Dystrophic cardiomyopathy: amplification of cellular damage by Ca\(^{2+}\) signalling and reactive oxygen species-generating pathways

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**Aims** Cardiac myopathies are the second leading cause of death in patients with Duchenne and Becker muscular dystrophy, the two most common and severe forms of a disabling striated muscle disease. Although the genetic defect has been identified as mutations of the dystrophin gene, very little is known about the molecular and cellular events leading to progressive cardiac muscle damage. Dystrophin is a protein linking the cytoskeleton to a complex of transmembrane proteins that interact with the extracellular matrix. The fragility of the cell membrane resulting from the lack of dystrophin is thought to cause an excessive susceptibility to mechanical stress. Here, we examined cellular mechanisms linking the initial membrane damage to the dysfunction of dystrophic heart.

**Methods and results** Cardiac ventricular myocytes were enzymatically isolated from 5- to 9-month-old dystrophic mdx and wild-type (WT) mice. Cells were exposed to mechanical stress, applied as osmotic shock. Stress-induced cytosolic and mitochondrial Ca\(^{2+}\) signals, production of reactive oxygen species (ROS), and mitochondrial membrane potential were monitored with confocal microscopy and fluorescent indicators. Pharmacological tools were used to scavenge ROS and to identify their possible sources. Osmotic shock triggered excessive cytosolic Ca\(^{2+}\) signals, often lasting for several minutes, in 82% of mdx cells. In contrast, only 47% of the WT cardiomyocytes responded with transient and moderate intracellular Ca\(^{2+}\) signals. On average, the reaction was 6-fold larger in mdx cells. Removal of extracellular Ca\(^{2+}\) abolished these responses, implicating Ca\(^{2+}\) influx as a trigger for abnormal Ca\(^{2+}\) signalling. Our further experiments revealed that osmotic stress in mdx cells produced an increase in ROS production and mitochondrial Ca\(^{2+}\) overload. The latter was followed by collapse of the mitochondrial membrane potential, an early sign of cell death.

**Conclusion** Overall, our findings reveal that excessive intracellular Ca\(^{2+}\) signals and ROS generation link the initial sarcolemmal injury to mitochondrial dysfunctions. The latter possibly contribute to the loss of functional cardiac myocytes and heart failure in dystrophy. Understanding the sequence of events of dystrophic cell damage and the deleterious amplification systems involved, including several positive feed-back loops, may allow for a rational development of novel therapeutic strategies.

1. Introduction

Dystrophinopathies result from mutations of the dystrophin gene (reviewed in Ervasti and Campbell\(^1\)). Dystrophin is a 427 kDa protein linking the cytoskeleton to a complex of transmembrane proteins, which interact with the extracellular matrix. Duchenne and Becker muscular dystrophy (DMD and BMD) are the two most common and severe forms and are characterized by progressive and disabling muscle weakness (one case per 3500 boys). Cardiac manifestations are present in essentially all patients by 20 years of age. The most common abnormality is dilated cardiomyopathy. Around 20% of the patients succumb from the cardiac manifestations. Clinically, the disease initially shows ECG abnormalities. Later, echocardiographic examinations reveal ventricular wall motion abnormalities in regions of fibrosis. With spreading of fibrosis, ventricular dysfunction and arrhythmias occur, ultimately leading to heart failure and sudden death.\(^2\) With improving medical care for the skeletal muscle weakness, the cardiac disease becomes more and more limiting for the survival of the patients.

In the skeletal muscle field, there is a significant body of data describing the cellular pathophysiological events (for review see Allen et al.\(^3\)). It is generally believed that the
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2. Methods

2.1 Cell isolation

Ventricular myocytes were enzymatically isolated from 5- to 9-month-old mice as previously described. The study conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). C57BL/10ScSn-mdx and wild-type (WT) mice with identical genetic background (5–10 months old) were provided by Drs M. Ruegg (University of Basel, Switzerland), U. Ruegg (University of Geneva, Switzerland), or purchased from the Jackson Laboratory (USA).

2.2 Experimental solutions

The isotonic superfusion solution contained (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl\textsubscript{2}, 1.1 MgCl\textsubscript{2}, 5 HEPES, and 10 glucose. The osmolality was ~310 mosm/kg, and pH 7.3. The hypotonic solution contained 70 mM NaCl instead of 140 mM, had osmolality ~170 mosm. Zero Ca\textsuperscript{2+} solution contained 2 mM EGTA. Where indicated, apocynin or ryanodine and thapsigargin were added for at least 30 min before the experiment. Most chemicals were obtained from Sigma. Mn-cpx 3 and apocynin were from Calbiochem, ryanodine from Alamone. All experiments were performed at room temperature (20–22°C).

2.3 Confocal microscopy

Changes in cytosolic and mitochondrial [Ca\textsuperscript{2+}], ROS production, and mitochondrial membrane potential were monitored with fluorescent indicators fluo-3 AM (5 μM), rhod-2 AM (5 μM), fura-red AM (5 μM), 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H\textsubscript{2}DCFDA; 20 μM), and tetrakis(4-chlorophenyl)triphenylphosphonium (TMRo; 100 μM), as previously described. As a rule, cells were incubated with indicators for 30 min at room temperature, followed by at least 15 min for de-esterification. Resting [Ca\textsuperscript{2+}] and Ca\textsuperscript{2+} influx were measured with a double-indicator ratiometric procedure, which simultaneously utilizes fluo-3 and fura-red indicators to correct for dye dilution during osmotic swelling. Fluo-3 AM was from Biotium, other indicators were from Invitrogen. A laser-scanning confocal microscope (Radiance, Bio-Rad) was used to acquire confocal images. Fluo-3, fura-red, and CM-H\textsubscript{2}DCFDA were excited with the 488 nm line of an Argon laser. TMRE and rhod-2 were excited with a HeNe laser at 543 nm. The emitted light was collected above 500 nm and above 570 nm, respectively. The emission of fluo-3 and fura-red was simultaneously recorded with two PMTs using 515/30 and >600 nm emission filters and the ratio calibrated according to.

2.4 Data analysis

Analysed data are represented as mean ± SEM. Where stated, statistical significance was determined using Student’s t-test or the log-rank test. In the figures, single asterisk indicates P < 0.05, double asterisks indicate P < 0.01.

3. Results

3.1 Osmotic shock triggers excessive cytosolic Ca\textsuperscript{2+} signals in mdx but not wild-type cardiomyocytes

We applied osmotic shocks to mimic some of the pathophysiological conditions of mechanical stress encountered by the cell in vivo. Hypotonic solution induces cell swelling and mechanical deformation of the cell membrane. Cytosolic Ca\textsuperscript{2+} transients were recorded in both WT and mdx cardiomyocytes loaded with fluo-3. As a rule, series of 100 XY confocal scans were acquired at 0.5 Hz. Exposure of the cardiomyocytes to hypotonic solution (170 mosm) for 40 s consistently resulted in reversible cell swelling (by 15.8 ± 1.5%, n = 34). In 82% of mdx myocytes (19 out of 23 cells), the return to isotonic solution caused extreme cytosolic Ca\textsuperscript{2+} signals such as bursts of Ca\textsuperscript{2+} sparks and repetitive Ca\textsuperscript{2+} waves exhibiting amplitudes comparable with normal Ca\textsuperscript{2+} transients (as shown in Figure 1A, left panel). In the majority of mdx cells, these Ca\textsuperscript{2+} signals lasted for the full duration of the recording (up to 3 min). In contrast, in only 47% of the WT cardiomyocytes, osmotic shock produced Ca\textsuperscript{2+} signals (nine out of 19 cells) and these were mostly moderate and short-lived (Figure 1A, right panel). The typical Ca\textsuperscript{2+} responses in mdx and WT.
cells are also visualized in movies in the online supplement. Figure 1B shows the averaged changes in intracellular fluorescence in all studied WT and mdx cardiomyocytes, normalized to the resting values before osmotic shock. The stress-induced increase in fluorescence was larger and longer lasting in mdx cells compared with WT myocytes. The mean cellular fluorescence signal recorded during the first 60 s after returning to the iso-osmotic solution was 6-fold larger in mdx cells (Figure 1C).

3.2 A small Ca\(^{2+}\) influx is required for stress-induced Ca\(^{2+}\) signals in mdx cardiomyocytes

The above observation together with the recent results by Yasuda et al.\(^\text{15}\) suggest that mdx myocytes are more susceptible to membrane damage than WT cardiomyocytes. To test whether these Ca\(^{2+}\) signals depend on extracellular Ca\(^{2+}\), we briefly removed Ca\(^{2+}\) from the extracellular solution. Figure 2 shows that switching to a solution containing 0 mM Ca\(^{2+}\) completely abolished the response to osmotic shock. This observation confirms that Ca\(^{2+}\) entry is required, although the precise pathway(s) leading to Ca\(^{2+}\) influx into dystrophic cells are yet to be established and seems to involve multiple molecular entities.\(^3\)

Nevertheless, the increase in plasmalemmal permeability to Ca\(^{2+}\) and the slightly elevated resting Ca\(^{2+}\) concentration in mdx myocytes (see Figure 2E and Williams and Allen\(^\text{19}\)) alone are unlikely to explain the sustained Ca\(^{2+}\) signals in mdx cells subjected to osmotic shock. In cells treated with ryanodine (5 μM) and thapsigargin (500 nM) to eliminate the function of the sarcoplasmic reticulum (SR), a substantial Ca\(^{2+}\) influx during the superfusion with hypotonic solution would be visible as an increase of the cytosolic Ca\(^{2+}\) concentration. However, when applying a ratiometric indicator technique involving fluo-3 and fura-red to compensate for dye dilution due to osmotic swelling, only a modest elevation of the Ca\(^{2+}\) concentration was observed, suggesting that the Ca\(^{2+}\) influx was small (Figure 2C and D, gray circles).

3.3 Osmotic shock stimulates reactive oxygen species production

Because we were not able to detect a substantial Ca\(^{2+}\) influx during the shock, we suspected some intracellular mechanisms by which even small increases in [Ca\(^{2+}\)]\(_i\) due to Ca\(^{2+}\) influx were amplified. One possibility could be oxidation of the cytosolic environment due to excessive ROS production,\(^\text{26}\) which could affect several proteins involved in intracellular Ca\(^{2+}\) homeostasis, most notably the ryanodine receptors (RyRs), thereby enhancing the sensitivity of SR Ca\(^{2+}\) release.

To test whether osmotic shock enhances ROS generation, we loaded myocytes with CM-H\(_2\)DCFDA. In cells, this non-fluorescent compound is hydrolysed to DCFH and ROS generation is detected as a result of DCF oxidation to DCF. Figure 3A shows images from one mdx cardiac myocyte before (image at 20 s), during (50 s), and after osmotic shock (80, 110, 140, and 170 s). Insets illustrate the banded fluorescence typical of a mitochondrial distribution. Figure 3B represents averaged and normalized
changes in DCF fluorescence in mdx and WT cells. To quantify the stress-induced changes in ROS production, we performed a linear fit to the fluorescence signal in each cell before and after osmotic shock. The slope of the DCF signal reflects the rate of ROS production. Before the osmotic shock, the ROS-related signals rose monotonically, presumably due to a basal generation of ROS. The initial slope was significantly larger in mdx myocytes than in WT cells (0.70 ± 0.09, n = 12 and 0.40 ± 0.07, n = 9, respectively) indicating an oxidative stress in these cells, as also suggested in Williams and Allen.27 Furthermore, we observed that the slope increased after the shock in both WT and mdx cells, but more in mdx myocytes than in WT cells [ratio of slopes before to after the shock was 1.72 ± 0.2 (n = 12) and 1.34 ± 0.12 (n = 9) in mdx and WT, respectively].

To confirm the involvement of ROS-dependent mechanisms in the generation of stress-induced Ca$^{2+}$ signals, we incubated mdx myocytes with 10 μM Mn-cpx3, a superoxide dismutase mimetic. Figure 4A shows that this ROS scavenger nearly eliminated cytosolic Ca$^{2+}$ transients produced by osmotic shock. After washing out the scavenger for 30 min, five out of six mdx cardiomyocytes recovered their exacerbated response. Figure 4B shows a significant decrease in the mean intracellular fluorescence recorded during the first 60 s after returning to an iso-osmotic solution in the presence of the scavenger.

There are several possible sources of ROS in cardiac myocytes that may be activated in response to mechanical stress. NAD(P)H oxidase (NOX) is known to be overexpressed in dystrophic heart27 and was found to be rapidly activated by osmotic swelling in astrocytes.28 Therefore, we tested whether NOX could be a source for a rapid increase in the ROS production in response to the mechanical stress in mdx myocytes. Cells were incubated for 30 min with the NOX inhibitor apocynin (0.2–1 mM). Figure 4C and D shows that the drug nearly eliminated the intracellular Ca$^{2+}$ signals induced by osmotic shock, suggesting NOX as a possible source of acute ROS production.

### 3.4 Excessive cytosolic Ca$^{2+}$ is taken up by the mitochondria

Mitochondria are well-established sources of ROS in myocardium subjected to acute or chronic mechanical stress.29
Mitochondrial ROS could be responsible for the sustained cellular Ca\(^{2+}\) signals observed in mdx myocytes (note the gradual increase in ROS-related fluorescence in the mitochondrial regions later during the experiment shown in Figure 3). It is known that muscle cells continuously generate ROS, as a by-product of the mitochondrial respiratory chain, and that enhanced mitochondrial Ca\(^{2+}\) uptake stimulates further mitochondrial ROS production. We tested whether and to what extent mitochondria take up cytotoxic Ca\(^{2+}\) after osmotic shock.

Mdx and WT myocytes were loaded with the Ca\(^{2+}\) indicator rhod-2 and imaged with the identical experimental protocol. Rhod-2 is a charged molecule that preferentially partitions into the mitochondria. Figure 5A shows the mitochondrial rhod-2 distribution and the fluorescence signal in mdx myocytes before (image at 1 s), during (image at 28 s), and after the shock. Following 40 s of osmotic shock, fluorescence gradually increased within all mitochondria. Figure 5B shows the time course of rhod-2 signals in three groups of mitochondria indicated by regions of interest (1–3 in A). (C) Pooled data from mdx (black circles) and wild-type (white circles) cells studied with the same protocol. At the end of the recording, the mitochondrial Ca\(^{2+}\) accumulation is significantly more pronounced in mdx myocytes (data from n = 6 and 8 cells, N = 2 and 3 mice). See Supplementary Material online for colour version.

### 3.5 Osmotic shock induces mitochondrial depolarization in mdx cardiac myocytes

It is established that mitochondrial Ca\(^{2+}\) overload and the subsequent oxidative stress can lead to collapse of the mitochondrial membrane potential (Δψ\(_{m}\)) and/or to the opening of the mitochondrial permeability transition pore (mPTP), ultimately causing necrotic/apoptotic cell death. Our results indicate that mitochondrial Ca\(^{2+}\) uptake is enhanced in mdx cardiac myocytes. We also found that some mitochondria lose Ca\(^{2+}\) after a period of initial accumulation, possibly as a result of irreversible mitochondrial depolarization. Therefore, we carried out experiments to directly monitor changes in mitochondrial membrane potential. TMRE is a charged dye, which distributes across the mitochondrial membrane in a voltage-dependent manner. Whenever the potential across the mitochondrial membrane dissipates, TMRE fluorescence is lost. Figure 6A shows one mdx cardiomyocyte loaded with 100 nM TMRE. A single mitochondrial (or a small group of mitochondria) can be visualized in each of the marked boxes. Subsequent to the osmotic shock, some mitochondria displayed irreversible loss of Δψ\(_{m}\) whereas others did not. Changes in TMRE fluorescence for four individual mitochondria are plotted in Figure 6B. Note that the final level of TMRE signal is essentially the same in all four organelles, indicating their complete depolarization. Figure 6C compares the incidence and timing of osmotic shock-induced mitochondrial depolarization in mdx and WT cardiomyocytes. For each time point, we
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4. Discussion

Dystrophin-deficient cardiomyocytes are vulnerable to mechanical stress. Recently published work connects increased membrane fragility and stretch-induced Ca\(^{2+}\) influx with death of dystrophic cells.\(^7\) In the present study, we identified two cellular signal amplification mechanisms that may link the fragility of the \textit{mdx} cardiomyocyte membrane to later cell damage. As detailed below, the simultaneous activation of these two signal amplification systems is detrimental, because of bidirectional cross-talk with positive feed-back. Thus, the cellular response proceeds beyond the physiological limits, culminating in a chain of events, which may later end in apoptotic and/or necrotic cell death.

4.1 Amplification mechanisms

The massive Ca\(^{2+}\) signals observed in \textit{mdx} myocytes after osmotic shock were manifest as a surge of abundant spontaneous Ca\(^{2+}\) sparks, regularly blending into Ca\(^{2+}\) waves travelling along the cell. In resting cardiomyocytes such signals are the hallmark of abnormal Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) activation and are commonly initiated by overloading the SR with Ca\(^{2+}\) in the course of various noxious conditions. The importance of CICR for the observed signal amplification was also emphasized by the absence of such Ca\(^{2+}\) signals in cells pretreated with ryanodine and thapsigargin. However, for Ca\(^{2+}\) overload to develop in a short period of time (i.e. during the exposure to hypotonic solution), Ca\(^{2+}\) influx has to be substantial. Surprisingly, such a massive Ca\(^{2+}\) influx could not be detected in our experiments, even though the behaviour of the myocytes in zero Ca\(^{2+}\) indicated an important role for at least some minor transsarcolemmal Ca\(^{2+}\) influx. The obvious discrepancy between massive and rapid CICR activation despite minimal Ca\(^{2+}\) entry and only slightly elevated resting Ca\(^{2+}\) levels led us to consider additional mechanisms that could make the RyRs rapidly more sensitive and provoke spontaneous Ca\(^{2+}\) signals without pronounced SR Ca\(^{2+}\) overload.

ROS have been implied in changing gating and Ca\(^{2+}\) sensitivity of the RyRs via redox modifications of the channels.\(^2\) Furthermore, increased ROS generation after mechanical stretch has been reported even in normal cardiac muscle.\(^2\) Our findings revealed an elevated basal rate of ROS production in \textit{mdx} cells. Furthermore, mechanical stress led to enhanced ROS production in all cardiomyocytes, but significantly more pronounced in \textit{mdx} cells. As one might expect based on these findings, ROS scavengers reversibly suppressed the excessive stress-induced Ca\(^{2+}\) signalling in \textit{mdx} cells. Taken together, our results suggest that the change of the cytosolic oxidative environment due to increased [ROS] promotes the abnormal CICR activation without pronounced Ca\(^{2+}\) overload.

ROS generation in response to mechanical forces may originate from several sources, including the NOX, xanthine oxidase, or other oxidase systems, such as nitric oxide oxidase, and mitochondria. Our results with the NOX inhibitor apocynin suggest an involvement of this source of ROS. Owing to its overexpression in dystrophic heart\(^6\) and due to its rapid activation by osmotic swelling,\(^2\) this source of ROS may be particularly important in the early phases of the response. It is also established that an elevation in mitochondrial [Ca\(^{2+}\)] subsequent to prolonged cytosolic Ca\(^{2+}\) signal activity stimulates mitochondrial ROS production.\(^2\) Consistent with this notion, we observed increased Ca\(^{2+}\) levels in cardiac mitochondria following the osmotic shock that were significantly larger in \textit{mdx} cells.
4.2 Cross-talk between Ca\(^{2+}\)-induced Ca\(^{2+}\) release and reactive oxygen species

CICR and ROS generation are two signalling pathways that exhibit a high degree of positive feed-back. Either mechanism could lead to the observed massive Ca\(^{2+}\) signals. However, the combination of both pathways being activated together may have even more severe consequences, as both pathways exhibit mutual and synergistic cross-talk with each other. For example, ROS could promote RyRs openings as well as further increase membrane fragility due to lipid peroxidation. In addition, ROS could stimulate Ca\(^{2+}\)-influx into the cells via SAC and/or SOC, as some TRP channels (potential candidates for SOC and SAC) have been shown to be sensitive to the redox potential of the cytosol.\(^3\) On the other hand, CICR and elevated [Ca\(^{2+}\)]\(_i\) favour mitochondrial ROS production, as discussed above. Taken together, the simultaneous activation of these signalling pathways is particularly destructive for the cells because it has a tendency to escalate into mutually synergistic positive feed-back signalling loops.

4.3 Link between abnormal cellular signalling and pathology of the heart

Although WT myocytes survive the initial Ca\(^{2+}\) overload, the strong activation of several pathways may overwhelm the cellular defence mechanisms in mdx cells. It has been postulated for many years that proteases are a downstream Ca\(^{2+}\)-activated target involved in protein degradation and necrosis of dystrophic skeletal muscle.\(^3\) Calpain is considered to be a likely candidate, as its expression is greater in mdx muscle.\(^3\) Because calpain is expressed in heart, and because calpain inhibitors protect from ischaemia-reperfusion injury,\(^32\) it is likely that this protease forms a part of the cellular death pathway in mdx heart.

Under pathological conditions, cytosolic and mitochondrial Ca\(^{2+}\) overload and/or excess in ROS can lead to opening of the mPTP, which is accompanied by a dissipation of the mitochondrial membrane potential and usually precedes necrotic/apoptotic cell death.\(^29\)\(^,\)\(^33\) Our data reveal multiple mitochondrial dysfunctions in mdx cardiac myocytes following osmotic stress. In particular, they show mitochondrial Ca\(^{2+}\) overload, increased ROS production, irreversible depolarization of the mitochondrial membrane, which can be a direct indication of mPTP opening. Apoptotic and necrotic cells have been observed in cardiac tissue of dystrophy patients and mdx mice. Lost cardiomyocytes cannot regenerate and are likely to be replaced by connective tissue, ultimately leading to the observed fibrosis.\(^2\)

At present, several studies are conducted to develop treatments of the cardiac dystrophic phenotype by either replacing the lost cardiomyocytes with stem cell approaches\(^34\) or to make the myocytes mechanically less vulnerable by gene transfer of dystrophin, utrophin, or functional dystrophin fragments (microdystrophins).\(^35\) Notably, mdx mice can partly compensate for the lack of dystrophin by adaptive upregulation and redistribution of the protein utrophin.\(^34\) An mdx mouse lacking utrophin presents with a more severe phenotype,\(^37\) particularly in skeletal muscle, but also in cardiac muscle.\(^38\) Although cell replacement therapies will obviously only be able to treat late stages of the disease, curative gene therapy may require a long time to develop for routine application. In the meantime, it would be helpful to implement pharmacological treatment options which are able to slow down the progression of the disease.\(^4\) Considering the early cellular stress responses reported here, this might be accomplished by interfering with any of the early cellular events leading to excessive Ca\(^{2+}\) and ROS signals.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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