulation and the stability of the thin filament: cofilin 2 (*CFL2*) [8] regulates actin filament dynamics; kelch family member 40 (*KLHL40*) is implicated in myogenesis [9] and in the stabilization of thin filament proteins [9,10] and kelch family member 41 (*KLHL41*) modulates ubiquitination of thin filament proteins [10,11]. The role of kelch repeat and BTB (POZ) domain containing 13 (*KBTBD13*) is unclear, but might involve a role in the ubiquitin-proteasome pathway [12]. The most recent implicated gene, *MYO18B*, codes for an unconventional myosin heavy chain expressed in cardiac and skeletal muscles. Its function is incompletely understood [13].

NM affects skeletal muscles, including the muscles of respiration. Consequently, patients with NM have reduced spirometric values such as forced vital capacity, peak expiratory flow, forced expiratory volume in 1 second [14–16], and they may suffer from the sensation of dyspnea [15–19]. Respiratory failure is the main cause of death in NM [20], and occurs even in ambulant patients who otherwise appear to be only mildly affected; respiratory failure may even be the presenting feature. This suggests that weakness of respiratory muscles may be much greater than that of peripheral skeletal muscles. The pathophysiology of respiratory muscle weakness is not completely understood, but might involve a dysfunctional diaphragm. The diaphragm is the main muscle of inspiration, and weakness of the diaphragm impairs respiration and causes dyspnea [21]. Thus, insights in diaphragm contractility in NM are important for developing treatment strategies.

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The little information available regarding diaphragm contractility in NM is a consequence of technical and ethical limitations in assessing diaphragm contractility *in vivo* and in obtaining diaphragm muscle biopsies from patients. Therefore, in the present study we took advantage of a mouse model for nebulin-based NM that we recently developed [22]. Mutations in *NEB* account for more than 50% of the gene mutations reported in NM patients [23], and in this mouse model exon 55 of *Neb* is deleted, a mutation frequently found in patients [22]. To assess diaphragm contractility, we isolated permeabilized muscle fibers from the diaphragm and activated the fibers with calcium. Muscle fiber contractility was determined and compared to the contractility of muscle fibers of three peripheral skeletal muscles: soleus, extensor digitorum longus and gastrocnemius. Our results indicate that diaphragm muscle fibers of $Neb^{\Delta Exon55}$ mice exhibit severe contractile weakness, a weakness that was more pronounced than that observed in soleus muscle, but not more pronounced than that in extensor digitorum longus (EDL) and gastrocnemius muscles.

2. Materials and methods

2.1. Generation of $Neb^{\Delta Exon55}$ mice

The procedure for the generation of the $Neb^{\Delta Exon55}$ mice has been previously described [22].

2.2. Muscle preparations

For contractility experiments we used diaphragm, soleus (slow-twitch), EDL (fast-twitch) and gastrocnemius (fast-twitch) muscles from wild-type (Wt) and knock-out littermate $Neb^{\Delta Exon55}$ mice. The number of mice used is summarized in Table 1. Muscles were harvested at day 6, as $Neb^{\Delta Exon55}$ mice die one week after birth [22].

2.3. Permeabilized muscle fiber contractility

The procedures for permeabilized muscle fiber contractility experiments were as described previously [24], with small modifications [22]. Small fiber bundles (diameter ~70 μm) were dissected from the triton-X permeabilized muscles, and attached to aluminum foil clips. The clips were attached to a force transducer (model 403A, Aurora Scientific, Ontario, Canada) and length controller (model 315-CI, Aurora Scientific, Ontario, Canada). We used a setup (model 802D, Aurora Scientific) that was mounted on top of an inverted microscope (Zeiss Axio Observer A1, Zeiss, Thornwood, NY, USA). Sarcomere length was determined using a fast Fourier transformation on a region of interest on the real-time camera image using ASI 900B software (Aurora Scientific Inc., Ontario, Canada).

Table 1
Number of animals used, for each muscle-type, in Wt and $Neb^{\Delta Exon55}$ mice.

	Diaphragm	Soleus	EDL	Gastrocnemius
Wt	6	6	7	6
$Neb^{\Delta Exon55}$	9	6	9	5

Experiments were performed at sarcomere length 2.3 μm. The width and depth of the muscle bundle were measured with a 40× objective. The cross-sectional area (CSA) was calculated from the average of three consecutive measurements made along the length of the muscle bundle. The temperature was kept constant at 20 °C using a TEC controller (ASI 825A, Aurora Scientific Inc. Ontario, Canada).

Various bathing solutions were used during the experimental protocols: a relaxing solution (pCa 9.0), a pre-activating solution with low EGTA concentration and several incremental Ca^{2+} solutions (pCa 7.0–4.5). Composition of these solutions has been described previously [24].

The preparations were activated at pCa 4.5 to obtain maximal Ca^{2+}-activated force. Maximal active tension was obtained by divided the force generated at pCa 4.5 by CSA.

2.3.1. Cross-bridge cycling kinetics (K_{tr})

Muscle bundles were first isometrically activated at pCa 4.5, and when a steady tension was reached, cross-bridges were disengaged by performing a quick release of 30% of the initial length, which reduced tension to zero. This was followed by unloaded shortening lasting 30 ms. The remaining bound cross-bridges were mechanically detached by rapidly (1 ms) re-stretching the muscle fiber bundles to its original length, after which tension redevelops. The K_{tr} was determined by fitting a double exponential through the force redevelopment curve (note that only the fast rate constant is reported as this is considered to reflect cross-bridge cycling kinetics).

2.3.2. Active stiffness

Following the K_{tr} protocol, active stiffness was determined by length variations of 0.3, 0.6 and 0.9% on the fiber, resulting in a force response. Stiffness is represented as the slope of the linear regression of the relationship between tension and length. A typical force trace, including the force response during the K_{tr} and active stiffness protocol, is shown in Fig. 1.

2.3.3. Calcium sensitivity of force generation

Preparations were in relaxing solution and exposed to pre-activating solution (pCa 9.0). Then, they were activated with solutions with a pCa ranging from 7.0 to 4.5 (Fig. 2A). The obtained force–pCa relation was fitted with a Hill equation, providing pCa_{50} (pCa giving 50% of maximal active tension) and the Hill coefficient, n_H, an index of myofilament cooperativity (Fig. 2B).

2.4. Fiber typing

As contractility is dependent on the myosin heavy chain (MyHC) isoforms expressed in the muscle fibers, a specialized sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the (MyHC) isoform composition of the muscle fiber preparations that we used in our contractility experiments. Muscles fibers were de-natured by boiling at 80 °C for 2 min in SDS sample buffer. The stacking gel contained a 4% acrylamide concentration (pH 6.7), and the separating gel contained 7% acryl-amide (pH 8.7) with 30% glycerol (v/v). The gels were run for 24 h at 15 °C and a constant voltage of 275 V. Finally, the gels were silver-stained,