The heart in Duchenne muscular dystrophy: early detection of contractile performance alteration

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Abstract

Progressive cardiomyopathy is a major cause of death in Duchenne muscular dystrophy (DMD) patients. Coupling between Ca\(^{2+}\) handling and contractile properties in dystrophic hearts is poorly understood. It is also not clear whether developing cardiac failure is dominated by alterations in Ca\(^{2+}\) pathways or more related to the contractile apparatus. We simultaneously recorded force and Ca\(^{2+}\) transients in field-stimulated papillary muscles from young (10–14 weeks) wild-type (wt) and dystrophic mdx mice. Force amplitudes were fivefold reduced in mdx muscles despite only 30 % reduction in fura-2 ratio amplitudes. This indicated mechanisms other than systolic Ca\(^{2+}\) to additionally account for force decrements in mdx muscles. pCa-force relations revealed decreased mdx myofibrillar Ca\(^{2+}\) sensitivity. 'In vitro' motility assays, studied in mdx hearts here for the first time, showed significantly slower sliding velocities. mdx MLC/MHC isoforms were not grossly altered. Dystrophic hearts showed echocardiography signs of early ventricular wall hypertrophy with a significantly enlarged end-diastolic diameter 'in vivo'. However, fractional shortening was still comparable to wt mice. Changes in the contractile apparatus satisfactorily explained force drop in mdx hearts. We give first evidence of early hypertrophy in mdx mice and possible mechanisms for already functional impairment of cardiac muscle in DMD.

Keywords: papillary muscle - muscular dystrophy - calcium - force transients - motility assay

Introduction

Duchenne muscular dystrophy is a progressive, wasting muscle disease that originates from numerous point mutations in the X-chromosomal DMD gene. Its product, dystrophin, is predominantly expressed in skeletal, cardiac and smooth muscle. Lack of dystrophin renders muscle fibres more susceptible to membrane damage during mechanical stress, especially for stretched contractions. Although skeletal muscle wasting clinically predominates, heart failure from progressive dilative cardiomyopathy (DCM) accounts for at least 30 % of deaths. However, many individuals show subclinical cardiac involvement presumably because of reduced cardiac workload in the mobility compromised patients [1]. Also, a direct cardiac correlate with eccentric contractions found in skeletal muscle is usually not present during pump cycles. A distinct effect of dystrophin deficiency can be expected in the heart, as its distribution is different from the one in skeletal muscle, i.e. a large proportion at the t-system level. It has long been thought that clinical manifestations of dystrophic cardiomyopathy were primarily a matter in aged DMD patients [1]. Accordingly, studies in young dystrophic mdx mice (2–3 mo) showed low levels of cardiac fibrosis, but a several-fold increase at older age (9–12 mo, [2]; 17 mo, [3]). Although no signs of heart failure were observed in young mdx mice [2], the disruption of the cardiac and skeletal muscle patterning process priming the dystrophic phenotype already begins early in embryonic development [4]. In the post-natal phase, mdx mice undergo a sudden onset of massive skeletal muscle

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degeneration (necrotic phase) at ~3–5 weeks [5] followed by a stable phase of degeneration/regeneration cycles and a terminal phase of exhausted regenerative capacity from ~15 mo [6]. Such systematic studies are not available for cardiac muscle. Echocardiography studies have detected beginning signs of ventricular hypertrophy in mdx hearts from 29 weeks which were not seen in 8 weeks old mice [3]. At 42 weeks, dilated cardiomyopathy with decreased fractional shortening was already apparent [3].

A couple of recent studies point towards altered cardiac Ca\(^{2+}\) handling in adult mdx mice (5–9 months [‘mo’], [7]; 9–12 mo, [2]), and in mdx mice in the early post-necrotic phase (2–3 mo, [2]). Surprisingly, only few studies addressed contractile properties of cardiac muscle from mdx mice, where specific force in young (8–14 weeks) animals was compromised [8, 9]. Likewise, there are even less studies that have addressed the frequency response of Ca\(^{2+}\) dynamics or force responses in stimulated dystrophic cardiac muscle [8]. This is important to know, as diastole progressively shortens with heart rate and may compromise intracellular Ca\(^{2+}\) removal.

To our knowledge, there is no study available that explains compromised cardiac contractile performance in direct correlation with myoplasmic Ca\(^{2+}\) fluctuations or provides further details on the molecular level of the contractile apparatus. Here, we simultaneously recorded force and Ca\(^{2+}\) transients in paced papillary muscle from 10 to 14 weeks old mdx and wt mice to test the hypothesis that impaired Ca\(^{2+}\) amplitudes were the main determinant of compromised force in mdx preparations. Reduced force output of mdx preparations was only partially explained by reduced Ca\(^{2+}\) transients, but rather by reduced myofibrillar Ca\(^{2+}\) sensitivity. Moreover, we detected slower sliding velocities in cardiac ‘in vitro’ motility assays. Although morphological signs of hypertrophy can be anticipated at this early stage, ‘in vivo’ echocardiography still showed a compensated situation with balanced fractional shortening.

**Materials and methods**

**Papillary muscle preparations**

Papillary heart muscle from young 10–14 weeks old mdx and wt mice were used. All experiments complied with guidelines laid down by the Local Animal Care Facilities (Universities Heidelberg & Essen; TG-27/09; IFORES 107-55604). Details on preparation procedures are given in the supporting information (SI Methods).

**Force and Ca\(^{2+}\) fluorescence recordings in papillary muscle preparations during external field stimulation**

After fura-2 loading, muscle strips were horizontally mounted in a temperature-controlled (37°C) recording chamber with a built-in force transducer (OPT1L; Scientific Instruments, Heidelberg, Germany). Muscle strips were field stimulated by trains of pulses at frequencies from 1 to 4 Hz. Prior to the first stimulation, papillary muscles were allowed to equilibrate for ~20 min. Initial stimulations were performed at 1 Hz with a pulse duration of 6–10 ms at 3 V. At the time of stimulation, resting metabolism was expected to be already at a very low steady-state level [10]. The muscle was gradually stretched until force transient amplitudes reached a maximum. All subsequent recordings were made at this length.

Repetitive trains of stimuli starting at 1 Hz and increasing frequency at 0.5 Hz intervals up to 4 Hz were applied and force and fura-2 twitch transients recorded for 10 sec. at each stimulation frequency. In between each train, a 5 min. rest period was allowed to ensure sufficient oxygenation to the muscle core [11]. Maximum twitch force amplitudes, time-to-peak (TTP) and the time constant (τ) of exponential force relaxation were evaluated (10–90% rise time or exponential fit to the decay of the traces, respectively).

Fura-2 Ca\(^{2+}\) transients were simultaneously recorded via an epifluorescence system (PH1A; Scientific Instruments) by dual wavelength excitation. The collected net fluorescence covered a region of interest ~0.3 mm\(^2\) in the middle of the muscle. Fura-2 ratio amplitudes, systolic and diastolic levels, activation kinetics (time-to-peak) and inactivation kinetics (τ) were analysed for each frequency. As we were primarily interested in the frequency dependence of Ca\(^{2+}\) dynamics in the same papillary muscle, intracellular [Ca\(^{2+}\)]\(_{i}\) is presented by the fura-2 ratios rather than converting to absolute Ca\(^{2+}\) concentrations which normally requires careful ‘in situ’ calibration [12]. As buffer properties are not supposed to change within the same preparation for different stimulation regimes, it is practical to represent Ca\(^{2+}\) levels by fura-2 ratios, for which we did not attempt to perform further calibration. Even if there was some difference in fura-2 Kd between genotypes [12], where appropriate, we compare frequency dependence of fura-2 signals normalized to the values obtained at 1 Hz to eliminate such a difference.

**Myofibrillar Ca\(^{2+}\) sensitivity in skinned papillary muscle preparations**

pCa-force experiments were carried out in skinned papillary muscles bathed in solutions mimicking the intracellular environment of varying pCa and recordings of steady-state force. Further details are given in the supporting information (SI Methods).

**In vitro motility assay of ventricular muscle myosin extracts**

Ventricles were dissected from the heart of mdx or wt mice and ~150 mg pieces transferred to a Guba-Straub phosphate extraction buffer at pH 6.5, immediately frozen in liquid N\(_{2}\) and stored until experimentation. The pyrophosphatase-based myosin extraction followed a multi-step high-speed centrifugation procedure with solution composition described earlier [13]. Briefly, small pieces of heart were stirred for 20 min. in ice-cold protein extraction buffer (mM: NaCl 300, NaH\(_{2}\)PO\(_{4}\) 100, Na\(_{2}\)HPO\(_{4}\) 50, MgCl\(_{2}\) 1, Na\(_{2}\)HPO\(_{4}\) 10, EDTA 10, DTT 10, pH 6.5) containing 0.1% NaN\(_{3}\) and 10 μg/ml and then centrifuged at 30,000 g for 10 min. The supernatant was diluted 1:12 in low salt buffer LSB (EDTA 1, Tris 10, pH 7.0) and stirred for another 30 min. followed by a second spin down at 30,000 g for 10 min. Pellets were dissolved in ATP-LSB (EGTA 1, MgCl\(_{2}\) 2, ATP 1, DTT 1, Tris 10, pH 7.0), incubated on ice for 30 min. and spun down for another 10 min. The extracted myosin was pipetted onto a flow cell coated with nitrocellulose. The motility assay
procedure followed the one given in Svensson et al. [13]. After extraction and removal of rigor bridges, 100 ng/ml rhodamin-phalloidin labelled actin was flushed into the chamber and washed out after a 2 min. incubation period. Cross-bridge cycling was initiated by adding 3 mM ATP. The filament sliding was visualized on an inverted epifluorescence microscope. Recordings and automated analysis were performed as before [14]. Several independent recordings were made from each flow cell (total of XYT movies sequences: wt 24; mdx 10) from two mdx and wt animals. With several hundred filaments being tracked during each movie sequence, our analysis gives a robust representation of sliding filaments from more than 5000 single filaments. From each movie sequence, the velocity distribution was constructed and fitted by a Gaussian function from which the median velocity values were further analysed for statistical differences. Velocity bins <1 μm/sec. were not included in the fitting procedure as within this range also a number of myosin heads in the rigor configuration may be contained.

**SDS PAGE and myosin light and heavy chain distributions**

Gel electrophoresis on homogenates of either dissected heart ventricles or atria from wt or mdx mice was performed as described for skeletal muscle [15] with the exception that total protein content added to stacking gels was ~50 μg for light chain separation (12% polyacrylamide) and ~10 μg for heavy chains (8%). Samples from wt skeletal edl and soleus muscle were also run on the same gels.

**In vivo transthoracic echocardiography in conscious wt and mdx mice**

Transthoracic echocardiography was performed in young conscious wt and mdx mice (10–16 weeks) as described in Goehringer et al. [16]. Briefly, parasternal short-axis M-mode images were obtained using a 15 MHz linear transducer (Sonos 5500, Philips Medical Syst., Boeblingen, Germany). At least three consecutive beats were used to measure end-diastolic internal diameter (LVEDD), end-systolic internal diameter (LVESD), diastolic posterior wall thickness and diastolic septum thickness. Fractional shortening (FS) is given as (LVEDD-LVESD)/LVEDD.

**Statistical analysis**

Details on the statistical analysis are given in the supporting information (SI Methods).

**Results**

**Simultaneously recorded force and Ca\(^{2+}\)-frequency relations in field-stimulated papillary muscles of young wt and mdx mice**

Figure 1A shows recordings of force and fura-2 Ca\(^{2+}\) transients in a papillary muscle from a 12-week-old wt mouse stimulated at 1 and 4 Hz. At the higher frequency, force amplitudes drop by 40% while Ca\(^{2+}\) transients are still comparable to low frequency stimulation. The three consecutive force transients shown at 1 and 4 Hz were taken from a series of 10 and 40 successive transients during the 10 sec. stimulations train, respectively, which are both shown in the insets. As can be seen, during the train force amplitudes remained fairly constant arguing against the development of hypoxic cores even at highest stimulation frequencies. Already from 1.5 Hz, force was significantly reduced and further dropped in a sigmoidal manner (Fig. 1B). Ca\(^{2+}\) transients showed a different behaviour: although absolute fura-2 ratio amplitudes were about 40% reduced in mdx papillary muscles, their frequency dependence was rather flat, whereas in wt they showed a positive bell shape (Fig. 1C). For smaller frequencies, decay time constants of fura-2 transients were somewhat larger in mdx versus wt preparations but approached the wt values for larger frequencies (Fig. 1C). In contrast, there was virtually no difference on transient rise time between strains in the frequency range (not shown).

**Myofibrillar Ca\(^{2+}\) sensitivity is reduced in mdx papillary muscles**

To search for possible explanations for vastly reduced force despite only a 40% decrease in Ca\(^{2+}\) amplitudes in the mdx samples, we tested a set of different possible contractile parameters. Myofibrillar Ca\(^{2+}\) sensitivity of papillary muscle strips was assessed in different pCa-containing solutions. The steady-state pCa-force relations from 8 wt and 20 mdx preparations clearly showed a marked right shift towards smaller pCa values for papillary muscles from dystrophic mice in the early pre-fibrotic phase (Fig. 2). pCa\(_{50}\) values were significantly smaller in mdx muscles: 5.548 ± 0.04 for wt and 5.310 ± 0.03 for mdx (P < 0.001), whereas Hill coefficients were 2.572 ± 0.02 for wt and 2.795 ± 0.27 for mdx (P = 0.63). Maximum forces at pCa = 4.28 were 2.17 ± 0.51 mN in wt and 2.42 ± 0.18 mN in mdx papillary muscles (P = 0.22, one-way ANOVA on ranks).

**In vitro motility assays reveal slower actomyosin filament sliding in mdx hearts**

Recordings of the molecular interactions of fluorescently labelled actin with ventricular myosin extracts in an *in vitro motility assay* showed a markedly impaired molecular interaction of the motor proteins. Figure 3A shows image sequences from representative recordings tracking two individual filaments in successive images from a wt and an mdx ventricular myosin extract. Velocity histograms from individual recordings showed a marked left shift in mdx hearts that was statistically highly significant, as judged from the much decreased mean sliding velocities in mdx hearts (Fig. 3B).

**Myosin heavy and light chain distributions are unchanged in mdx ventricles**

To test whether the slower propelling velocities in the mdx ventricles were also reflected by changes in the myosin isoforms
expression, SDS PAGE analysis on several mdx and wt hearts was performed. In ventricles and atria extracts, the ventricular and atrial light chain isoforms were clearly distinguishable (ALC, VLC) showing the two well-known isoforms [13, 17] (Fig. 4A). In MHC gels, a clear single band of ~185 kD was seen reflecting the overall prominent \( \alpha \)-MHC in mouse atria and ventricles [17]. Densitometric analysis of gels from three wt and four mdx hearts of similar age showed unchanged MHC expression (given as atria-to-ventricle signal density ratios) and VLC profiles in the ventricles. Interestingly, atrial ALC isoforms were significantly increased in mdx cardiac ventricles (Fig. 4B).

Mdx hearts already show morphological signs of cardiomyopathy in the pre-fibrotic phase with preserved fractional shortening ‘\textit{in vivo}’

\textit{In vivo} echocardiography sequences of beating hearts in M-mode were obtained from three conscious wt and four mdx mice. Quantification of ventricular wall diameters already showed significantly increased thickness of the posterior wall (wt: 0.57 ± 0.02 mm, mdx: 0.87 ± 0.05 mm; \( P < 0.01 \)) and septum (wt: 0.75 ± 0.05 mm, mdx: 1.09 ± 0.10 mm; \( P < 0.05 \)) and an increased left ventricular end-diastolic...
Altered Ca\(^{2+}\) homeostasis may be a strong candidate, however, none of those studies provided a mechanistic explanation. Compared with wt muscles stimulated between 4 and 12 Hz [8], how-
declines at higher stimulation frequencies, i.e. a negative ‘treppe’ in wt mouse papillary-like muscle from 8 Hz stimulation or in rat papillary muscle already at < 6 Hz [24]. However, as stated by Redel et al. [23], FFRs in mouse papillary muscles can range from negative to strongly positive relations. At ∼2 mM external Ca²⁺, force-frequency relations were negative [26, 27] and supposedly are flat under conditions that most closely resemble the physiological situation in mouse papillary muscle [25]. To further rule out possible artefacts from potential hypoxic cores that could result in production of reactive oxygen species [7], we concentrated on stimulation frequencies between 1 and 4 Hz only.

The most obvious reason for compromised force in mdx hearts would be apparent from altered Ca²⁺ handling. In failing hearts, it is well known that a negative FFR is often reflected by alteration in Ca²⁺ handling and/or ec-coupling [21]. Ca²⁺ handling abnormalities in mdx mice are related to aberrant mechanosensitive channel activity (e.g.

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Fig. 3 In vitro motility assay in ventricular myosin extracts from young wild-type (wt) and mdx mice. (A) Example images from two motility assay XYT stacks of wt and mdx myosin extracts. Arrows and arrowheads point towards individual actin filaments tracked in successive images. Image frames and times relative to first frame are indicated. The asterisk points towards a standing filament that does not move in successive images indicative of rigour bridges that introduce artificially high counts velocities < 1.5 µm/sec. (B) Velocity distributions from two individual XYT recording sequences (n = 24 for wt; n = 10 for mdx) showing the Gaussian fits for the velocities from several hundreds of tracked filaments. There is a prominent left shift of the mdx curve. The results from several thousand filaments confirmed a significantly shifted velocity towards smaller values in mdx hearts.

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Fig. 4 Myosin heavy (MHC) and light chain (MLC) distributions are similar in mdx and wild-type (wt) hearts. (A) 8% (MHC) and 12% (MLC) SDS PAGE gels from atrial and ventricular preparations of two wt and mdx mice, each. For the MLC gels, the atrial ALC and ventricular VLC isoforms are shown. Protein loading was ∼10 µg (MHC) or 50 µg (MLC) for each lane. (B) Densitometric analysis shows unaltered atrial-to-ventricular MHC ratios in mdx papillary muscle. For MLCs, signals were doubled in atria but the slower ventricular isoforms in the ventricles were similar in wt and mdx hearts. S.D.: standard marker (17 kD band, MLC; 200 kD, MHC). Analysis from three to four wt and mdx hearts. *: P < 0.05.
TRPC1, [2]), increased Na+/Ca2+ exchanger function [2], ROS production and inflammatory activity [7, 18] that maintain Ca2+ overload and fibrotic remodelling in dystrophic heart. Several Ca2+ handling proteins, e.g., expression patterns of SERCA2, are known to be altered in mdx mice also in an age-dependent manner [2, 28]. In the age group used in our study, Williams and Allen [2] found a slight reduction in SERCA2 expression that turned into a marked increase in old mice (12 mo). Alongside with hypernitrosylation of RyR2 and increased SR leakage [19], all these Ca2+ handling mechanisms compete to favour or limit the increase in myoplasmic Ca2+ that was found in resting or stressed cardiomyocytes [2]. The strong t-tubular localization of dystrophin in cardiomyocytes might also point towards its involvement in tubular mechanosensitive channels that could contribute to aberrant mdx cardiac Ca2+ homeostasis [7]. As the time for Ca2+ extrusion shortens with higher stimulation frequencies, it was found markedly increased Ca2+-transient amplitudes at basal 1 Hz stimulation which would be detectable in our pCa-force experiments [33, 34]. Such an increased PKA-mediated TnI phosphorylation would also explain a disproportionate decrease of force in mdx papillary muscle despite a much lesser drop in Ca2+-transient amplitude.

**Implications for dystrophic hearts: in vivo recordings show signs of hypertrophy in the pre-fibrotic phase**

So far, the mechanisms found that would reduce isometric force five-fold in papillary muscle preparations would predict a rather non-sustainable situation in vivo. However, although mdx mice have a reduced lifespan, signs of beginning cardiomyopathy were established as soon as week 16 [38]. No differences between wt and mdx hearts were recently found from echocardiography recordings in 8-week-old mice, but there was a significantly increased LV mass at 29
-42 weeks [3]. Our echocardiography data in young conscious mdx mice showed unaltered fractional shortening which is considered a global clinical parameter for cardiac contractile function [16]. This seems in contrast to the vast reduction in force production in the isolated mdx preparations. However, in a very recent clinical follow-up study of 11 DMD patients who developed severe DCM over 9 years, all patients showed clear LVEFD enlargements within the observation period with almost no impairment of left ventricular function and only modest reduction in the fractional shortening index, as assessed by echocardiography [39]. This indicates that despite clear signs of DCM ‘in vivo’, presumably paralleled by force decrements on the organ muscular level, cardiac output may appear balanced for long times. One explanation, which still awaits further experimental evidence, could be reflected by biophysical considerations. Using Laplace’s law and our values for the significantly increased ventricular wall diameters and enlarged ventricular cavity, a force (tension) decrement of ~1.5 still yields the same pressure level for mdx hearts. Therefore, early hypertrophy in the pre-fibrotic phase as soon as ~10–16 weeks in our study can be an attempt to compensate for an already decreased wall tension. However, such calculations are not static and fractional shortening can still appear normal despite substantially suppressed contractility on the cellular level. For example, in a study on cardiac performance following aortic banding hypertrophy in mice, a decrease in fractional shortening also showed some time lag to the increase in left ventricular wall thickness suggesting that despite already developed morphological signs of cardiac hypertrophy, global function can remain well compensated [40].

In summary, we present the very first evidence for early hypertrophy in mdx hearts in the pre-fibrotic phase at <16 weeks and possible cellular mechanisms for impaired force output in dystrophic hearts.

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Conflicts of interest

We declare that there are no potential conflicts of interest. All funding agencies had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Supporting information

Additional Supporting information may be found in the online version of this article:

Data S1 Supporting Materials and Methods related to preparation, in vivo motility assays and biochemistry.

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