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Sexually dimorphic myofilament function in a mouse model of nemaline myopathy

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ABSTRACT

Nemaline myopathy, the most common congenital myopathy, is characterized by mutations in genes encoding myofilament proteins such as skeletal α-actin. These mutations are thought to ultimately lead to skeletal muscle weakness. Interestingly, some of the mutations appear to be more potent in males than in females. The underlying mechanisms remain obscure but may be related to sex-specific differences in the myofilament function of both limb and respiratory muscles. To verify this, in the present study, we used skeletal muscles (tibialis anterior and diaphragm) from a transgenic mouse model harbouring the His40Tyr amino acid substitution in skeletal α-actin. In this animal model, 60% of males die by 13 weeks of age (the underlying causes of death are obscure but probably due to respiratory insufficiency) whereas females have a normal lifespan. By recording and analysing the mechanics of membrane-permeabilized myofibres, we only observed sex-related differences in the tibialis anterior muscles. Indeed, the concomitant deficits in maximal steady-state isometric force and stiffness of myofibres were less exacerbated in transgenic females than in males, potentially explaining the lower potency in limb muscles. However, the absence of sex-difference in the diaphragm muscles was rather unexpected and suggests that myofilament dysfunction does not solely underlie the sexually dimorphic phenotypes.

Introduction

Nemaline myopathy is the most common congenital myopathy [1,2]. The clinical spectrum is very broad and ranges from adult-onset cases with slow progression to severe cases with antenatal or neonatal onset associated with early death [1,2]. It is caused by mutations in various genes such as ACTA1, which encodes skeletal α-actin [3] and is most of the time associated with weakness affecting limb and respiratory muscles [4,5]. Interestingly, some sex-related differences may exist. For instance, for the His40Tyr skeletal α-actin mutation, the most severely affected individual was a male who died at 2 months of age while the female patient is still alive at 51 years of age [6]. Similarly, in the transgenic mouse model expressing the same mutation, 60% of the males die by week 13 whereas females have a normal lifespan [7]. Even though the underlying causes of death are totally unknown, Nguyen et al. [7], proposes respiratory failure as a potential contributing event. Altogether, these observations suggest that this specific mutation is more potent in males than in females. The pathophysiological mechanisms, which underlie this sexual dimorphism, remain to be investigated.

In male mice carrying the His40Tyr skeletal α-actin defect, we have reported a myofilament dysfunction inducing a myofibre weakness in both limb and respiratory muscles [8]. Indeed, in limb muscles, the mutants are "poison-protein". They prevent proper myosin cross-bridge binding and reduce the number of actomyosin interactions in the strong binding state, limiting the maximal steady-state isometric force-generating capacity of myofibres [8]. In the diaphragm, in addition, the presence of a large amount of non-contractile areas further decreases the number of functional myofilaments [8]. In the present study, we initially hypothesized that in females expressing the same mutation, the above molecular and cellular mechanisms also occur but are less exacerbated than in males, thus, partly explaining the sexually dimorphic phenotypes. To verify this, we used skeletal muscles (tibialis anterior

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and diaphragm) from females harbouring the His40Tyr skeletal α-actin mutation. We then recorded and analysed the mechanics of single membrane-permeabilized myofibres.

Material and methods

Animals

Five 2- to 3-month old female wild-type mice as well as five age- and gender-matched TG(ACTA1)^{H40Y} mice expressing the His40Tyr mutation in skeletal α-actin, were included in the analyses. The results were compared with the data previously obtained from males [8]. For a complete description of the mice, please see [7]. Mice were killed by cervical dislocation under deep isoflurane sedation and skeletal muscles (tibialis anterior and diaphragm) were dissected and separated into three portions.

Muscle preparation

One portion was slightly overstretched longitudinally and fixed in 2.5% glutaraldehyde. Another was directly frozen in liquid nitrogen-chilled propane and stored at −80 °C. The final portion was placed in relaxing solution at 4 °C, and bundles of ~50 fibres were dissected free and then tied with surgical silk to glass capillary tubes at slightly stretched lengths. The muscle bundles were then treated with skinning solution (relaxing solution containing glycerol; 50:50 v/v) for 24 h at 4 °C, after which they were transferred to −20 °C. The muscle bundles were treated with sucrose, a cryoprotectant, within 1–2 weeks for long-term storage [9]. After the sucrose treatment, muscle bundles were detached from the capillary tubes and snap frozen in liquid nitrogen-chilled propane and stored at −80 °C. All procedures involving animal care, welfare and handling were performed according to institutional guidelines and were reviewed and approved by the Uppsala Local Ethical Committee on Animal Research.

Mechanical recordings

On the day of an experiment, a muscle bundle was de-sucrosed and fibres isolated. Each fibre segment 1–2 mm long was then left exposed to the experimental solution between connectors leading to a force transducer (model 400A, Aurora Scientific) and a lever arm system (model 308B, Aurora Scientific) [10]. While the fibre was in relaxing solution, the sarcomere length was set to 2.50 μm by adjusting the overall segment length [11]. Fibre cross-sectional area (CSA)[1] was calculated from the diameter and depth, assuming an elliptical circumference, and was corrected for the 20% swelling that is known to occur during skinning [10]. Mechanical experiments were performed at 15 °C and included force measurements (normalized to CSA) after various length steps at saturating [Ca^{2+}] (pCa 4.50).

Solutions

For the mechanical recordings, relaxing and activating solutions contained (in mM) 4 Mg-ATP, 1 free Mg^{2+}, 20 imidazole, 7 EGTA, 14.5 creatine phosphate, and KCl to adjust the ionic strength to 180 mM and pH to 7.0. The pre-activating solution was identical to the relaxing solution except that the EGTA concentration was reduced to 0.5 mM. The concentrations of free Ca^{2+} were 10^{-9} M (relaxing and pre-activating solutions) and $10^{-4.50}$ M (activating solution), expressed as pCas (i.e., −log10 [Ca^{2+}]). The rigor activating solution was similar as the regular activating solution except that MgATP and creatine phosphate were absent.

Protein expression and quantification

After the mechanical recordings, each myofibre was placed in urea buffer (120 g urea, 38 g thiourea, 70 ml H_2O, 25 g mixed bed resin, 2.89 g dithiothreitol, 1.51 g Trizma base, 7.5 g SDS, 0.004 % bromophenol blue) in a plastic microcentrifuge tube and stored at −80 °C. Samples were then diluted and MyHC isoform composition of fibres was determined by 6% SDS–PAGE. The acrylamide concentration was 4% (wt/vol) in the stacking gel and 6% in the running gel, and the gel matrix included 30% glycerol. Sample loads were kept small (equivalent to approximately 0.05 mm of fibre segment) to improve the resolution of the myosin heavy chain bands (types I, IIa, IIx and IIb). Electrophoresis was performed at 120 V for 24 h with a Tris–glycine electrode buffer (pH 8.3) at 15 °C (SE 600 vertical slab gel unit, Hoefer Scientific Instruments). The gels were silver-stained and subsequently scanned in a soft laser densitometer (Molecular Dynamics) with a high spatial resolution (50 μm pixel spacing) and 4096 optical density levels.

The relative contents of MyHC, actin and total protein were measured from frozen muscle cross-sections, i.e., the samples were cut at their greatest girth perpendicular to the longitudinal axis of muscle fibres into 10-μm-thick cross-sections with a cryotome (2800 Frigocut E, Reichert-Jung) at −20 °C. The 10-μm cross-sections of each muscle were dissolved into 100 μl of urea buffer and stored at −80 °C. On the day of experiment, samples were diluted, centrifuged and heated (90 °C for 2 min). Total protein quantification of the samples was performed using the NanoOrange® Protein Quantification Kit (Invitrogen), the fluorescence of the samples was measured using a Plate Chameleon™ Multilabel Platereader (Hidex Oy) and the software MikroWin2000, version 4.33 (Microtek Laborsysteme GmbH). The fluorescence of the samples was related to a standard curve made using bovine serum albumin (Invitrogen) at concentrations ranging from 10 μg ml^{-1} to 0.1 μg ml^{-1} [12]. MyHC quantification was determined by 12% SDS–PAGE. Volumes of 5 μl of the samples were loaded along with 5 μl of the standard dilutions. The standard was prepared by pooling cross-sections from wild-type mice. Linear myosin curves were observed within the 5–200 μg ml^{-1} range. MyHC content was then normalized to total protein content. In addition, the MyHC to actin ratio was calculated from 12% SDS–PAGE gels. The gels were stained with Coomassie blue and subsequently scanned to determine the MyHC to actin ratio [11,13].

Ultrastructural analysis

Longitudinal sections were processed for electron microscopy according to standard procedures [14]. In brief, samples were slightly stretched and fixed in 2.5% (vol/vol) glutaraldehyde for 48 h and 1% (wt/vol) osmiumtetroxide for 1 h, dehydrated with ethanol, and embedded into plastic. Sections were then collected on 300-mesh Formvar-coated nickel grids. These sections were examined in a Jeol-1200EX electron microscope. Micrographs were taken from a large number of randomly selected fields at constant calibrated magnifications.

Statistical analyses

Data are presented as means ± standard error of the means (SEMs). SigmaStat (Jandel Scientific) and Microsoft Excel were used to generate descriptive statistics. Differences between the groups were determined by two-way ANOVAs with specific references to genotype, sex and genotype × sex interactions (p-value set at 0.05).

[1] *Abbreviations used:* CSA, cross-sectional area; SEMs, standard error of the means.