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Altered Gene Expression Pathways in Duchenne Muscular Dystrophy

Nevenka Juretić, Francisco Altamirano, Denisse Valladares and Enrique Jaimovich
Centro de Estudios Moleculares de la Célula, Facultad de Medicina, Universidad de Chile, Santiago, Chile

1. Introduction

Duchenne muscular dystrophy (DMD) is caused by the absence of functional dystrophin (Blake et al. 2002). Dystrophin is a cytoskeleton protein normally expressed in the inner face of the plasma membrane (Ahn and Kunkel 1993). In normal skeletal muscle, dystrophin is associated with a complex of glycoproteins known as dystrophin-associated proteins (DAPs), providing a linkage between the extracellular matrix (ECM) and cytoskeleton (Batchelor and Winder 2006). Lack of dystrophin in dystrophic muscle results in loss of the complex integrity and allegedly impairs the stability of the plasma membrane causing mechanical stress fragility, and an increase in Ca^{2+} permeability (Alderton and Steinhardt 2000). But the pathophysiology of muscular dystrophy is not only explained by this increased mechanical fragility and a role for dystrophin and DAPs has been suggested as being part of a protein signaling complex involved in cell survival (Rando 2001). In this chapter we discuss evidence of such a role, which may evidence possible interactions between dystrophin and proteins other than those involved in DAP and possible cell location of dystrophin in regions other than the sarcolemma cytoskeleton.

2. Calcium homeostasis

Ca^{2+} is a highly versatile second messenger that can regulate several cellular functions. Skeletal muscles use Ca^{2+} for contraction process and as regulatory signaling molecule. Subsequently, muscle plasticity is closely related with calcium signals (Berchtold et al. 2000).

Under resting conditions, wild type *(wt)* skeletal muscle cells maintain the cytosolic calcium concentration ($[Ca^{2+}]_i$) around 100-120 nM (Lopez et al. 1987; Eltit et al. 2010). Since the chemical gradient between $[Ca^{2+}]_i$ and extracellular medium or sarcoplasmic reticulum (SR) is about 10,000 fold, to constantly keep the $[Ca^{2+}]_i$ in the nM range, skeletal muscle cells uses a complex machinery to finely regulate calcium concentration. Plasma membrane Ca^{2+}-ATPase (PMCA), Na^+/Ca^{2+} exchanger (NCX) in the plasma membrane and the SR Ca^{2+}-ATPase (SERCA) extrudes the Ca^{2+} to extracellular space or to SR, respectively. These functions are opposed, under resting conditions, for the SR Ca^{2+} leak type-1 ryanodine receptor (RyR1) channels and the basal sarcolemma Ca^{2+} influx (Eltit et al. 2010).

2.1 Altered resting calcium in DMD

Several reports demonstrate that the $[Ca^{2+}]_i$ is elevated in *mdx* mice and DMD human fibers (Lopez et al. 1987; Yeung et al. 2005; Allen et al. 2010). Lopez et al. (1987) have shown that the $[Ca^{2+}]_i$ in DMD muscle fibers is 370 nM, while in normal muscle fibers was around 100 nM (Lopez et al. 1987). Similar results were obtained in *mdx* adult fibers compared with the *wt* counterpart (Yeung et al. 2005; Allen et al. 2010). The authors demonstrated that $[Ca^{2+}]_i$ was elevated under resting conditions in *mdx* fibers and when the fibers were exposed to stretch-induced damage, $[Ca^{2+}]_i$ increased to higher levels, around 700 nM (Yeung et al. 2005; Allen et al. 2010).

Increased $[Ca^{2+}]_i$ has been related with necrosis through calpain activation and mitochondrial permeability transition pore (MPTP) (Turner et al. 1988; Spencer et al. 1995; Millay et al. 2008).

The mechanism that has been proposed for dystrophin function involves a role in sarcolemma stabilization, so in muscle fibers that lack this protein, membrane damage would be recurrent (Petrof et al. 1993; Mokri and Engel 1998). These evidences suggested the hypothesis of Ca^{2+} leak into the cell through damaged membrane. There are several evidences in *mdx* muscle fibers that relate the calcium entry with the transient receptor potential channels (TRPC1) and the store-operated calcium entry (SOCE) mechanism. TRPC1-dependent calcium entry is increased in *mdx* muscle fibers (Vandebrouck et al. 2002; Yeung et al. 2005; Gervasio et al. 2008). The blockage of these cationic channels with streptomycin or spider venom toxin (GsMTx4) reduced $[Ca^{2+}]_i$ and prevented the rise of the $[Ca^{2+}]_i$ following stretch (eccentric) contractions. This maneuver, partially reduced the decline in both the tetanic Ca^{2+} increase and force (Yeung et al. 2005; Allen et al. 2010). Gervasio et al. 2008 showed that TRPC1, caveolin-3 and Src-kinase protein levels are increased in *mdx* muscle (Gervasio et al. 2008). The authors propose that the stretch-induced muscle damage and the increase in the $[Ca^{2+}]_i$ is produced by the ROS production, activation of Src-kinase and TRPC-induced Ca^{2+} entry. Furthermore, administration of streptomycin reduced muscle damage and increased myofiber regeneration (Yeung et al. 2005).

More recently, store-operated calcium entry has been implicated in the exacerbated resting Ca^{2+} entry observed in *mdx* fibers (Boittin et al. 2006; Vandebrouck et al. 2006; Edwards et al. 2010). These Ca^{2+} entries are modulated by a Ca^{2+}-independent phospholipase A_2, which is overexpressed in dystrophic fibers (Boittin et al. 2006). Vandebrouck et al. (2005) demonstrate that the high store-operated Ca^{2+} transients observed in dystrophin-deficient myotubes were associated with sustained cytosolic Ca^{2+} transients and high intra-mitochondrial entries, that can be reduced by mini-dystrophin expression or FCCP (uncoupler of oxidative phosphorylation) (Vandebrouck et al. 2006). In addition, the thresholds for SOCE activation and deactivation occur at higher $[Ca^{2+}]_{SR}$ and the proteins levels of STIM1 and Orai1 was 3-fold increased in *extensor digitorum longus* (EDL) muscles from *mdx* mice (Edwards et al. 2010).

2.2 SR Ca^{2+} loading capacity

There is a controversy about the loading capacity of the SR $[Ca^{2+}]_{SR}$ in dystrophic skeletal muscle cells compared with normal skeletal muscle cells. Roberts et al. (2001), using a Ca^{2+}-sensitive photoprotein aequorin chimera with SR destination sequence, show that after SR

Ca^{2+} depletion, the re-addition of Ca^{2+} to the media increases the $[Ca^{2+}]_{SR}$ rapidly up to a steady state that is 50% higher that the *wt* myotubes (Robert et al. 2001). In contrast, Culligan et al. (2002) shows a reduction in Ca^{2+} binding in the SR microsomes from *mdx* mice, associated with a drastic reduction in the calsequestrin-like proteins and normal SERCA1 expression and activity (Culligan et al. 2002). However, a reduction in SERCA activity has been observed in dystrophic muscle (Kargacin and Kargacin 1996; Divet et al. 2005), which could account for the increased $[Ca^{2+}]_i$. SERCA1a overexpression in *mdx* diaphragm muscle by adeno-associated virus gene transfer, resulted in a reduction of centrally located nuclei and reduced susceptibility to eccentric contraction-induced damage (Morine et al. 2010). More recently, δ-sarcoglycan-null and *mdx* mice transgenic animals that overexpress SERCA1, show a reduction in myofiber central nucleation, tissue fibrosis and serum creatine kinase levels. In addition SERCA1 overexpression enhances excitation-contraction (E-C) coupling and restore the $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SR}$ in both dystrophic models (Goonasekera et al. 2011).

2.3 Excitation-Contraction (E-C) coupling

The proteins involved in E-C coupling are normally expressed in dystrophic muscle. The expression of α1-, α2- and β-subunits of the dihydropyridine receptor (DHPR) are similar in microsomes from control and *mdx* mice (Culligan et al. 2002). RyR1 and SERCA1 are also found in comparable amounts in control and dystrophin-deficient muscles (Culligan et al. 2002).

In skeletal muscle cells, membrane depolarization induces a conformational change in Cav1.1 DHPRs that is transmitted to the ryanodine receptor (RyR1), causing it to release Ca^{2+} from the SR, that it is necessary for the contraction process.

Several evidences indicate that the dystrophic skeletal muscle cells have an unpaired E-C coupling. Comparisons of the cytosolic calcium transients evoked by single action potential have shown that the calcium transients are reduced in *mdx* fibers compared with *wt* fibers (Woods et al. 2004; Hollingworth et al. 2008). Recently, similar results have been found in fibers from *utr -/- mdx* mice (Capote et al. 2010). Muscle weakness observed in isolated fibers from *mdx* mice and DMD patients has not been fully explained. The reduction in the Ca^{2+} transient evoked by single action potential, reduction in $[Ca^{2+}]_{SR}$ and increased $[Ca^{2+}]_i$ could provide a mechanism for contractile dysfunction and impaired force production in DMD patients.

3. Excitation-Transcription (E-T) coupling

We have previously described that membrane depolarization of skeletal myotubes evokes a fast Ca^{2+} transient during the stimuli, that promotes a contractile response through "E-C coupling", and a slow Ca^{2+} transient peaking 60-100 seconds later, mostly associated to cell nuclei (Jaimovich et al. 2000; Estrada et al. 2001; Powell et al. 2001; Araya et al. 2003; Cardenas et al. 2005). Slow Ca^{2+} transients are involved in the "E-T coupling" mechanism, which relates membrane depolarization with gene expression (Powell et al. 2001; Araya et al. 2003; Carrasco et al. 2003; Juretic et al. 2006; Juretic et al. 2007). The signaling pathway begins at the DHPR, which by a mechanism involving G protein (Eltit et al. 2006), activates

PI3 kinase and PLC to produce inositol 1,4,5-trisphosphate (IP_3) that diffuses in the cytosol and reaches IP_3 receptors (IP_3Rs) located both at the SR membrane and at the nuclear envelope, promoting Ca^{2+} release (Araya et al. 2003). IP_3 mediated Ca^{2+} signals induce both a transient activation of ERK½ and transcription factor CREB, and an increase in early genes (*c-fos, c-jun* and *egr-1)* and in late genes (troponin I, interleukin-6, hmox and hsp70) mRNA levels after depolarization of normal skeletal muscle cells (Carrasco et al. 2003; Juretic et al. 2006; Juretic et al. 2007; Jorquera et al. 2009). Moreover, in electrically stimulated adult muscle fibers, slow Ca^{2+} signals mediate the frequency-dependent activation of slow-phenotype muscle fiber genes (slow troponin I, TnIs) and repression of fast-phenotype ones (TnIf) (Casas et al. 2010). These evidences link slow Ca^{2+} transients with muscular effects of nerve activity and with the process of muscle cell plasticity.

Recently we described a new role for ATP signaling in skeletal muscle in a process called "E-T" coupling (Buvinic et al. 2009, see Fig.1). We were able to show that the main ATP efflux pathway is through pannexin 1 hemichannels. We know that DHPR receptors and pannexin 1 interact with each other but it is not clear whether it is a direct interaction. The ATP released will locally activate the purinergic receptors P2X and P2Y localized in the membrane. This activation induces a transient increase in intracellular Ca^{2+} with specific kinetics. We demonstrated that ATP participates in the fast calcium transient related to contraction because apyrase (catalyses the hydrolysis of ATP) reduced the depolarization-evoked Ca^{2+} transient by about 20%. We can speculate that activation of P2X receptors may contribute to improve the skeletal muscle cells Ca^{2+} availability needed to sustain contractions. Moreover, we could also show that ATP participates in "E-T" coupling due to the total inhibition by apyrase of the second Ca^{2+} transient induced by depolarization. Additionally, the use of apyrase during the electrical stimulation completely abolished the increase in gene expression related with muscle plasticity (unpublished data). We can conclude that gene expression is regulated through activation of P2Y receptors mediated by the ATP released during depolarization.

3.1 Extracellular ATP: a major mediator for signal transduction

ATP for a long time was considering as a molecule that was involved with energy and metabolism of many cells. Nevertheless in the last few years ATP has been considered as an extracellular messenger for autocrine and paracrine signaling (Corriden and Insel 2010). It has been described as a regulator of inflammation, in embryonic and stem cell development, ischemia, among others (Bours et al. 2006; Burnstock and Ulrich 2011). In skeletal muscle ATP has been implicated in the regulation of proliferation, differentiation and regeneration (Ryten et al. 2002; Ryten et al. 2004) and also promoting the stabilization of the neuromuscular junction (Jia et al. 2007).

ATP release is induced in response to several kinds of stress in many cells type, including hypoxia, ischemia, osmotic swelling and mechanical stimulation (Corriden and Insel 2010). ATP can exit cells using several different purinergic signal efflux pathways (Fitz 2007). The main source of extracellular ATP is cell lysis, which occurs when massive cell death takes place during trauma, injury or inflammation. A non-lytic source of ATP is the release of secretory granules during stimulated exocytosis, which occurs in secretory cell types like epithelial cells of the liver, lung, kidney, neurons and astrocytes (Volonte and D'Ambrosi

2009). A non-lytic, and also non-exocytotic release of ATP can occur by channel- or transporter-mediated mechanisms, such as: (a) hemichannels, such as connexins and pannexin (Dubyak 2009); (b) anion channels, such as plasmalemma voltage dependent anion channel, voltage-dependent maxi-anion channel, volume sensitive Cl^- channel and P2X7 receptor (Sabirov and Okada 2005; Suadicani et al. 2006; Liu et al. 2008); (c) ATP-binding cassette transporters, such as cystic fibrosis transmembrane conductance regulator Cl^- channel and P-glycoprotein (Campbell et al. 2003; Sabirov and Okada 2005); and (d) exchange carriers such as ADP/ATP exchange carrier (Sabirov and Okada 2005; Volonte and D'Ambrosi 2009). Several studies have recently demonstrated that ATP can be released by pannexin hemichannels in a variety of cells types that include myotubes (D'Hondt et al. 2011). Pannexin is widely distributed among tissues with cell communication via calcium waves (Shestopalov and Panchin 2008). The channel formed by this protein can be opened by mechanical perturbation at the resting membrane potential. The channel is permeable for ATP and it can be opened at physiological calcium concentration (Barbe et al. 2006). These properties make pannexin 1 (Panx1) a very attractive candidate for an ATP-releasing channel. The widespread distribution of Panx1 has been confirmed in a variety of human tissues, with the highest levels being found in skeletal muscle (Baranova et al. 2004). Results of our laboratory indicate that this hemichannel is expressed in myotubes and adult fibers of rat and mouse.

Once released, ATP acts as an extracellular signal trough the binding to purinergic receptors expressed in most cell types. Purinergic receptors comprise both ionotropic P2X receptor subtypes and G-protein-coupled P2Y receptor subtypes (Burnstock 2004). Between the purinergic receptors and the purine-generating reactions, there exist purino-converting enzymes. These enzymes named ectonucleotidases, consist of several different families with well-characterized molecular and functional features (Yegutkin 2008). They operate to metabolize nucleotides down to the respective nucleoside analogues, thus having the potential to decrease the extracellular concentrations of nucleotides. Consequently these enzymes modulate ligand availability at both nucleotide and nucleoside receptors (Yegutkin 2008). The contribution of the diverse ectonucleotidases to the modulation of purinergic signaling depends on their availability of different ectonucleotidases and their selectivity for substrates, but also on their abundance and cell distribution (Volonte and D'Ambrosi 2009).

ATP signaling has been implicated in many cell functions ranging from proliferation, differentiation, toxic actions, neurotransmission, smooth and cardiac muscle contraction, vasodilation, chemosensory signaling and secretion, to complex phenomena such as immune responses, male reproduction, fertilization, embryonic development, and so on (Burnstock 2004). This vast heterogeneity of their biological responses is influenced by different parameters such as the presence of endogenous ligands at receptor sites and the time and distance from the source of release; the concentration gradient of a ligand that simultaneously can activate more than one receptor subtype; the different composition of purinergic receptors in a given cell, or even more the composition in the diverse sub membrane compartments in which each ligand operates (Volonte and D'Ambrosi 2009).

3.2 Purinergic receptors

Purinergic receptors are subdivided into two major groups: eight G-protein-coupled seven-transmembrane P2Y subunits ($P2Y_{1,\ 2,\ 4,\ 6,\ 11\text{–}14}$), and seven P2X ligand-gated ion channels

($P2X_{1-7}$). These two types of receptor have larger differences in their aminoacid sequences, molecular/physiological properties and relative sensitivities to ATP, with ranges of nanomolar for P2Y, low micromolar for most P2X, to high micromolar for P2X7. Moreover the complexity of these receptors is augmented because both subtypes can form homomers and heteromers and these different combinations can change the agonist and antagonist selectivity, transmission signaling, channel and desensitization properties (Nakata et al. 2004).

P2X receptors are ATP-gated ion channels that mediate sodium influx, potassium efflux and, to varying extents, calcium influx, leading to depolarization of the cell membrane. Membrane depolarization subsequently activates voltage-gated calcium channels, thus causing accumulation of calcium ions in the cytoplasm. The predicted structure of the P2X subunits is a transmembrane protein with two membrane spanning domains that are involved in gating the ion channel and lining the ion pore (Surprenant and North 2009). Functional P2X receptor ion channels are now thought to consist of three subunits that could be homomers and heteromers (North 2002). The different combinations present different desensitization and permeability properties, as well as agonist and antagonist specificities. P2X receptors are widely distributed, and in neurons, glial cells, bone, muscle, endothelium, epithelium, and hematopoietic cells, they have functional roles. Moreover, several studies have implicated these receptors in the pathophysiology of Parkinson's disease, Alzheimer's disease, and multiple sclerosis (Jarvis and Khakh 2009).

P2Y receptors are G-protein-coupled receptors (GPCRs) that are activated by purine and/or pyrimidine nucleotides. Like other members of the GPCR superfamily, they are composed of seven transmembrane spanning regions that assist in forming the ligand binding pocket and also the purinergic receptor (Abbracchio et al. 2006). Stimulation leads to activation of heterotrimeric G proteins and their dissociation into α and $\beta\gamma$ subunits that can then interact with a variety of effector proteins. Some of P2Y receptors are activated mainly by nucleoside diphosphates ($P2Y_{1,6,12}$), while others are activated mainly by nucleoside triphosphates ($P2Y_{2,4}$). Otherwise, some P2Y receptors are activated by both purine and pyrimidine nucleotides ($P2Y_{2,4,6}$), and others only by purine nucleotides ($P2Y_{1,\ 11,\ 12}$) (Jacobson et al. 2009). Each individual P2Y receptor subtypes can couple to distinct G proteins that are specific for each cell type or tissue. The abilities to activate different G proteins were inferred from their capability to induce increases in inositol tris-phosphate, cytoplasmic Ca^{2+}, or cyclic AMP levels, and determination of sensitivity to the Gi/o protein inhibitors pertussis toxin (PTX) (Abbracchio et al. 2006). P2Y receptors can also be coupled to the activation of monomeric G proteins like Rac and RhoA. Even more, in the last few years many studies have revealed that a cross-talk exist between different GPCRs and their downstream effectors as well as between GPCRs and other signaling proteins, such as ion channels, integrins, and receptor and non-receptor tyrosine kinases (von Kugelgen 2006). These properties explain how the activation of particular P2Y receptors can lead to the induction of more than one signaling pathway in the same cell type. These receptors are able to regulate many different functions in a variety of cell types, and for that reason an intense effort has been developed to design selective agonist and antagonist ligands, both as pharmacological tools and as potential therapeutic agents (Abbracchio et al. 2003; Brunschweiger and Muller 2006). For cystic fibrosis, dry eye disease, and thrombosis the application of P2Y receptor ligands has been tested as drug candidates. The development of

new chemical compounds will provide new opportunities for therapeutics of several diseases, including cardiovascular diseases, inflammatory diseases, and neurodegeneration (Jacobson and Boeynaems 2010).

Between the many functions that P2 receptors can regulate is ion channel activity. The studies have been performed mainly in neurons, in which specific P2 subtype can regulate the N-type Ca^{2+} channel and the M-current K^+ channel. Nevertheless, recent studies have demonstrated that P2 receptors can induce fast inhibitory junction potential in rat colon (Grasa et al. 2009), membrane hyperpolarization in vascular endothelial cells (Raqeeb et al. 2011), Ca^{2+} influx mediated contraction in intestinal myofibroblasts (Nakamura et al. 2011), and contraction induced by electrical field stimulation in smooth muscle (Cho et al. 2010). These data suggest that ATP signaling is important in excitable cells for their normal function. In skeletal muscle there are many evidences of the importance of ATP signaling. The activation of P2 receptors has been associated with modulation of Ca^{2+} influx and signaling (Sandona et al. 2005; May et al. 2006), activation of the ERK½ (May et al. 2006), muscle contractility (Sandona et al. 2005; Grishin et al. 2006), and regulation of excitability of muscle fibers (Voss 2009; Broch-Lips et al. 2010). Also extracellular nucleotides play important functions during skeletal muscle development and regeneration (Ryten et al. 2002; Ryten et al. 2004). Importantly, it has been shown that ATP promotes differentiation of rat skeletal muscle satellite cells (Araya et al. 2004; Banachewicz et al. 2005).

4. Alterations in both IP_3Rs and E-T coupling in DMD models

We have described that the amount of IP_3Rs, as well as the total mass of IP_3, are largely increased in both an *mdx* mice derived cell line and in a human DMD derived cell line compared to normal cells (Liberona et al. 1998). In dystrophic skeletal muscle, it has been suggested that an alteration of Ca^{2+} homeostasis occurs and might be responsible for muscle degeneration (Turner et al. 1988; Turner et al. 1991). Several studies indicate that IP_3 pathways could be involved in the DMD pathophysiology (Liberona et al. 1998; Balghi et al. 2006a; Balghi et al. 2006b). We recently found that both expression and localization of IP_3Rs are different in normal and dystrophic human skeletal muscle and cell lines (Cárdenas et al. 2010). On the other hand, experiments performed using two types of myotubes originated from the same Sol8 cell line – dystrophin deficient myotubes, SolC1(-), and myotubes transfected to express the minidystrophin, SolD(+) - show that Ca^{2+} rise evoked by potassium depolarization was higher in SolC1(-) than in SolD(+) myotubes (Balghi et al. 2006a). Analysis of the kinetics of the Ca^{2+} rise, reveals that the slow IP_3-dependent release may be increased in the SolC1(-) as compared to the SolD(+), suggesting an inhibitory effect of mini-dystrophin on IP_3R-dependent K^+-evoked Ca^{2+} release (Balghi et al. 2006a). Moreover, it has been described that IP_3 production after membrane depolarization is significantly elevated in dystrophin-deficient myotubes and that the presence of mini-dystrophin under the membrane leads to reduced IP_3 production (Balghi et al. 2006a). In fact, we have recently demonstrated, using normal (RCMH) and dystrophic (RCDMD) human skeletal muscle cell lines, that IP_3 dependent, slow Ca^{2+} transients evoked by electrical stimulation are faster in dystrophic cells, compared to normal myotubes (Cárdenas et al. 2010). Electrical stimulation induced an important phosphorylation of ERK½ in normal but not in dystrophic cells, and a differential pattern of gene expression between cell lines.

In normal adult mice skeletal muscle, we observed that IP_3R immuno-labeling follows distinctive patters resembling the SR (types 1, 2 and 3 IP_3Rs), sarcolemmal (types 1 and 3 IP_3Rs) or nuclear localizations (types 1 and 3 IP_3Rs) (Casas et al. 2010). The labeling for both type 1 and type 2 IP_3Rs subtypes showed a fiber type-specific distribution with much higher expression in fast (type II) muscle fibers, whereas type 3 IP_3R showed a uniform distribution in both fiber types, as shown by co-labeling with slow myosin heavy chain antibody. Likewise, mice muscle fibers show a characteristic mosaic pattern for type 1 IP_3R (Casas et al. 2010). When human muscle was studied, type II muscle fibers showed a much more intense labeling for the IP_3R subtype 1 compared to type I (slow) fibers. In biopsies from DMD patients, we found that 24 ± 7% of type II fibers have totally lost type 1 IP_3R labeling, compared to age-matched control biopsies (Cárdenas et al. 2010). On the other hand, RCDMD cells show a five-fold over expression of type 2 IP_3Rs and down regulation of type 3 IP_3Rs compared to normal RCMH cells (Cárdenas et al. 2010). Unlike normal muscle cells, type 2 IP_3R locate in the nucleus in RCDMD cells, while type 1 and type 3 IP_3Rs also display a particular subcellular location for each line (Cárdenas et al. 2010). These results showed that IP_3Rs expression and localization are different in muscle affected by DMD.

5. Signaling by extracellular nucleotides in dystrophic skeletal muscle

A number of skeletal muscle pathologies have been associated with alterations in the metabolism of extracellular ATP, changes in the sensitivity towards ATP and altered expression of purinergic receptors; among these pathologies we have DMD. In recent works, ATP signaling has been implicated in abnormal calcium homeostasis in dystrophic muscle and proposed to have implications in the pathogenesis of muscular dystrophies. Moreover, in myoblasts of a dystrophin negative muscle cell line, exposure to extracellular ATP elicited a strong increase in cytoplasmic Ca^{2+} concentrations. This increased susceptibility to ATP was due to changes in expression and function of P2X receptors and proposed to be a significant contributor to pathogenic Ca^{2+} entry in dystrophic mouse muscle (Yeung et al. 2006). The plasma membrane Na^+/H^+ exchanger (NHE) has been proposed to be involved in the pathogenesis of muscular dystrophy, most probably through the sustained increase in intracellular Ca^{2+}. The mechanism by which NHE is constitutive activated appears to be through stimulation of P2 receptors with ATP being continuously released in response to stretching (Iwata et al. 2007).

Nevertheless, these works failed to explain the mechanism by which ATP is released from skeletal muscle. ATP in skeletal muscle was proposed to be co-released with acetylcholine from motor nerve terminals during nerve activation (Smith 1991; Silinsky and Redman 1996) and released from muscle fibers during contraction (Cunha and Sebastiao 1993; Hellsten et al. 1998). Dystrophic muscle would be expected to contain high levels of extracellular ATP due mainly to fiber injury.

We propose now that in skeletal muscle, ATP is released upon contraction or electrical stimulation mainly through activation of pannexin 1 hemichannels. Any disturbance in either pannexin 1 channels or changes in P2 receptors expression or activity will have implications in skeletal muscle normal function. The possibility that this system is altered in muscular dystrophies raises new possibilities of therapeutic strategies in the treatment of diseases like DMD.

In addition to the structural role for dystrophin and its known associated proteins, there is clear evidence for signal transduction roles. The best studied signaling protein linked is the nNOS pathway. In DMD nNOS appears to be either drastically reduced or even absent (Niebroj-Dobosz and Hausmanowa-Petrusewicz 2005). It has been propose that part of muscle degeneration in DMD may result from the reduction in the production of nNOS/NO (Niebrój-Dobosz, 2005). Lately many additional signaling pathways have been demonstrated to be altered in dystrophy, such as: nuclear factor kappa-B (NF-kB), tumor necrosis factor (TNF)-alpha and interleukin (IL)-6 (Messina et al. 2011). The precise role of these signaling pathways remains mysterious, it is interesting to investigate whether the abnormal regulation of one (or more) of these pathways contributes to skeletal muscle pathogenesis in dystrophy.

To address the different pathways that could be altered in muscular dystrophy, many studies have compared gene expression profile between normal and dystrophic muscle based on microarray analysis. These analysis have been done in patients with DMD and in *mdx* mice. These studies include different types of muscle and in different times of the human disease (Chen et al. 2000) or in different life periods of *mdx* mice (Porter et al. 2003b; Lang et al. 2004; Porter et al. 2004; Dogra et al. 2008). In DMD patients biopsies that were individually analyzed, the upregulated genes are related with ECM and cytoskeleton, muscle structure and regeneration, immune response, signal transduction and cell-cell communication (Chen et al. 2000). In the mouse model there are many gene expression studies. The main muscles studied are diaphragm, extraocular muscles and leg muscle groups (Porter et al. 2003b; Lang et al. 2004; Dogra et al. 2008). Among the results, it is worth mentioning that the response to the lack of dystrophin varies in different muscle groups of human and *mdx* mice, and it was proposed that changes in gene expression could be related with the progression of the disease (Porter et al. 2003b; Lang et al. 2004; Porter et al. 2004; Dogra et al. 2008). Moreover, some groups studied the profile of gene expression in skeletal muscle implicated in specific pathways such as regeneration (Turk et al. 2005), inflammation (Evans et al. 2009a), immune system (Evans et al. 2009b) and specific transcription factors (Dogra et al. 2008). Also there are some studies that propose that expression of utrophin in the *mdx* mouse muscle results in a gene expression profile that is similar to that seen for the *wt* mouse (Baban and Davies 2008).

The analysis performed by Porter et al. (2002) established that numerous pathogenic pathways in *mdx* skeletal muscles are closely related and share features with DMD (Porter et al. 2002). Among the genes that were increased in *mdx* muscle is purinergic receptor P2X. The $P2X_4$ up regulation in dystrophic muscle has been attributed to vascular permeability changes and to inflammatory responses (Porter et al. 2002). Later, Yeung et al. (2004) demonstrated that P2X4 were expressed in infiltrating macrophages in dystrophic human and mouse muscle, and could be related with the inflammatory process (Yeung et al. 2004). Jiang et al. 2005 demonstrated that there is a differential expression of P2X receptors that change during the progression of the disease in both human and mouse dystrophic muscle (Jiang et al. 2005). They found that the $P2X_1$ and $P2X_6$ receptors are expressed during the process of regeneration in mouse muscular dystrophy, and the expression of $P2X_2$ is associated with type 1 fibers. Nevertheless, the work of Yeung et al. (2006) demonstrated that increase in P2X receptors increased the susceptibility of dystrophic myoblasts to extracellular ATP (Yeung et al. 2006). They proposed that changes in P2X will significant contribute to pathogenic Ca^{2+} entry.

Moreover, studies of Ryten et al. (2002, 2004) identified a role for ATP in the regulation of skeletal muscle formation, through inhibiting the proliferation and increase the rate of differentiation of satellite cells (Ryten et al. 2002; Ryten et al. 2004) Later, they show that the $P2X_2$, $P2X_5$ and $P2Y_1$ receptors were strongly expressed in *mdx* skeletal muscle and in the cells known to be important for muscle regeneration.

As previously described, P2 receptors have been implicated in the alteration on intracellular calcium. This could also be releated with some of the signaling pathways that are dependent on calcium homeostasis, like the activation of proteases. It has been demonstrated that changes in intracellular calcium can activate calpain and proteolytic damage to sarcomer proteins, like titin (Goll et al. 2003; Zhang et al. 2008).

The original sarcoglycan (SG) complex has four subunits and comprises a subcomplex of the dystrophin-associated protein complex (Hack et al. 2000). Gene defects in α-sarcoglycan also lead to a severe muscular dystrophy, type 2D limb-girdle muscular dystrophy (Roberds et al. 1994). The role of sarcoglycans in dystrophin complex function is not entirely understood. The α-sarcoglycan was described as an ecto-ATPase with distinctive enzymatic properties *in vitro* (Betto et al. 1999). Later on, α-sarcoglycan was demonstrated to significantly contribute to total ecto-nucleotidase activity of C2C12 myotubes and during the differentiation of this cell type (Sandona et al. 2004). As a result, mutations of the α-sarcoglycan gene causing the loss of its enzymatic function could represent an important mechanism to explain the pathogenesis mechanisms leading to dystrophy.

Taken these studies together, we can conclude that modifications in ATP signaling, due to changes in ATP release mechanism or receptors expression and availability, could be implicated in several mechanisms potentially involved in diseases. For these reasons ATP signaling has been considered as a good candidate for therapeutic targets for the treatment of muscle diseases

6. Gene expression in DMD

Microarrays analysis has been the basis of a number of publications in which dystrophic muscle is compared with unaffected muscle. Gene expression comparison of human biopsies from DMD and normal skeletal muscle has shown that many of the differentially expressed genes reflect in histo-pathology changes. For example, immune response signals and ECM genes are overexpressed in DMD muscle, an indication of the infiltration of inflammatory cells and connective tissue (Haslett et al. 2002). cDNA analysis of individual DMD patients have shown that genes related to immune response, sarcomere, ECM and signaling/cell growth were increased. Up-regulation of these genes accompanies dystrophic changes in DMD muscles such as myofiber necrosis, inflammation and muscle regeneration (Noguchi et al. 2003). Up-regulated inflammatory gene expression and activated immune cells are present in dystrophic muscle and play a critical role in muscle wasting (Evans et al. 2009b). The pro-inflammatory cytokines TNF-alpha, IL-1beta and IL-6 are up-regulated in Duchenne patients and *mdx* mice (Porreca et al. 1999; Porter et al. 2002; Kumar and Boriek 2003; Acharyya et al. 2007; Hnia et al. 2008). The fact that a number of chemokines are expressed directly by the muscle cell suggests that muscle tissue may contribute to chemotaxis process (Porter et al. 2003a). Using microarray technology we have shown that membrane depolarization induces expression and repression of a number of genes in both

normal (RCMH) and DMD (RCDMD) human skeletal muscle cell lines. Importantly, modulated genes are mostly different for these two cell lines (Cárdenas et al. 2010). Nevertheless, the expression of only 44 of them is modified in both cell lines. The pattern of expression (up- or down-regulation) of these common genes is strikingly different between cell lines, and they appear to be regulated in opposite ways (Cárdenas et al. 2010).

Within these 44 genes we identified genes related to the immune response (HLA-DQB1), cytoskeleton/ECM proteins (ADD1, KRT1, and FBLN1), and signaling (NRG and POU2F2), among others. We found that 18 of these 44 genes are related to processes associated with Ca^{2+}, and 10 of them have been related in some way to dystrophy (Cárdenas et al. 2010).

Within the genes whose expression increases in RCDMD cells, particularly interesting in relation to muscle function and development, are those coding for the two isoforms of neuregulin (NGR1-β2 and NRG1-γ) and the POU2F2 gene (Cárdenas et al. 2010). NRG1 is a growth factor that potentiates myogenesis and may play an important role in differentiation of satellite cells in muscle regeneration (Hirata et al. 2007). Moreover, NRG stimulates Ca^{2+}-induced glucose transport during contraction (Canto et al. 2006) and is implicated in the metabolic and proliferative response of muscle to exercise (Lebrasseur et al. 2003). POU2F2 has been described as a transcription factor expressed in developing mouse skeletal muscle (Dominov and Miller 1996).

In addition, we found variations in the expression of ICEBERG, HLA-DQB1, ADD1, FBLN1 and TRIO genes that also have been associated with Ca^{2+} and dystrophy (Cárdenas et al. 2010). Considering that changes observed in DMD muscle biopsies have been related to elevation of intracellular Ca^{2+} concentration, which could activate Ca^{2+}-dependent degradation pathways, resulting in myofibril disruption and muscle necrosis (Turner et al. 1993). It will be interesting to analyze the roles described for the above mentioned genes. To our knowledge, there are no studies describing the role of membrane depolarization on the expression of these genes, and further studies are needed to explore the involvement of IP_3-mediated slow Ca^{2+} signals in the expression of some of these particular genes in skeletal muscle cells (Cárdenas et al. 2010).

Gene expression profiling at different stages in *mdx* models have also evidenced the highly dynamic process of the disease onset. These works, show that dystrophy in *mdx* models have an onset at 3 weeks of age, with a peak in pathology around 8 weeks. Interestingly, at this stage, there is a marked upregulation of almost 9 fold of the purinergic receptor $P2X_4$ (Porter et al. 2003b).

Although no therapy described to date can effectively slow or halt muscle degeneration in dystrophic patients (Kapsa et al. 2003), a promising pharmacological treatment for DMD aims to increase levels of utrophin and to identify molecules that modulate utrophin expression (regulatory pathways) by activation of its promoter (Dennis et al. 1996), in muscle fibers of affected patients to compensate for the absence of dystrophin (Miura and Jasmin 2006).

Indeed, utrophin is considered the autosomal homolog of dystrophin because it shares structural and functional motifs throughout the length of the molecule (Love et al. 1989; Khurana et al. 1990; Nguyen et al. 1991; Ohlendieck et al. 1991; Tinsley et al. 1992). It is capable of associating with members of the DAPs with similar affinity to dystrophin as well (Matsumura et al. 1992; Winder et al. 1995). Studies in the dystrophin-deficient *mdx* mice have established that the elevation of utrophin levels in dystrophic muscle fibers can restore

sarcolemmal expression of DAPs members and alleviate the dystrophic pathology (Miura and Jasmin 2006). Direct evidence for the ability of utrophin to functionally substitute for dystrophin comes from experiments demonstrating that transgene-driven utrophin overexpression can effectively rescue dystrophin-deficient muscle in *mdx* mice (Tinsley et al. 1996; Deconinck et al. 1997; Tinsley et al. 1998).

6.1 Electrical stimulation induces calcium-dependent up-regulation of neuregulin-1β in dystrophic skeletal muscle cell lines

Neuregulin (NRG) is one of many factors that increase utrophin expression (Miura and Jasmin 2006). It belongs to a family of proteins structurally related to the epidermal growth factor (EGF) that are synthesized in and secreted from motoneurons and muscle (Falls 2003). Four members of NRG proteins, NRG-1 to NRG-4, have been identified. The best-studied and most characterized products are those encoded by NRG-1 gene.

Neuregulin-1 (NRG-1) was initially described as a neurotrophic factor involved in neuromuscular junction formation in skeletal muscle, but recently it has emerged as a myokine, with relevant effects on myogenesis, muscle metabolism and regeneration, and has been considered as a strong candidate to transduce muscle adaptation to chronic exercise (Lebrasseur et al. 2003; Guma et al. 2010).

Interestingly, NRG-1 treatment increases utrophin mRNA levels and transcriptional activity in mouse and human myotubes (Gramolini et al. 1999; Khurana et al. 1999). Moreover, Krag et al. (2004) described that intraperitoneal injection of a small peptide region of NRG-1 ectodomain increases utrophin expression in *mdx* mice (Krag et al. 2004). Observed increase was accompanied by a reduction in muscle degeneration and inflammation, and by decreased susceptibility to the damage induced by lengthening contractions. Improvement in muscle function was deemed to result specifically from the up-regulation of utrophin because NRG-1 administration has no beneficial effect in dystrophin/utrophin double-knockout animals (Krag et al. 2004).

However, regardless the evidences supporting such important roles for NRG-1 in skeletal muscle, the molecular mechanisms involved in its expression are still unclear.

When we investigated the effect of membrane depolarization on global gene expression in dystrophic RCDMD cells using microarrays technology, our data revealed that membrane potential changes, induced by electrical stimulation, resulted in significant up or down regulation of 150 genes after 4 h. Interestingly, two NRG-1 isoforms (β and γ) appear within the ten highest up-regulated genes (Cárdenas et al. 2010).

Taking into account the important biological effects of NRG-1 in the muscle and its potential clinical implication in DMD, we focused our study on the regulation of muscle NRG-1 expression, specifically on NRG-1β isoform, that displays a higher affinity for NRG receptor (Juretić et al. n.d.). NRG-1β increased expression was confirmed by quantitative PCR. We observed that electrical stimulation induces a significant increase of NRG-1β mRNA level in RCDMD cells, with a maximun at 4 h post-stimuli, but has no effect on NRG-1β expression in RCMH cells treated with the same procedure, suggesting that activation of molecular pathways involved in the regulation of NRG-1β gene expression are different in normal and dystrophic cells. Western blot analysis of stimulated RCDMD cells demonstrates that

observed increase in NRG-1β mRNA levels was followed by actual enhancement of the corresponding protein (Juretić et al. n.d.).

Accumulating evidence suggests that integral dystrophin-DAPs complex components are also implicated in signaling in DMD, and that mutations in non-DAP protein encoding genes may lead to the muscular dystrophy phenotype, supporting the idea that more than one molecular pathway is implicated in the disease (Haslett et al. 2002). Thus, it is likely that the lack of DAP proteins in the cell membrane will somehow affect the regulation of Ca^{2+} transients and gene expression in dystrophic cells after electrical stimulation. In fact, Balghi et al. (2006) have demonstrated that IP_3 production after depolarization is significantly elevated in SolC1(-) dystrophin deficient myotubes and that the presence of mini-dystrophin under the membrane leads to reduced IP_3 production (Balghi et al. 2006b).

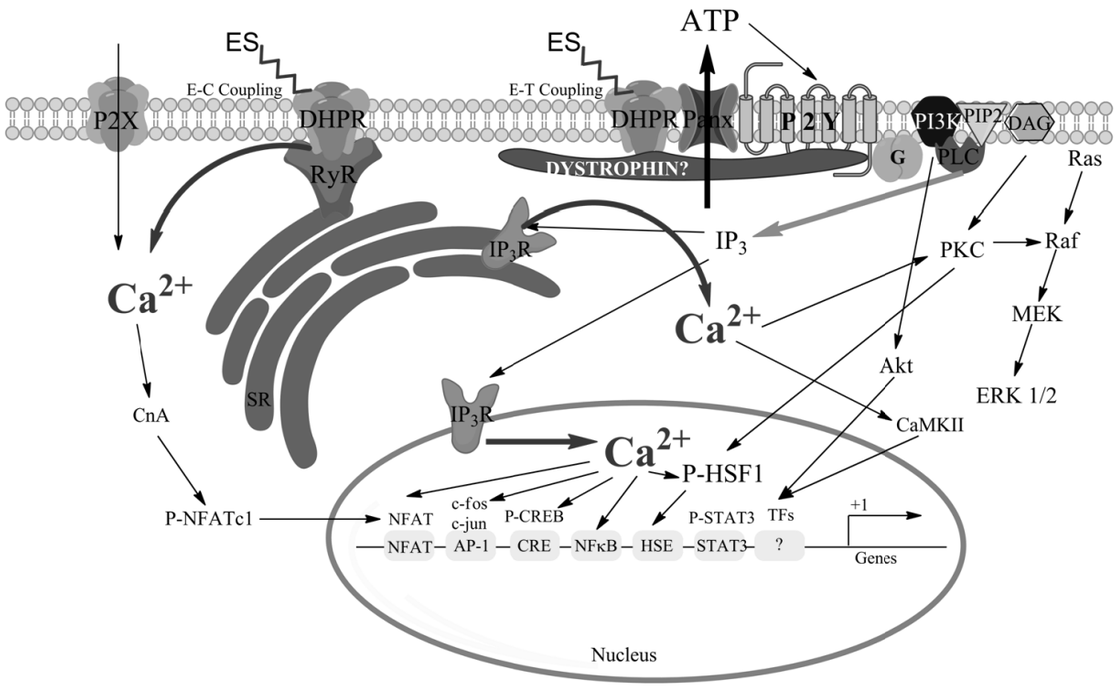

Fig. 1. **Diagram for the model for excitation-transcription coupling in skeletal muscle.** Two protein complexes are proposed to be present in the transverse tubule (T-T) membrane. The first one is the excitation-contraction (E-C) complex, comprising the voltage sensing dihydropyridine receptor (DHPR, Cav1.1) and the ryanodine receptor (RyR). We propose that purinergic P2X receptors also contribute to the fast calcium transient associated to E-C coupling. The excitation-transcription (E-T) coupling complex comprises also the DHPR, pannexin1 (Panx), the purinergic receptor P2Y linked to a G protein and possibly the phosphatidyl inositol 3 kinase (PI3K) and phospholipase C (PLC). It is likely that dystrophin is playing a role stabilizing this complex in the membrane. Upon electrical stimulation (ES), membrane depolarization will trigger a conformational change in DHPR which somehow will induce opening of Panx channel and ATP will be released. ATP acting on P2Y receptors will activate PI3K via G protein and in turn PLC will be recruited to the membrane producing inositol (1,4,5) trisphosphate (IP_3) and diacyl glycerol (DAG). IP_3R- mediated calcium signals will be responsible for activation of kinases (PKC, CaMK II, ERK½) and transcription factors leading finally to gene expression.

7. Final remarks

Results discussed here point out to the important role of slow Ca^{2+} transients evoked by electrical stimulation in the activation of the pathways that couple excitation to gene expression in dystrophin-deficient muscle cells (a putative role for dystrophin is schematized in Fig. 1). If we find ways to intervene such pathways in a manner that can compensate dystrophin dysfunction, the understanding of this new role of dystrophin will give new insights to the design of a therapeutic strategy in order to potentiate muscle survival and regeneration in DMD.

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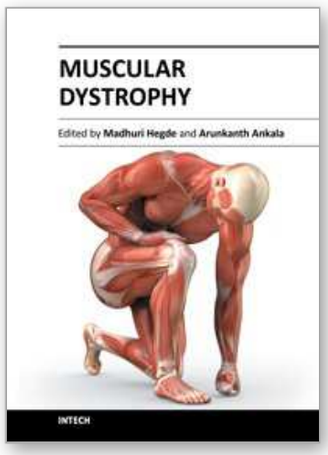

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With more than 30 different types and subtypes known and many more yet to be classified and characterized, muscular dystrophy is a highly heterogeneous group of inherited neuromuscular disorders. This book provides a comprehensive overview of the various types of muscular dystrophies, genes associated with each subtype, disease diagnosis, management as well as available treatment options. Though each different type and subtype of muscular dystrophy is associated with a different causative gene, the majority of them have overlapping clinical presentations, making molecular diagnosis inevitable for both disease diagnosis as well as patient management. This book discusses the currently available diagnostic approaches that have revolutionized clinical research. Pathophysiology of the different muscular dystrophies, multifaceted functions of the involved genes as well as efforts towards diagnosis and effective patient management, are also discussed. Adding value to the book are the included reports on ongoing studies that show a promise for future therapeutic strategies.

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