Changes in cytosolic Ca^{2+} dynamics in the sarcoplasmic reticulum associated with the pathology of Duchenne muscular dystrophy

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Abstract Duchenne muscular dystrophy (DMD) is a life-limiting X-linked genetic disorder caused by a lack of the membrane-associated protein dystrophin. The absence of dystrophin increases the susceptibility of muscle fibers to damage. Repeated damage results in ineffective muscle repair and the development of pseudo-hypertrophied muscles; these bulky muscles are weak despite their size. The mechanisms underlying the functional impairments in dystrophic muscle have not yet been fully determined. However, several recent studies indicate that elevated intracellular Ca^{2+} homeostasis is a cause or facilitator of the development of muscle weakness in DMD. This review focuses on abnormalities of Ca^{2+} homeostasis and the possibilities for treatment by counteracting the Ca^{2+} dysregulation.

Keywords : Duchenne muscular dystrophy, Ca^{2+} homeostasis, sarcoplasmic reticulum (SR), ryanodine receptor (RyR1), sarcoplasmic endoplasmic reticulum ATPase (SERCA)

Introduction

DMD is the most severe form of muscular dystrophy, affecting about 1 in 3500 male births. Loss of dystrophin leads to disruption of the dystrophin glycoprotein complex (DGC) that crosses the sarcolemma and connects the cytoskeleton and contractile apparatus to the extracellular matrix and basement membrane^{1,2)}. Intracellular Ca^{2+} homeostasis ($[\text{Ca}^{2+}]_i$) is compromised in dystrophic muscle fibers, which triggers chronic inflammation, repeated cycles of degeneration with progressively ineffective regeneration^{3,4)}, and fibrosis¹⁾. Mechanisms mediating the influx of Ca^{2+} into dystrophic muscle include membrane tears¹⁾, stretch-activated channels⁵⁾, Ca^{2+} leak channels⁶⁾, and leaky Ca^{2+} release channels⁷⁾ that cause increased entry via store-operated channels⁴⁾.

Skeletal muscle activation at the neuromuscular junction induces a membrane depolarization that propagates along the plasma membrane to invaginations called transverse tubules (T-tubules). In the T-tubules, depolarization activates the L-type Ca^{2+} channel, also called the dihydropyridine receptor (DHPR)⁸⁾. Activation of the DHPR stimulates the opening of a sarcoplasmic reticulum (SR) Ca^{2+} channel, the type 1 ryanodine receptor (RyR1). This causes the release of massive amounts of Ca^{2+} from the SR into the cytosol resulting in an increase in the $[\text{Ca}^{2+}]_i$ concentration responsible for myofibril gliding and muscle contraction. In contrast, muscle relaxation occurs after closing of the RyR1 and Ca^{2+} is taken up into the SR by

the sarcoplasmic endoplasmic reticulum ATPase (SERCA) Ca^{2+} pump⁴⁾.

Although maintaining Ca^{2+} homeostasis is critical for controlling muscle contraction and relaxation, Ca^{2+} is equally important as a second messenger that triggers long-term functions such as muscle adaptation or degradation⁴⁾. In resting non-contracting muscle, minor elevations in low nanomolar concentrations of $[\text{Ca}^{2+}]_i$ can induce beneficial muscle adaptations including upregulation of muscle thermogenesis⁹⁾, increased mitochondrial biogenesis, and improved resistance to fatigue¹⁰⁾. However, elevations in $[\text{Ca}^{2+}]_i$ above 100 nM can be deleterious to muscle function^{11,12)}. Indeed, excessively elevated $[\text{Ca}^{2+}]_i$ has been observed in muscles of dystrophic mice, leading to increases in calpain activation¹³⁾, impaired autophagy¹¹⁾ and mitochondrial function¹³⁾.

The role of RyR1 in dystrophic muscle

RyR1 is the major isoform of the ryanodine receptor expressed in skeletal muscle. It is composed of 106 exons of which two are alternatively spliced (exons 70 and 83); the final transcript produces a protein of 5038 amino acids. The functional RyR1 channel is a massive 2.5 MDa homotetramer whose structure has been recently elucidated at near-atomic resolution using cryo-electron microscopy (cryo-EM)¹⁴⁻¹⁷⁾. The major part of the monomer, namely the first 4000 amino acid residues of RyR1, are exposed to the cytosol, forming the “feet” of the molecule observed in cryo-EM. The C-terminal 1000 amino acid residues contain the four transmembrane helices responsible

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for SR membrane anchoring and formation of the channel pore.

In *mdx* mice, a commonly used animal model of DMD, an approximately 80% reduction in nitric oxide synthase (NOS) mRNA and protein have been reported^{17,18}. Furthermore, this reduction has been implicated in the pathophysiology of DMD¹⁹. RyR1 contains multiple cysteine residues that can be modified at physiological pH by either S-nitrosylation²⁰ or S-glutathionylation²¹. Nitric oxide-mediated regulation of RyR1 increases channel activity in vesicles and in single-channel measurements²⁰. It is well known that RyR1 channel function may be regulated by S-nitrosylation but, until recently, the functions of the nitrosylated form *in vivo* have not been well understood. In skeletal muscle, neuronal NOS (nNOS) is the principal source of NO. It is localized at the plasma membrane, where an N-terminal glycine-leucine-glycine-phenylalanine motif binds to the DGC via interaction with α -syntrophin^{17,22}. Disruption of the DGC in DMD results in selective loss of the nNOS catalytic activity that is associated with its down-regulation at the transcriptional level^{17,18}. Recently, Campbell and colleagues showed that reduced sarcolemmal-localized nNOS is linked to decreased activity following mild exercise in mouse models, including *mdx* mice²³. In contrast, Bellinger et al. reported that inducible NOS (iNOS) is increased in the skeletal muscle of both DMD patients⁷ and *mdx* mice, and that the *mdx* phenotype could be rescued by dystrophin or utrophin over-expression²⁴. The concurrent down-regulation of nNOS and the up-regulation of iNOS in *mdx* mice suggest that the latter could be a compensatory response, but the differences in subcellular localization of the two NOS isoforms could account for some of the pathology observed in *mdx* mice, including “leaky” RyR1 channels⁷. In muscular dystrophy, both the effects of increased nitrosylative stress on the RyR1 complex and increased Ca^{2+} influxes across the plasma membrane, due to disruption of the DGC, may activate RyR1 channels. This may occur, in particular, because depletion of the stabilizing subunit calstabin1 (FKBP12) from the RyR1 channel, due to nitrosative stress, may render it particularly sensitive to Ca^{2+} -mediated activation. Preventing calstabin 1 depletion from RyR1 using S107, a compound that binds to the RyR1 channel and enhances the binding affinity of calstabin1 to the nitrosylated RyR1 channel, inhibited Ca^{2+} leakage from the SR, reduced damage, improved muscle function, and increased exercise performance in *mdx* mice⁷.

The role of SERCAs in dystrophic muscle

SERCAs are major regulators of $[\text{Ca}^{2+}]_i$ in skeletal muscle, using the energy released from the hydrolysis of ATP to transport Ca^{2+} ions from the cytosol into the lumen of the SR²⁵. SERCAs are 110 kDa integral membrane proteins belonging to the P-type family of cation transporters,

characterized by phosphorylation of a critical aspartate residue during their catalytic cycle²⁶. In vertebrates, there are three distinct genes for SERCA (*ATP2a1/Serca1*, *ATP2a2/Serca2*, *ATP2a3/Serca3*) that produce more than 10 distinct isoforms as a result of alternative splicing²⁷. In adult skeletal muscle, two SERCA isoforms, SERCA1a and SERCA2a, predominate, and they are differentially expressed in specific muscle fiber types²⁷. Specifically, the SERCA1a isoform, which exhibits faster kinetics²⁷, is co-expressed with the fast myosin heavy chain (MHC) isoforms MHC IIb, MHC Iix, and MHC IIa, while the slower SERCA2a isoform²⁷ is co-expressed with the slow MHC isoform MHC I²⁸. SERCA serves as a central regulator of striated muscle performance and the pathological signaling pathway that drives cardiovascular and skeletal muscle disease¹³. Interestingly, a reduction in SERCA activity has been observed in dystrophic muscle²⁹, which is likely responsible for some of the aspects of defective Ca^{2+} handling in muscular dystrophy.

Because SERCAs control the depletion of Ca^{2+} from the cytosol and back into the SR, Mazala et al. investigated whether SERCA1 overexpression in the fast-twitch muscle fibers of *mdx* mice could positively affect the pseudo-hypertrophy and weakness that develops as a result of elevated $[\text{Ca}^{2+}]_i$ ³⁰. *Serca1* overexpression rescued phenotypic changes in dystrophic mice, including a reduction in pseudo-hypertrophy, decreased spinal curvature (i.e., kyphosis), fewer muscle fibers with centrally located nuclei, less necrosis, normalized serum creatine kinase levels, and reduced susceptibility to stretch-induced muscle damage. These data suggest that elevated intracellular Ca^{2+} may be responsible for a delay in repair processes, whereas decreasing Ca^{2+} by overexpression of *Serca1* reduces muscle damage and pseudo-hypertrophy in the muscle of dystrophic mice. On the other hand, the maximal isometric force of the quadriceps normalized to muscle mass was not improved in *mdx* mice by *Serca1* overexpression. Thus, muscle weakness was not completely prevented by *Serca1* overexpression in dystrophic muscle.

Regulatory SERCA activities

Recently it was discovered that the small peptides phospholamban (PLN) and sarcolipin (SLN) regulate muscle contractility by diminishing the rate of Ca^{2+} reuptake into the SR by direct modulation of SERCA pump activity³¹⁻³³.

Monomeric, unphosphorylated PLN, a 52-amino acid peptide³¹, binds to SERCAs at low ($<1 \mu\text{M}$) Ca^{2+} concentrations and inhibits SERCAs from cycling by affecting the rate of SERCA Ca^{2+} uptake as well as net ATP hydrolysis³². However, under higher Ca^{2+} concentrations, PLN dissociates from SERCAs, allowing the pump to become fully activated³¹. Similarly, during adrenergic activation, phosphorylation of PLN at Ser16 and/or Thr17 in the heart relieves inhibition of SERCAs, accelerating Ca^{2+} uptake and muscle relaxation.

SLN is a 31-amino acid peptide with a molecular weight of 3733 Da³³. The closely related gene structure, and the similarity in protein sequences of the transmembrane regions of PLN and SLN led to the conclusion that these peptides originated from the same family. Similar to PLN, the N-terminus of SLN is exposed to the cytosol and the C-terminus is located on the luminal side³³. However, there are conflicting views on the regulation of SERCAs by SLN that emerged from *in vivo* studies in skeletal and cardiac muscles. Nonetheless, recent *in vivo* studies of *SLN* gene transfer in wild-type mice as well as *SLN*-transgenic mice showed that *SLN* by itself is capable of regulating SERCAs and affecting muscle contraction^{34,35}. However, PLN and SLN are not expressed in most adult skeletal muscles in mice, suggesting the possible existence of other SERCA regulatory factors in adult muscles. Anderson et al. reported that a newly identified micropeptide, myoregulin (MLN), forms a stable complex with SERCAs in the membrane of the SR, and that MLN also directly influences Ca²⁺ levels in the SR as well as maximal exercise performance³⁶. This 46-amino acid peptide had been unnoticed for years, hidden in an uncharacterized vertebrate transcript annotated as a long non-coding RNA. The tiny 138-bp open reading frame is encoded in the third exon of the *MLN* transcript, is highly conserved between mouse and human, and is expressed exclusively in skeletal muscle during embryogenesis and in adulthood. MLN, as well as PLN and SLN, comprises a single transmembrane α -helix that interacts specifically with SERCAs. This gene is controlled in both fast and slow fibers by a core muscle transcription circuit, with functional binding sites in its promoter for MyoD and MEF2, both of which regulate skeletal myogenesis.

In addition, Nelson et al., have discovered another novel conserved peptide of 34 amino acids encoded by a muscle-specific RNA that was previously also annotated as a putative long non-coding RNA³⁷. This peptide was named DWORF (Dwarf Open Reading Frame) and it shows structural similarity with the SERCA modulator family members PLN, SLN, and MLN. DWORF is also localized to the SR membrane, where it enhances SERCA activity by displacing all three SERCA inhibitors PLN, SLN, and MLN. In line with this mechanism, cardiomyocytes from DWORF-overexpressing mice display increases in peak Ca²⁺ transient amplitude and Ca²⁺ load in the SR compared to wild types, plus a reduced time constant for cytosolic Ca²⁺ decay during each contraction-relaxation cycle. Conversely, slow skeletal muscle lacking DWORF exhibits delayed Ca²⁺ clearance and relaxation because of reduced SERCA activity. DWORF is the only known endogenous peptide to activate the SERCA pump by physical interaction, thereby enhancing muscle contractility.

Of these factors modulating SERCA activity, *SLN* is expressed at an abnormally high level in dystrophic muscles and its levels correlate with a decreased maximal velocity

of SR Ca²⁺ uptake in the skeletal muscles of mild (*mdx*) and severe (*mdx:utr*^{-/-}) dystrophic mice³⁸. *SLN* upregulation in all skeletal muscles of *mdx* and *mdx:utr*^{-/-} mice highlights novel insights into SERCA dysregulation and abnormal [Ca²⁺]_i handling in the dystrophic environment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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