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Medicine in focus

Duchenne muscular dystrophy – What causes the increased membrane permeability in skeletal muscle?

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ABSTRACT

Duchenne muscular dystrophy is a severe muscle wasting disease caused by a mutation in the gene for dystrophin – a cytoskeletal protein connecting the contractile machinery to a group of proteins in the cell membrane. At the end stage of the disease there is profound muscle weakness and atrophy. However, the early stage of the disease is characterised by increased membrane permeability which allows soluble enzymes such as creatine kinase to leak out of the cell and ions such as calcium to enter the cell. The most widely accepted theory to explain the increased membrane permeability is that the absence of dystrophin makes the membrane more fragile so that the stress of contraction causes membrane tears which provide the increase in membrane permeability. However other possibilities are that increases in intracellular calcium caused by altered regulation of channels activate enzymes, such as phospholipase A_2, which cause increased membrane permeability. Increases in reactive oxygen species (ROS) are also present in the early stages of the disease and may contribute both to membrane damage by peroxidation and to the channel opening. Understanding the earliest phases of the pathology are critical to therapies directed at minimizing the muscle damage.

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1. Introduction

Duchenne muscular dystrophy (DMD) is a severe degenerative disease of muscle which affects boys who have a mutation in the dystrophin gene leading to absence of the dystrophin protein in muscle. Dystrophin is a cytoskeletal protein which links intracellular γ-actin of the cytoskeleton to a group of proteins in the cell membrane, the dystrophin-associated protein complex (DAPC). The DAPC is further linked to the extracellular matrix through laminin (Fig. 1A). In DMD not only is dystrophin absent, but the proteins of the DAPC are also greatly reduced (Ervasti and Campbell, 1991) while several other proteins normally associated with the DAPC show increased expression (Gervasio et al., 2008) (Fig. 1B). While the primary cause of the disease is the absence of dystrophin, the complex pathways which link the absence of dystrophin to the profound muscle wasting, inflammation and fibrosis observed at the end stage of the disease are unclear.

A cardinal feature of the disease, present from birth and before physical symptoms, is a very large elevation of plasma creatine kinase suggesting that there is increased permeability of the muscle surface membrane allowing soluble muscle enzymes to leak out of the cell. Early electron microscopy studies on DMD described focal disruptions of the surface membrane and noted contracture of the neighbouring myofibrils (Mokri and Engel, 1975). This first led to the hypothesis that damage to the surface membrane was an early feature of the disease and the suggestion that Ca^{2+} influx through a membrane defect might contribute to the disease. Experimentally the increased membrane permeability has been repeatedly confirmed by studies in which markers which are normally membrane impermeant, such as albumin and Evans Blue dye, can be found inside muscle fibres.

In order to understand the earliest phase of the disease, a key question is the mechanism whereby the absence of dystrophin exacerbates the increase in membrane permeability membrane. A popular view is that contraction can cause mechanical injury (membrane tears) and that, in the absence of dystrophin, the sarcolemma is more fragile and therefore predisposed to membrane tears (Petrof et al., 1993; Davies and Nowak, 2006). The purpose of this article is to review the evidence for the hypothesis that membrane tears are the cause of the increased membrane permeability. We believe the evidence for this hypothesis is weak and discuss alternative mechanisms for the increased membrane permeability.

2. Evidence for membrane tears

Muscles are subjected to stress and strain during normal contractions and these are exacerbated when the muscle is stretched

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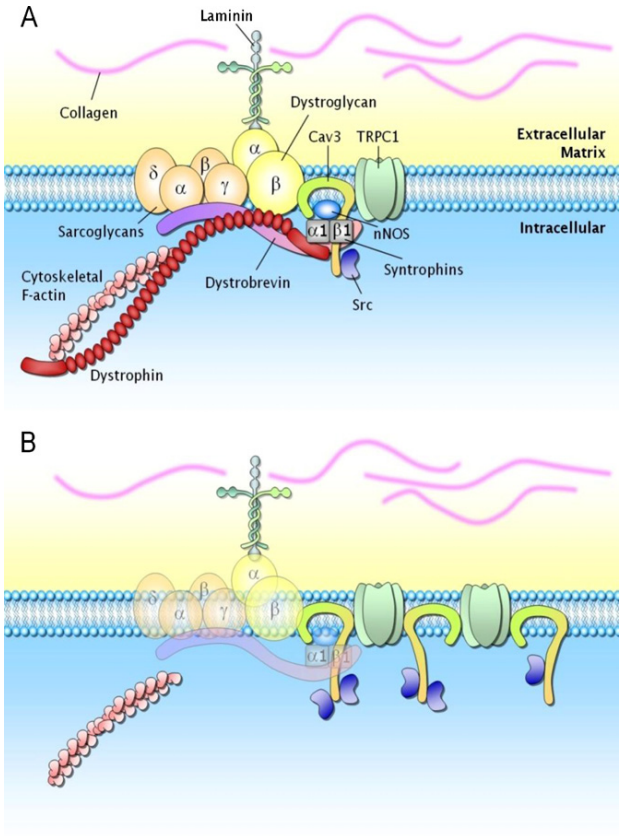

Fig. 1. Diagram showing the relationship between dystrophin, the dystrophin-associated protein complex, the surface membrane and the extracellular matrix. Panel A shows the arrangement in a wild-type muscle. Panel B shows some of the protein changes observed in dystrophic muscle.
From Allen et al. (2010)

by a large external force during a contraction (eccentric contraction). It has been known for many years that eccentric contractions in normal people lead to a mild form of muscle damage, characterised by weakness and delayed onset of swelling, stiffness and soreness. It is also known that leakage of creatine kinase from the muscle occurs during this delayed damage so that membrane permeability must be increased. An important observation, confirmed in many laboratories, is that muscles from the *mdx* mouse, which also lacks dystrophin, are much more susceptible to eccentric damage (Petrof et al., 1993). McNeil and Khakee (1992) used the entry of extracellular albumin to detect increased membrane permeability following eccentric contractions in normal muscles and found a large increase in the number of fibres containing albumin. They concluded that the most likely cause of this increase in permeability was 'focal membrane disruption caused by the imposition of mechanical force on the fragile membrane'. They later showed that in the *mdx* mouse this type of increased membrane permeability was greatly enhanced (Clarke et al., 1993). Note that these two papers, though often cited as evidence for the membrane tears, only provide evidence for stretch-induced membrane permeability; no direct evidence of membrane tears was offered.

How can membrane tears be experimentally distinguished from other causes of increased membrane permeability? Membrane defects can be produced artificially in muscles to examine the properties and repair of defects. A recent approach to this issue is to burn holes in the membrane with a powerful laser (Bansal et al., 2003). Using fluorescent markers they were able to show that repair occurs in less than one minute. Importantly in this study they found that repair was not different in *mdx* muscle fibres. These considerations suggest that the 'signature' of membrane tears would be (i) appearance of increased permeability synchronous with the eccentric contraction and (ii) the increased permeability would disappear after repair, which appears to be a minute or so. Judged by these criteria the study by McNeil and Khakee (1992) is inconclusive since the exercise period was 1 h so that there was a delay of between 0 and 1 h between contractions and the assessment of permeability. We have imaged Ca^{2+} and Na^{+} inside fibres during and immediately after a single eccentric contraction to determine whether we could detect highly localized regions of Ca^{2+} or Na^{+} as a consequence of membrane tears (Yeung et al., 2005). We have never successfully observed such events but this negative result does not rule out small or transient tears.

3. Alternative explanations for increased membrane permeability after stretched contractions

As noted above, if the cause of increased permeability were membrane tears one would predict increases in permeability starting immediately after the stretched contraction and persisting only for a minute or so. Instead there is a slow increase in intracellular Na^{+} ($[Na^{+}]_i$) and $[Ca^{2+}]_i$ starting after the contractions and reaching a maximum after 10–20 min (for review see Allen et al., 2010). Furthermore the increase in ion levels is eliminated by drugs which block stretch-activated channels suggesting that the rise in $[Ca^{2+}]_i$ and $[Na^{+}]_i$ following stretched contraction is caused by channel activation rather than membrane tears (Sonobe et al., 2008; Yeung et al., 2005). A similar conclusion was reached by (McBride et al., 2000) from studies of membrane depolarization in muscles subjected to stretched contractions. These results establish that opening of stretch-activated channels can explain increases in $[Na^{+}]_i$, $[Ca^{2+}]_i$ and depolarization.

What then is the cause of the membrane permeability to large molecules if it is not membrane tears? Early studies showed that increasing $[Ca^{2+}]_i$ with ionophores or Ca^{2+} channel opening drugs led to loss of enzymes from muscle and that protection was provided by phospholipase A_2 inhibitors and by ROS scavengers (Duncan and Jackson, 1987; Howl and Publicover, 1990). Thus it appears that elevated $[Ca^{2+}]_i$ permeabilises the membrane either through activation of phospholipase A_2 or by excessive production of ROS leading to lipid peroxidation. A feature of this pathway is that the increased permeability will be delayed at least until a sufficient rise in $[Ca^{2+}]_i$ has occurred which on current evidence would seem likely to be many minutes. In support of the channel activation theory, we have shown that dye uptake increased progressively over 60 min after stretched contractions in *mdx* muscle and that blockers of stretch-activated channels prevented most of this increased membrane permeability (Whitehead et al., 2006). In addition we have shown that minimizing the ROS increase with ROS scavengers reduces the membrane permeability (Whitehead et al., 2008).

4. Role of absence of dystrophin

On the interpretation described above key events in the development of increased membrane permeability are (i) the elevation of $[Ca^{2+}]_i$ and (ii) the production of ROS. The evidence discussed above suggests that the elevated $[Ca^{2+}]_i$ arises through activation of a Ca^{2+} permeable channel, probably a stretch-activated channel. A series of electrophysiological studies have demonstrated increased channel activity in *mdx* muscles but the identity of the channel remains unclear (for review see Allen et al., 2010). A key issue is why these channels are more active in DMD or, more specifically,

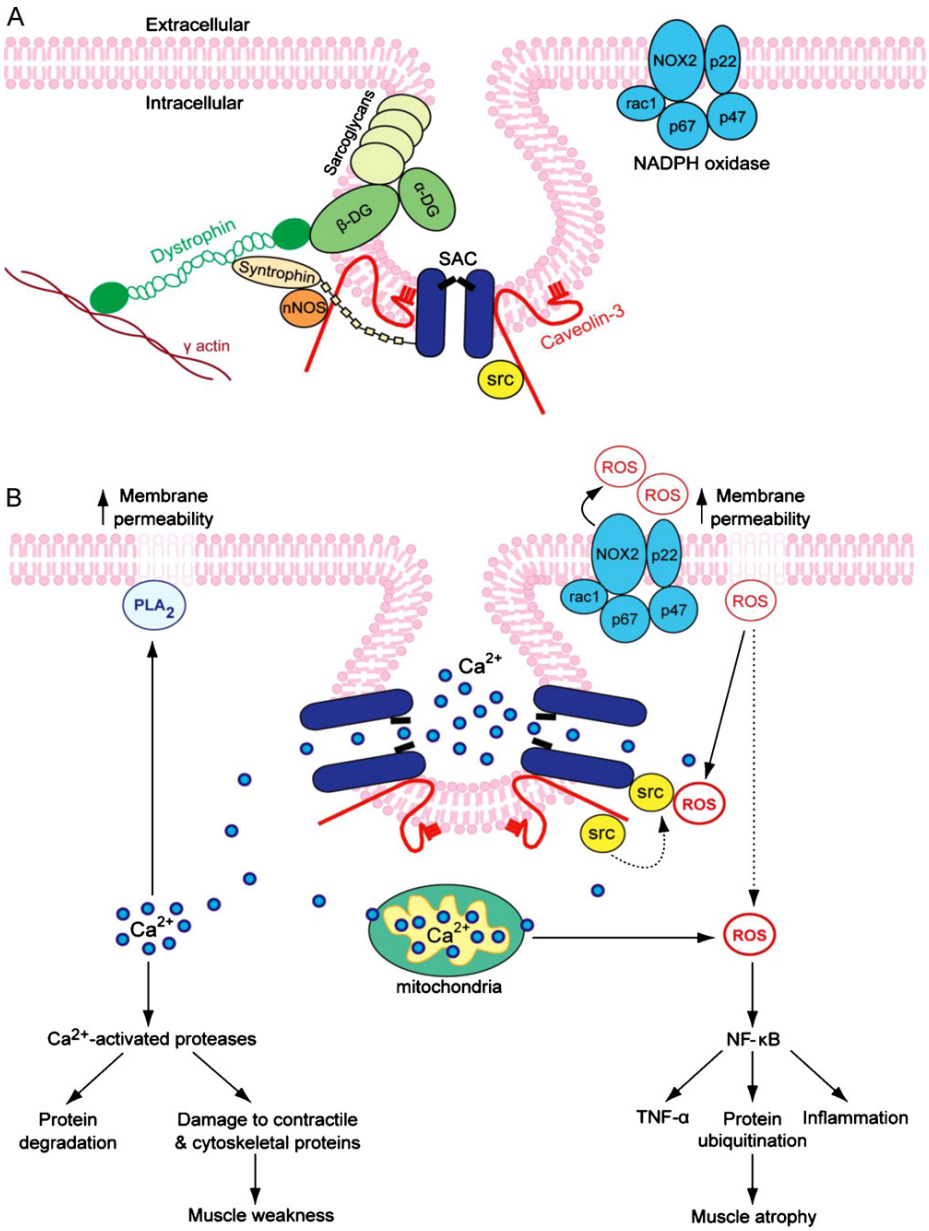

Fig. 2. Schematic diagram illustrating the relation between dystrophin, the dystrophin-associated protein complex, the caveolae and proteins associated with the caveolae. Panel A shows relationships in a normal muscle. Panel B illustrates some of the changes found in dystrophic muscle and shows some of the damage pathways activated in dystrophic muscle. Abbreviations; α and β DG, dystrophoglycans; nNOS, neuronal nitric oxide synthase; SRC, the sarcoma tyrosine kinase; NOX2, the catalytic unit of NADPH oxidase and associated sub-units; SAC, stretch-activated channel; PLA_2, phospholipase A_2.

the pathway by which the absence of dystrophin triggers channel activity. Fig. 2 indicates one scheme involving abnormal activity in the caveolae.

Dystrophin connects the cytoskeletal actin to the DAPC and there is preferential connection at the site of the costamere, where multiple connections between the contractile machinery and membrane proteins are located (Porter et al., 1992). Dystrophin binds to β-dystroglycan which also binds caveolin-3, the scaffolding protein of the caveolae. In DMD dystrophin is absent and all the DAPC are down-regulated whereas caveolin-3 shows increased expression (for review see Davies and Nowak, 2006). Perhaps as a further consequence, caveolae are increased in frequency but disordered in location in DMD (Shibuya et al., 2002). Caveolins have an intimate role in muscle disease; mutations of caveolin-3, the muscle specific caveolin are associated with several muscle diseases and overexpression of caveolin-3 leads to a severe dystrophic disease (for review see Gazzerro et al., 2010).

The mechanism by which caveolin-3 is associated with muscle disease is unclear. Caveolae concentrate many key proteins in a limited region and this proximity is thought to facilitate interaction and regulation. Thus L-type Ca^{2+} channels, Na^+ channels, various K^+ channels, the Na/Ca exchanger, SRC kinase, PLC, nNOS, and TRPC1 have all been shown to locate to caveolae or bind caveolin-3 (Balijepalli and Kamp, 2008). Of these L-type Ca^{2+} channels (Friedrich et al., 2004), Na^+ channels (Hirn et al., 2008) and a variety of stretch-activated and store-activated channels have all been shown to function abnormally in dystrophic muscle (for review see Allen et al., 2010). Thus it seems that loss of dystrophin disturbs caveolin function and leads to abnormal activity of channels located in caveolae.

Assuming that TRPC1 protein forms or contributes to the stretch-activated channel (Maroto et al., 2005), its known binding partners include α-syntrophin, Homer-1 and caveolin-3 (Sabourin et al., 2009a). Thus the stretch-sensitivity of the channel could arise through mechanical connections with any of these. Why is the stretch-activated channel more active in the absence of dystrophin? Currently this is unknown though there are many possibilities. For instance, absence of dystrophin may modulate the mechanical properties of the above TRPC1 binding proteins. Another possibility is that increased ROS production sensitizes the channel and this is supported by the observations that ROS scavengers reduce stretch-induced Ca^{2+} entry in *mdx* muscles (Gervasio et al., 2008). The proposed mechanism was that ROS activated SRC kinase which phosphorylated TRPC1 leading to increased stretch-sensitivity (Fig. 2B). Many other possible pathways have been proposed (Boittin et al., 2006; Stiber et al., 2008; Sabourin et al., 2009b) and this is a very active research area.

There are also studies suggesting that TRPC1 may not be the source of additional Ca^{2+} entry in muscular dystrophies. Gottlieb et al. (2008) reinvestigated their earlier conclusion (Maroto et al., 2005) and were no longer able to express TRPC1 in the membrane of cell lines weakening their earlier conclusion that SAC_{NSC} was encoded by TRPC1. On a more positive note, Kanzaki et al. (1997) have identified a growth-factor regulated Ca^{2+} permeable channel which was later found to be stretch-activated (Nakamura et al., 2001). This channel appears to be encoded by TRPV2, is overexpressed in the *mdx* mouse and when inactivated in the *mdx* mouse leads to amelioration of the symptoms (Iwata et al., 2009). Thus the identity of the SAC_{NSC} and the Ca^{2+} entry pathway in DMD still remains uncertain.

The source of the ROS in normal and *mdx* muscle is another source of uncertainty. NADPH oxidase has recently been identified in muscle and the main catalytic sub-unit, NOX2, is usually found in lipid rafts, areas of the membrane enriched in cholesterol and sphingomyelins, which included caveolae (Ushio-Fukai, 2009). We have recently shown in *mdx* skeletal muscle, as previously demonstrated in *mdx* cardiac muscle (Williams and Allen, 2007) that NADPH oxidase is an important source of ROS (Whitehead et al., 2010; see Fig. 2). This data is consistent with the finding that NADPH oxidase contributed to Ca^{2+} spark production induced by hypotonic swelling in *mdx* muscle (Shkryl et al., 2009). There is also evidence that mitochondrial changes, triggered by increased Ca^{2+}, may cause increased production of ROS by mitochondria (Burelle et al., 2010). The increased production of ROS may have multiple consequences (Fig. 2B) and, in particular, activation of the transcription factor, NF-κB, which induces the expression of a series of pathway contributing to the inflammation, fibrosis and atrophy. These pathways may have a major role the end-stage of the disease (Kumar et al., 2004). The elevated $[Ca^{2+}]_i$ will also have many downstream consequences including the activation of calpains (Spencer and Mellgren, 2002).

5. Conclusions

Currently there is no treatment for DMD which prevents or reverses the inevitable progression of the disease. There are high hopes that gene or stem cell therapy in one of its many forms will eventually replace the missing dystrophin and lead to a definitive treatment. The most advanced clinical approach is anti-sense oligonucleotide treatment which allows synthesis of a shortened dystrophin with the mutated exon deleted (Kinali et al., 2009). However the best expectation for this treatment is to convert Duchenne MD to Becker MD, a serious disease but with a much better prognosis. Thus there remains scope, both now and even after the possible introduction of gene therapy, for therapies which are capable of minimizing the muscle damage and slowing progression of the disease. We argue that the earliest common events after dystrophin loss would be the most effective stage to target. For this reason improved understanding of the early consequences of dystrophin loss are critical. In our view the focus on 'membrane tears' has impeded attempts to understand in detail how the increased membrane permeability occurs. There is substantial agreement that a number of channels located in the caveolae malfunction in DMD and that a calcium-permeable channel is a critical early stage in the disease. Identification of this channel and improved understanding of its regulation appear essential to better understanding of the disease and possible approaches to therapy.

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