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Altered myofilament function depresses force generation in patients with nebulin-based nemaline myopathy (NEM2)

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ABSTRACT

Nemaline myopathy (NM), the most common non-dystrophic congenital myopathy, is clinically characterized by muscle weakness. However, the mechanisms underlying this weakness are poorly understood. Here, we studied the contractile phenotype of skeletal muscle from NM patients with nebulin mutations (NEM2). SDS–PAGE and Western blotting studies revealed markedly reduced nebulin protein levels in muscle from NM patients, whereas levels of other thin filament-based proteins were not significantly altered. Muscle mechanics studies indicated significantly reduced calcium sensitivity of force generation in NM muscle fibers compared to control fibers. In addition, we found slower rate constant of force redevelopment, as well as increased tension cost, in NM compared to control fibers, indicating that in NM muscle the rate of cross-bridge attachment is reduced, whereas the rate of cross-bridge detachment is increased. The resulting reduced fraction of force generating cross-bridges is expected to greatly impair the force generating capacity of muscle from NM patients. Thus, the present study provides important novel insights into the pathogenesis of muscle weakness in nebulin-based NM.

1. Introduction

Nemaline myopathy (NM) is the most common non-dystrophic congenital myopathy, with an estimated incidence of ~1 per 50,000 live births (Wallgren-Pettersson, 1990). NM is characterized at the muscle's ultrastructural level by the presence of rod-shaped structures (nemaline rods) in affected muscle fibers (Morris et al., 1990). Clinically, the most prominent feature of NM is generalized muscle weakness that greatly affects the daily-life activities, and the quality of life of these patients (North et al., 1997).

NM is a genetically heterogeneous disorder of the skeletal muscle thin filament caused by mutations in any one of at least six different genes, all encoding thin filament proteins of the sarcomere: *ACTA1* (actin), *TPM3* and *TPM2* (α- and β-tropomyosin), *TNNT1* (troponinT), *CFL2* (cofilin-2), and *NEB* (nebulin), for a review see Sanoudou and Beggs (2001). Despite detailed knowledge of the underlying genetic basis for NM in many patients, the mechanisms underlying muscle weakness in NM patients are poorly understood.

Mutations in the nebulin gene are the most common cause of NM (patients referred to as NEM2), accounting for ~50% of all NM cases (Pelin et al., 1999). Nebulin is a giant protein (MW ~800 kDa) expressed at high levels in skeletal muscle. A single nebulin molecule spans the thin filament with its C-terminus anchored at the Z-disk and its N-terminal region directed towards the thin filament pointed end (Wang and Wright, 1988). Previous studies (Witt et al., 2006; Bang et al., 2006) revealed that nebulin-deficient murine muscle fibers have thin filaments that vary in length (Witt et al., 2006) and that are on average shorter than in wildtype muscle (Witt et al., 2006; Bang et al., 2006), supporting the notion that nebulin is important in establishing thin filament length. Thin filament length is an important aspect of muscle function as the extent of overlap between thick and thin filaments determines the sarcomere's force generating capacity: short thin filaments reduce overlap and impair force generation. In accordance with a role for nebulin in establishing thin filament length, we have shown that similar to the nebulin knockout (KO) mouse model, human NM patients with nebulin-deficiency also have shorter and non-uniform thin filament lengths, which can partly account for the observed muscle weakness in nebulin-based NM (Ottenheijm et al., 2009).

Recent studies on nebulin knockout mouse models suggest that nebulin's role in muscle function extends beyond a purely structural one, and involves a role in the regulation of cross-bridge cycling kinetics and thin filament activation. It was found that nebulin increases the fraction of force generating cross-bridges that is available in the overlap zone (Chandra et al., 2009; Bang

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et al., 2009) and enhances the force response to submaximal calcium concentrations (Chandra et al., 2009). Thus, in the nebulin KO mouse model reduced active tension and calcium sensitivity of force generation has been reported. Here we tested whether similar characteristics are present in muscle from NM patients with nebulin gene mutations. We found a reduced rate of force development as well as increased tension cost in nebulin-deficient muscle fibers from NM patients. Moreover, force generation in response to submaximal calcium concentrations was significantly decreased. These findings suggest altered cross-bridge cycling kinetics and thin filament activation in nebulin-deficient fibers from NM patients, and provide a novel mechanism for muscle weakness in nebulin-based NM.

2. Methods

2.1. Muscle biopsies from nemaline myopathy patients

Skeletal muscle specimens, remaining from diagnostic procedures or obtained during clinically indicated surgical procedures, were collected from four nemaline myopathy patients following informed consent supervised by the Children's Hospital Boston institutional review board, and from four unaffected control subjects, and stored frozen and unfixed at −80 °C until use (Table 1). All four NM patients had mutations in the nebulin gene, including three patients who were homozygous for the previously described deletion of exon 55 (Anderson et al., 2004) and one patient with a heterozygous single base deletion (p.Ser1908AlafsX8) resulting in a premature stop codon and an unidentified second mutation. Two of the three patients with the exon 55 deletion (biopsies T11 and T12) were included in the original report of this mutation by Anderson et al. (2004) and all three patients with this deletion (biopsies T11, T12, and T124) were previously described by Lehtokari et al. (2009).

2.2. Gel electrophoresis and Western blotting

For nebulin expression, muscle samples were homogenized and analyzed on 2.6–7% SDS–acrylamide gels (Ottenheijm et al., 2009). To prevent protein degradation, all buffers contained protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), 0.5 mM; leupeptin, 0.04 mM; E64, 0.01 mM). Gels were scanned and analyzed with One-D scan EX (Scanalytics Inc., Rockville, MD, USA) software. The integrated optical density of nebulin, myosin heavy chain (MHC), and actin was determined. For Western blot analysis of the thin filament-based regulatory proteins, 3.75–12% acrylamide gels were used. For troponin expression patterns, Western blotting was performed using fast-skeletal and slow-skeletal specific antibodies (ss-TnI: cs-20645; fs-TnI: sc-8120; ssTnT: sc-28269; fs-TnT sc-8123), and a troponinC antibody (sc-8117) that recognizes both slow- and fast-skeletal troponinC (Santa Cruz Biotechnology Inc, USA). For tropomyosin expression, Western blotting was performed using an antibody directed against both α- and β-tropomyosin (CH1, Hybridoma Bank, University of Iowa). Secondary antibodies conjugated with fluorescent dyes with infrared excitation spectra were used for detection. One or two-color infrared Western blots were scanned (Odyssey Infrared Imaging System, Li-Cor Biosciences, NE. USA) and the images analyzed with One-D scan EX. For myosin heavy chain isoform composition, skeletal muscles were denatured by boiling for 2 min. The stacking gel contained a 4% acrylamide concentration (pH 6.7), and the separating gel contained 7% acrylamide (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, scanned, and analyzed with One-D scan EX software.

2.3. Immunofluorescence confocal scanning laser microscopy

Small strips were dissected from the biopsies and skinned overnight at ∼4 °C in relaxing solution (in mM; 20 BES, 10 EGTA, 6.56 $MgCl_2$, 5.88 NaATP, 1 DTT, 46.35 K-propionate, 15 creatine phosphate, pH 7.0 at 20 °C) containing 1% (v/v) Triton X-100. To prevent protein degradation, the solutions contained protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), 0.5 mM; Leupeptin, 0.04 mM; E64, 0.01 mM). Immuno-labeling and confocal scanning laser microscopy was performed essentially as described previously (Ottenheijm et al., 2009), using Alexa Fluor 488 conjugated phalloidin (A12379, Invitrogen). Images were produced using a Bio-Rad MRC 1024 confocal laser scanning microscope using the LaserSHARP 2000 software package (Hercules, CA, USA). From the acquired images, thin filament lengths were determined using ImageJ software (National Institutes of Health).

2.4. Muscle mechanics

Small strips dissected from muscle biopsies were skinned overnight (as described above). The skinning procedure renders the membranous structures in the muscle fibers permeable, which enables activation of the myofilaments with exogenous Ca^{2+}. Preparations were washed thoroughly with relaxing solution and stored in 50% glycerol/relaxing solution at −20 °C for up to ∼8 weeks. Small muscle bundles (diameter ∼0.07 mm) were dissected from the skinned strips, and were mounted between a displacement generator and a force transducer element (AE 801, SensoNor, Norway) using aluminum T-clips. Sarcomere length (SL) was set using a He–Ne laser diffraction system. Mechanical experiments on contracting muscle were carried out at an SL of ∼2.5 μm for control muscle, and at just over slack length for NM muscle: a length selected for the following reason. By constructing force-SL relations we (Ottenheijm et al., 2009) previously showed that at an SL of 2.5 μm human muscle fibers from controls produced maximal force, whereas nebulin-deficient muscle fibers from NM patients produced maximal force just over slack length, due to their shorter thin filaments (Ottenheijm et al., 2009). Thus, by performing our mechanical studies on NM muscle set just over slack length, we aimed to minimize force differences due to shorter thin filament lengths. Fiber width and diameter were measured at three points along the fiber and the cross-sectional area was determined assuming an elliptical cross-section. Three different bathing solutions were used during the experimental protocols: a relaxing solution, a pre-activating solution with low EGTA concentration, and an activating solution. The composition of these solutions was as described previously (Stienen et al., 1996).

Force-pCa relations. To determine the force-pCa relation (pCa = −log of molar free Ca^{2+} concentration), skinned muscle fiber bundles were sequentially bathed in solutions with pCa values ranging from 4.5 to 9.0 and the steady-state force was measured. Measured force values were normalized to the maximal force obtained at pCa 4.5. The obtained force-pCa data were fit to the Hill equation, providing pCa_{50} (pCa giving 50% maximal active tension) and the Hill coefficient, n_H, an index of myofilament cooperativity.

K_{tr} measurements. To measure the rate of tension redevelopment (K_{tr}), we used the large slack/release approach (Brenner and Eisenberg, 1986), to disengage force generating cross-bridges from the thin filaments, which were isometrically activated. Fast activation of the fiber was achieved by transferring the skinned muscle fibers from the pre-activation solution containing a low concentration of EGTA (pCa 9.0) to a pCa 4.5 activating solution. Once the steady-state was reached, a slack equivalent to 10% of the muscle length was rapidly induced at one end of the muscle using the motor. This was followed immediately by an unloaded shortening lasting 30 ms. The remaining bound cross-bridges were