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## The variably spliced ASI region of RyR1 contains a basic $\alpha$ -helix domain that modifies EC coupling

A.F. Dulhunty,<sup>1</sup> T. Kimura,<sup>2</sup> J.D. Lueck,<sup>3</sup> P.J. Harvey,<sup>1</sup> S.M. Pace,<sup>1</sup> N. Ikemoto,<sup>4</sup> M.G. Casarotto<sup>1</sup> and R.T. Dirksen,<sup>3</sup> <sup>1</sup>JCSMR, ANU, PO Box 334, ACT, 2601, Australia, <sup>2</sup>Hyogo College of Medicine, 1-1 Mukogawa-cho Nishinomiya, HYOGO 663-8501, Japan, <sup>3</sup>University of Rochester Medical Center, PO Box 711, 601 Elmwood Avenue, Rochester, NY, 14642 USA and <sup>4</sup>Boston Biomedical Research Institute, Watertown, MA, USA.

The function of the alternatively spliced ASI residues (Ala<sup>3481</sup>-Gln<sup>3485</sup>) in the skeletal muscle ryanodine receptor (RyR1) Ca<sup>2+</sup> release channel has been examined. The residues are present in the adult (ASI(+))RyR1 isoform but absent in the juvenile (ASI(-))RyR1 splice variant. ASI(-)RyR1 is over-expressed in myotonic dystrophy type 1 (DM1) and is less active than ASI(+) (Kimura *et al.*, 2005). The ASI region contributes to an inhibitory inter-domain interaction which is stronger in ASI(-)RyR1 since its interruption by ASI domain peptides causes greater activation in ASI(-)RyR1 (Kimura *et al.*, 2007). We predicted that interruption of this ASI interaction may contribute to excitation-contraction (EC) coupling and if it did, EC coupling would be stronger in ASI(-)RyR1. The ASI domain peptides include the ASI residues and a sequence of 5 contiguous positively charged residues which bind to the  $\beta$ 1a subunit of the skeletal dihydropyridine receptor (DHPR) and whose deletion depresses EC coupling (Cheng *et al.*, 2005). We predicted that this sequence may contribute to ASI peptide activity. Interruption of an inhibitory inter-domain interaction by domain peptide DP4 also activates RyR1 (Yamamoto *et al.*, 2000). Based on their positions in the RyR1 sequence, we predicted that the ASI and DP4 regions must support different inter-domain interactions.

EC coupling was examined in RyR-null myotubes injected with cDNA for ASI(-)RyR1 or ASI(+)RyR1. Intracellular Ca<sup>2+</sup> was measured in intact myotubes loaded with fura-FF AM. Voltage-gated L-channel activity and SR Ca<sup>2+</sup> release were measured simultaneously using whole cell patch clamp. The structure of the ASI peptides was examined using nuclear magnetic resonance (NMR). Ca<sup>2+</sup> release from isolated skeletal SR vesicles was measured using spectrophotometry. RyR activity was assessed from [<sup>3</sup>H] ryanodine binding (which increases when channel open probability increases) or from RyR channels incorporated into artificial lipid bilayers.

Ca<sup>2+</sup> release during EC coupling was greater in myotubes expressing ASI(-)RyR1 than in those expressing ASI(+)RyR1. The L-type Ca<sup>2+</sup> current was similar in both ASI(-)RyR1 and ASI(+)RyR1 expressing myotubes (indicating similar DHPR expression, function and alignment with RyRs). As with caffeine (Kimura *et al.*, 2005), maximal 4-chloro-m-cresol induced Ca<sup>2+</sup> release was less for ASI(-)RyR1. Total SR Ca<sup>2+</sup> load and resting cytoplasmic Ca<sup>2+</sup> concentrations were the same in both cases. These results were consistent with ASI(-) region supporting a stronger inter-domain interaction with greater inhibition, which then allowed greater activation when the interaction was interrupted during EC coupling.

The NMR-derived structures of ASI(-) and ASI(+) peptides both have random coil N-terminal regions bracketing (Ala<sup>3481</sup>-Gln<sup>3485</sup>) and an  $\alpha$ -helical C-terminal part spanning the 5 contiguous basic residues. The activity of the ASI peptides on RyR1 activity was critically dependent on the basic residues and their inclusion in an  $\alpha$ -helical structure. The structure and action of the ASI peptides mimicked that of the basic  $\alpha$ -helical A region of the DHPR  $\alpha$ 1s II-III loop. In addition, the ASI peptides competed with peptide A for RyR1 activation. It remains to be determined whether this similarity between the DHPR  $\alpha$ 1s II-III loop and the ASI region is coincidental or whether it has a functional significance in the intact cell. RyR activation by the ASI peptides and DP4 exhibited different Ca<sup>2+</sup>-dependence and the effects of the two domain peptides were additive, suggesting that they acted at separate sites on RyR1 and interrupted distinctly different inter-domain interactions.

The results show that the ASI residues have a strong influence on the efficacy of EC coupling which is stronger when they are deleted. Our findings further indicate that disruption of an inter-domain interaction involving the ASI region may play a role in EC coupling. Overall, the data suggest that enhanced Ca<sup>2+</sup> release during EC coupling may contribute both to developmental changes in Ca<sup>2+</sup> release and to the myopathy in DM1.

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## Reduced $\text{Ca}^{2+}$ -activated force explains increased skeletal muscle fatigability in heart failure

E. Verburg,<sup>1,2</sup> M. Munkvik,<sup>1</sup> P.K. Lunde,<sup>1</sup> G.D. Lamb<sup>2</sup> and O.M. Sejersted,<sup>1</sup> <sup>1</sup>Institute for Experimental Medical Research, Ullevaal University Hospital, Oslo, Norway and <sup>2</sup>Department of Zoology, La Trobe University, Bundoora, VIC 3086, Australia.

Congestive heart failure (CHF) is accompanied by a skeletal muscle myopathy causing increased exertional fatigability. The underlying cellular mechanisms are unclear but the myopathy seems to affect predominantly slow-twitch fibres. We have investigated whether the intrinsic  $\text{Ca}^{2+}$ -activating properties of the myofilaments are altered in CHF in resting and fatigued skeletal muscle *soleus* fibres.

CHF was induced by coronary artery ligation in male wistar rats while under general anaesthesia (ventilated with isofluoran 2.5% in 30%  $\text{O}_2$  and 70%  $\text{N}_2\text{O}$ ). Sham-operated rats were used as controls (SHAM). Postoperatively the animals were given 0.2 mg  $\text{kg}^{-1}$  buprenorphine and kept under daily surveillance. Six weeks after ligation the degree of heart failure was assessed measuring left ventricle end diastolic pressure (LEVDP, with Millar catheter) under general anaesthesia (again isofluoran) and lung weight (post mortem, at the end of the experiment). The right soleus muscle was prepared *in situ* with the distal tendon attached to a force transducer. Blood supply to the muscle was left intact. The muscle was kept at  $\sim 38^\circ\text{C}$  by dripping warmed saline on the muscle. Fatigue was induced by subtetanic stimulation resulting in repeated partially fused trains of isometric contractions with a peak force of about 30% of maximum tetanic force (1 ms pulses at 5 Hz in trains of 6 s on, 4 s off for 30 minutes). Within 10s after completion of the fatigue-protocol the fatigued muscle was dissected out, subsequently pinned at resting length in a petri dish under paraffin oil and cooled on ice. The non-stimulated contralateral muscle served as resting control. The rats were then killed. From the muscles single fibres were dissected out and mechanically skinned. The skinned fibres were mounted between forceps and a force transducer, and stretched to 120% of slack length. Fibre diameter was measured while the fibre was still under oil. Subsequently the fibre was transferred to a series of baths containing cytosol-mimicking solutions with strongly EGTA-buffered free  $[\text{Ca}^{2+}]$  or  $[\text{Sr}^{2+}]$  ranging from 0.05 to 20  $\mu\text{M}$  or 200  $\mu\text{M}$ , pH 7.1. Maximal  $\text{Ca}^{2+}$ -activated force was calculated and relative force-pCa and -pSr plots were constructed and hill curves fitted. At last the fibres were put in 10  $\mu\text{l}$  SDS-buffer solution for later gel-electrophoresis analysis of myosin light chain phosphorylation (MLC) and myosin heavy chain (MHC) isoforms. The fatigue protocol resulted in a  $44 \pm 5\%$  (mean  $\pm$  SE) decrease in peak force produced by the whole muscle during the 5 Hz trains in CHF rats, whereas peak force was decreased by only  $24 \pm 9\%$  in SHAM rats ( $n = 5$  each in CHF and SHAM). In the resting contralateral skinned single fibres, no differences were found in maximum  $\text{Ca}^{2+}$ -activated force, fibre cross-sectional area (CSA) or  $[\text{Ca}^{2+}]$  eliciting 50% force ( $\text{pCa}_{50}$ ) between SHAM and CHF rats (each  $n = 10$  fibres from 5 rats). Fatigued fibres from both SHAM and CHF rats, tended to have lower maximum  $\text{Ca}^{2+}$ -activated force per CSA than the contralateral resting fibres (each  $n = 10$  fatigued and 10 contralateral fibres from 5 rats). However, in SHAM rats, fibre CSA was 28% higher in fatigued than in contralateral resting fibres ( $p < 0.02$ ), whereas CSA was slightly lower in fatigued CHF fibres ( $p < 0.05$ ). The lack of activity-induced cell-swelling in CHF is to be confirmed with microscopy analysis of whole muscle cross sections. When disregarding CSA, absolute maximum  $\text{Ca}^{2+}$ -activated force in fatigued fibres in CHF rats was  $23 \pm 10\%$  ( $n = 10$ ) lower than contralateral resting fibres ( $p < 0.02$ ), whereas in fatigued fibres from SHAM rats absolute  $\text{Ca}^{2+}$ -activated force was not altered ( $p > 0.1$ ).  $\text{Ca}^{2+}$ -sensitivity was not altered compared to contralateral resting fibres in either fatigued CHF or SHAM fibres.  $\text{Sr}^{2+}$ -activation curves indicated that two of in all 42 fibres dissected were either fast twitch or mixed fibre types (both from SHAM rat), and these fibres were not included. All other fibres had  $\text{Sr}^{2+}$ -activation curves typical for predominantly slow-twitch type MHC. The MHC isoforms in each included fibre are to be confirmed with gel electrophoresis.

In conclusion, increased fatigability in slow muscles in CHF seems to be due to intrinsic alterations in actin-myosin interaction of slow twitch fibres during activity leading to a decrease in absolute force produced at all  $[\text{Ca}^{2+}]$ . The lack of activity-induced cell swelling in CHF fibres may in some way play a role.

## Transgenic expression of cardiac $\alpha$ -actin rescues the lethal phenotype of skeletal $\alpha$ -actin knockout mice

G. Ravenscroft,<sup>1,2</sup> K.J. Nowak,<sup>1</sup> C. Jackaman,<sup>1</sup> E.M. Lim,<sup>1</sup> P.J. Morling,<sup>3</sup> L.M. Griffiths,<sup>3</sup> V. Fabian,<sup>3</sup> J.L. Lessard,<sup>4</sup> A.J. Bakker,<sup>2</sup> K.E. Davies<sup>5</sup> and N.G. Laing,<sup>1</sup> <sup>1</sup>University of Western Australia, Centre for Medical Research, B Block, QE II Medical Centre, Nedlands Western Australia, <sup>2</sup>University of Western Australia, Department of Physiology, 35 Stirling Highway, Crawley Western Australia, <sup>3</sup>Royal Perth Hospital, Neuropathology, PathWest Anatomical Pathology, Perth, Australia, <sup>4</sup>Cincinnati Children's Hospital Medical Center, Division of Developmental Biology, Cincinnati, United States and <sup>5</sup>MRC Functional Genetics Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom.

Over 130 different mutations have been identified to date in the skeletal muscle  $\alpha$ -actin gene (*ACTA1*). These mutations manifest as histologically distinct congenital myopathies, though most patients have clinically severe early onset disease. During skeletal muscle development, the cardiac  $\alpha$ -actin isoform is the predominant actin isoform, but it is downregulated by birth (Ilkovski *et al.*, 2005). The cardiac and skeletal actin isoforms differ by 4 of 375 amino acids, with the differences consistent in all species that have these two proteins (Vandekerckhove *et al.*, 1986). Recently it has been shown that certain patients have recessive *ACTA1* disease caused by an absence of skeletal muscle  $\alpha$ -actin. These patients all retained expression of cardiac  $\alpha$ -actin in their skeletal muscles, with the disease severity correlating inversely with the level of cardiac  $\alpha$ -actin expression (Nowak *et al.*, 2007). It was hypothesised that expression of cardiac  $\alpha$ -actin could serve as a functional replacement for skeletal muscle  $\alpha$ -actin. Transgenic mice expressing cardiac  $\alpha$ -actin in postnatal skeletal muscle were generated and crossed with heterozygous skeletal muscle  $\alpha$ -actin knock-out mice, producing mice with only cardiac  $\alpha$ -actin in their skeletal muscle (rescued nulls). Muscle morphology of the rescued nulls was examined via light and electron microscopy. The contractile properties of single skeletal muscle fibres from rescued null mice were investigated using the skinned fibre technique. Homozygous skeletal  $\alpha$ -actin knock-out mice all die by postnatal day 9 (Crawford *et al.*, 2002). This lethal phenotype was rescued by the transgenic expression of cardiac  $\alpha$ -actin, allowing survival into adulthood and procreation (mice we term "rescued nulls"). 94 % of the rescued null mice survived until at least 3 months and the oldest are over 15 months old. The skeletal muscle from the rescued null mice was morphologically similar to controls. However, the rescued null skinned EDL fibres did display a 25 % reduction in normalised maximum force production ( $p = 0.001$ ). Alterations in the  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus were also observed as indicated by significant differences in the slopes of the pCa-force curves ( $p = 0.013$ ) and the pCa10 values ( $p = 0.004$ ) compared with controls. This small force deficit may not be of major significance, as the rescued null mice were found to be functionally normal, as measured by a grip strength test ( $p = 0.777$ ) and Rota-rod testing ( $p = 0.472$ ). These studies show that cardiac  $\alpha$ -actin can functionally replace skeletal muscle  $\alpha$ -actin in adult skeletal muscle, thus offering a potential treatment for the skeletal muscle  $\alpha$ -actin myopathies.

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## Contractile and fatigue properties of $\alpha$ -actinin-3 knockout fast-twitch EDL muscle

S.I. Head,<sup>1</sup> S. Chan,<sup>1</sup> D.G. Mac Arthur<sup>2</sup> and K.N. North,<sup>2</sup> <sup>1</sup>Department of Physiology, University of New South Wales, Sydney, NSW 2052, Australia and <sup>2</sup>Faculty of Medicine, University of Sydney, The Children's Hospital at Westmead, Neurogenetics Research Unit Westmead, Westmead, NSW 2145, Australia.

The actin-binding protein  $\alpha$ -actinin-3 is one of the two isoforms of  $\alpha$ -actinin that are found in the Z-discs of skeletal muscle, and is specifically expressed in fast glycolytic (Type 2B) muscle fibres. Homozygosity for a common polymorphism in the ACTN3 gene results in complete deficiency of  $\alpha$ -actinin-3 in about 1 billion people worldwide. Although  $\alpha$ -actinin-3 deficiency does not cause disease, recent studies suggest that the absence of  $\alpha$ -actinin-3 is detrimental to sprint and power performance in elite athletes (Yang *et al.*, 2003). To determine the effect of  $\alpha$ -actinin-3 deficiency on the physiological properties of skeletal muscle, we studied isolated extensor digitorum longus muscles (EDL) from a specially developed  $\alpha$ -actinin-3 knockout mouse. Animals aged 8 to 10 weeks were sacrificed with an overdose of halothane (ethics approval UNSW). The EDL muscle was dissected from the hindlimb and tied by its tendons to a force transducer at one end and a linear tissue puller at the other. It was placed in a bath continuously superfused with Krebs solution, with composition (mM): 4.75 KCl, 118 NaCl, 1.18 KH<sub>2</sub>PO<sub>4</sub>, 1.18 MgSO<sub>4</sub>, 24.8 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub> and 10 glucose, with 0.1% fetal calf serum and continuously bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> to maintain pH at 7.4. The muscle was stimulated by delivering a supramaximal current between two parallel platinum electrodes. At the start of the experiment, the muscle was set to the optimum length  $L_0$  that produced maximum twitch force. All experiments were conducted at room temperature ( $-22^{\circ}\text{C}$  to  $24^{\circ}\text{C}$ ).

$\alpha$ -actinin-3-deficient muscles showed similar levels of damage to wild-type muscles following eccentric contractions of 20% strain,  $1.6 \pm 2.0\%$  in wild-types and  $2.6 \pm 1.5\%$  in knockouts, suggesting that the presence or absence of  $\alpha$ -actinin-3 does not influence mechanical stability of the sarcomere.  $\alpha$ -actinin-3 deficiency does not result in a loss of fast glycolytic fibres (expressing myosin 2B). However,  $\alpha$ -actinin-3-deficient muscles were 9% lighter than  $\alpha$ -actinin-3-positive muscles, with a corresponding 9% reduction in cross-sectional area. Knockouts displayed longer twitch half-relaxation times; the half-relaxation time of  $15.7 \pm 0.6$  ms in knockouts was 2.6 ms longer than the half-relaxation time of  $13.2 \pm 0.6$  ms in wild-types ( $p = 0.008$ ).  $\alpha$ -actinin-3-deficient muscles showed significantly better recovery from fatigue; 30 minutes following the fatigue protocol knockouts recovered to  $86.1 \pm 1.1\%$  of their original force, but wild-types recovered to only  $78.4 \pm 1.9\%$  of original ( $p = 0.013$ ). In combination, these data suggest that  $\alpha$ -actinin-3 deficiency results in fast-twitch, glycolytic fibres developing slower-twitch, more oxidative properties while not affecting the mechanical strength of the fibre. This alteration in the metabolic profile of the fast muscle would be detrimental to optimal sprint and power performance but beneficial for endurance activities.

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## Higher expression of caveolin-3 in mechanically-skinned single fibres from slow-twitch muscle compared with fast-twitch muscle

R.M. Murphy and G.D. Lamb, Department of Zoology, La Trobe University, VIC 3086, Australia.

Caveolin-3 is a muscle specific membrane protein which is important in the formation of caveolae (caveolin-containing lipid rafts) and which has been linked to membrane trafficking and signal transduction events. Mutations in caveolin-3 cause limb girdle muscular dystrophy type 1C (LGMD1C) and rippling muscle disease. Caveolin-3 is transiently associated with the t-tubular membrane during muscle development and it is widely reported to be localised exclusively at the sarcolemma in mature muscle fibres. Using microscopy, caveolin-3 was localized to the t-tubule in adult mouse soleus muscle fibres. Male Long-Evans hooded rats (6-8 months old) were sacrificed using a lethal overdose of fluothane in accordance with the La Trobe University Ethics Committee and the *extensor digitoralis longus* (EDL) and *soleus* (SOL) muscles were excised. From these muscles, segments of fibres were collected with either their sarcolemma present (intact) or with their sarcolemma removed by mechanically-skinning the fibres (skinned). We found a high expression of caveolin-3 in skinned fibres, supporting an intracellular pool of caveolin-3, likely localised to the t-tubular membrane. A higher expression of caveolin-3 was seen in both skinned and intact fibres from the predominantly type I *soleus* muscle compared to the fibres from the predominantly type II EDL muscle (eg., for intact fibres there was  $200 \pm 25\%$  caveolin-3 in SOL fibres relative to that in EDL fibres,  $p < 0.05$ ,  $n = 12$ , unpaired, two-tailed *t*-test). The relative amount of caveolin-3 in skinned and intact segments was quantified by comparing skinned and intact segments from the same fibre. For EDL and SOL fibres the amount of caveolin-3 in a skinned segment was 70-80% of that in the intact segment. The amount of caveolin-3 present in EDL skinned fibres was also analysed alongside their corresponding sarcolemmal portions and when expressed as a percentage of the total caveolin-3 present,  $29 \pm 9\%$  ( $n = 6$ ) of the caveolin-3 was found in the sarcolemma. As expected, caveolin-3 was found to be tightly bound in skeletal muscle fibres with none of the protein being present in various physiologically-based wash solutions (including very low and physiologically high  $\text{Ca}^{2+}$  concentrations) following washes of skinned fibres for up to 60 min. However, 80% or more of the detectable caveolin-3 became diffusible following a 30 min treatment with 1% Triton X-100 solution. These findings show that there is a greater expression of caveolin-3 in slow twitch muscle fibres compared with fast-twitch muscle fibres and that a greater proportion of the caveolin-3 is found inside muscle fibres than in the sarcolemma.

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## Contractile characteristics of permeabilized muscle fibres from dystrophic dogs

C. van der Poel,<sup>1</sup> K.T. Murphy,<sup>1</sup> J.C.S. Bizario,<sup>2</sup> M.C.R. Costa,<sup>2</sup> M.L. Pinto<sup>2</sup> and G.S. Lynch,<sup>1</sup> <sup>1</sup>Basic and Clinical Myology Laboratory, Department of Physiology, The University of Melbourne, VIC 3010, Australia and <sup>2</sup>Associação de Amigos dos Portadores de Distrofia Muscular, University of Ribeirão Preto, São Paulo, Brasil.

Duchenne muscular dystrophy (DMD) is an X-linked myopathy characterised by progressive muscular degeneration and weakness that ultimately results in premature death *via* cardiac or pulmonary failure. Like the *mdx* mouse model of DMD, the Golden Retriever muscular dystrophy (GRMD) dog model has a single mutation in the dystrophin gene. Similar to DMD patients, affected dogs with the mutant dystrophin gene have weak muscles and eventually die from the disease *via* cardiac or pulmonary failure. Although the *mdx* mouse provides a useful model for studying some aspects of the pathogenesis of muscular dystrophy, the GRMD dog provides a better model relevant to DMD (Childers *et al.*, 2005). Unfortunately, there are few GRMD dog colonies around the world making it a unique but not widely used model for understanding underlying mechanisms and investigating potential therapeutic approaches for DMD. Little information exists about many of the fundamental aspects of skeletal muscles from dystrophic compared with healthy dogs. The aim of this study was to identify the Ca<sup>2+</sup>- and Sr<sup>2+</sup>-activated contractile characteristics of single muscle fibres from dystrophic dogs.

Two-year old dystrophic dogs ( $n = 3$ ) and control litter mates ( $n = 3$ ) from a colony at the University of Ribeirão Preto were used in this experiment. Dogs were anaesthetised (Tiletamine Cloridrate 125.0 mg and Zolazepam Cloridrate 125.0 mg) and open muscle biopsies taken from the *biceps femoris* muscle. All the dogs recovered from the anaesthesia. The muscle samples were blotted on filter paper and bundles tied to a capillary tube and placed immediately in a vial containing skinning solution of the following composition (mM): potassium propionate (125), EGTA (5), ATP (2), MgCl<sub>2</sub> (2), imidazole (20) and 50 % v/v glycerol, adjusted to pH 7.1 with 4M KOH; and stored at -20°C for up to 12 weeks (Lynch *et al.*, 2000). Single permeabilized muscle fibres were isolated from the bundles, attached to a sensitive force transducer and activated by rapid immersion in buffered solutions of varying [Ca<sup>2+</sup>] and [Sr<sup>2+</sup>]. Based on their contractile characteristics during Ca<sup>2+</sup>- and Sr<sup>2+</sup>-activation, fibres were allocated into discrete populations: type I, type II and hybrid type I/II fibres. After the contractile properties had been determined, the fibre segments (volume 40nl) were placed in 10µl solubilising buffer, incubated at room temperature for 24h, boiled for 3 min and stored at -80°C, for later analysis of troponin-C isoforms by SDS-PAGE.

There was a significant (25%) decrease in specific force production of muscle fibres from dystrophic dogs ( $214.9 \pm 17.9$  kN/m<sup>2</sup>,  $n = 15$ ) compared with litter mate controls ( $159.6 \pm 8.7$  kN/m<sup>2</sup>,  $n = 15$ ). There was no difference in fibre sensitivity to Ca<sup>2+</sup> [pCa50; control  $6.41 \pm 0.03$ ; dystrophic  $6.36 \pm 0.023$  ( $n = 15$  both groups)]. A high incidence (65%) of fibres from dystrophic dogs were hybrid fibres as evident from their biphasic force-pSr relations and combination of slow and fast troponin-C isoforms (Lynch *et al.*, 1995).

Functional assessments of skinned fibres coupled with biochemical analysis of their contractile and regulatory proteins can provide important information about the progression of muscular dystrophy in the GRMD model and its relevance to DMD.

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## **Mitochondrial ATP production rate is severely impaired in dystrophic *mdx* skeletal muscle, and is not influenced by altered calcium concentration**

*E. Rybalka and A. Hayes, Exercise Metabolism Unit, Centre for Aging, Rehabilitation, Exercise & Sport and School of Biomedical & Clinical Sciences, Victoria University, PO Box 14428, Melbourne City MC, VIC 8001, Australia.*

Duchenne Muscular Dystrophy (DMD) is characterised by  $[Ca^{2+}]$ -induced hypercatabolism of skeletal muscle tissue. This is associated with reductions in high energy nucleotides of up to 50% of normal levels, which may be directly associated with elevated intracellular  $[Ca^{2+}]$ . We have thus examined cellular energy production by performing direct measurement of mitochondrial ATP production rate (MAPR) using substrates that mimic the major metabolic pathways in dystrophic *mdx* diaphragm, and assessed the effect of an extra-mitochondrial supraphysiological calcium spectra (0-400 nM) such to mimic “resting” dystrophic intracellular  $Ca^{2+}$  conditions in this *in vitro* setting.

Age-matched dystrophic *mdx* ( $n = 8$ ) and normal control ( $n = 10$ ) C57BL/10 mouse diaphragm were utilised to bioluminometrically quantify MAPR under each of carbohydrate, fat, protein, carbohydrate+fat+protein (total) and complex II metabolism. The effect of both nil and supraphysiological  $[Ca^{2+}]$  on MAPR was determined for total and complex II metabolism in both diaphragm and tibialis anterior (TA) for direct comparison between diaphragm and hind limb skeletal muscle. Citrate synthase (CS) activity was also assessed. In all cases, muscles were excised from anaesthetised animals, and mitochondria were isolated via step-wise centrifugation.

MAPR was significantly depressed across all metabolic pathways except for that of complex II in *mdx* compared to control diaphragm ( $p < 0.01$ ). The greatest depression was observed for total metabolism in which a 3-fold reduction was evident. MAPR under stimulation of isolated metabolic pathways was similarly reduced by 2.5-fold for carbohydrate, 2-fold for fat and 1.5-fold for protein metabolism.  $Ca^{2+}$  had no effect on MAPR under total or complex II metabolism at any of the concentrations assessed, in either diaphragm or TA. CS activity of *mdx* muscle was comparable to controls in both muscles, however the susceptibility of dystrophic mitochondria to mechanical damage during the mitochondrial extraction process (as determined by the ratio of  $CS_{after}$  to  $CS_{total}$  activity) was significantly greater in *mdx* compared to controls, and in diaphragm compared to TA ( $p < 0.01$ ).

These results demonstrate severely impaired mitochondrial function of dystrophic diaphragm and hind limb muscle. This depression may be associated with physical and/or functional reductions of enzymes associated with the TCA cycle, which reduces the flow of NAD-associated electrons to the electron transport chain and hence ATP production capacity. This observation seems to be directly related to the DMD phenotype as supraphysiological  $[Ca^{2+}]$  had no effect on MAPR, and dystrophic mitochondria were less resistant to mechanical damage as observed by CS analysis.

## Stretch-induced oxidative damage to *mdx* muscle: the role of NADPH oxidase

N.P. Whitehead,<sup>1</sup> E.W. Yeung,<sup>2</sup> C. Pham<sup>1</sup> and D.G. Allen,<sup>1</sup> <sup>1</sup>Discipline of Physiology, Bosch Institute, University of Sydney, NSW 2006, Australia and <sup>2</sup>Department of Rehabilitation Sciences, Hong Kong Polytechnic University, Hong Kong.

Duchenne muscular dystrophy (DMD) is a degenerative muscle disease caused by the absence of the protein dystrophin, which connects the cytoskeleton to the surface membrane. In the *mdx* mouse, an animal model of DMD, we have shown that increased stretch-activated channel (SAC) activity is the main source of Ca<sup>2+</sup> influx following stretched contractions (Yeung *et al.*, 2005). Canonical transient receptor potential 1 (TRPC1) is the putative SAC protein in mammalian cells (Maroto *et al.*, 2005). It is known that some TRPC channels are activated by reactive oxygen species (ROS) and given that *mdx* muscles show evidence of oxidative stress, we postulated that stretch-induced ROS might activate SACs in dystrophic muscles. NADPH oxidase produces ROS in smooth muscle subjected to stretch (Grote *et al.*, 2003), and so we also investigated whether this was a source of the stretch-induced ROS production in *mdx* skeletal muscle. The two hypotheses tested through these experiments were: 1) Stretch-induced ROS mediate Ca<sup>2+</sup> influx through SACs and cause muscle damage; 2) NADPH oxidase is the main source of the stretch-induced ROS production in *mdx* muscle.

In the first experiments, we investigated whether the antioxidant N-acetylcysteine (NAC) could reduce stretch-induced muscle damage in *mdx* muscle. *mdx* and wild type (C57Bl/10ScSn) mice were euthanased and the *extensor digitorum longus* muscles removed. Muscles were perfused with or without 20 mM NAC. Solutions also contained 0.02% Evans Blue Dye (EBD) for assessment of membrane permeability. Muscles underwent 3 stretched (eccentric) contractions at 35°C. Tetanic force was measured before and 60 min after eccentric contractions and then muscles were frozen and sectioned for EBD uptake. Following the stretched contractions, force fell to 35 ± 3% for *mdx* muscles and NAC significantly improved force to 51 ± 2% (*p* < 0.01). As expected, force was much greater for wild-type muscles (69 ± 5%) and NAC had no additional effect. The area of EBD uptake was 8.6 ± 1.8% in *mdx* muscle cross-sections and this was significantly reduced by NAC to 2.6 ± 0.8% (*p* < 0.01). Wild-type muscles had a value of 1.8 ± 0.7%, which was not significantly different to NAC.

Secondly, we tested if ROS could activate SACs. *mdx* and wild type mice were killed by cervical dislocation and single muscle fibres from the *flexor digitorum brevis* were dissected. Fibres were loaded with the fluorescent Ca<sup>2+</sup> probe Fluo-4 AM and subjected to 10 stretched contractions. Following the stretched contractions, there was a significant increase in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) up to 30 min for *mdx* fibres (*p* < 0.001) but not for wild type fibres. The increased [Ca<sup>2+</sup>]<sub>i</sub> in *mdx* fibres was prevented by the antioxidant tiron (5 mM), which also reduced the force deficit. We then showed, in resting *mdx* fibres, that 10 μM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), also increased [Ca<sup>2+</sup>]<sub>i</sub>, which could be returned to baseline levels by the SAC blocker, streptomycin. Taken together, these findings suggested that increased ROS production during stretched contractions activate SACs and allow Ca<sup>2+</sup> entry in *mdx* muscle.

Finally, we explored whether NADPH oxidase was the main source of the enhanced ROS production during stretch. As with tiron, the increased [Ca<sup>2+</sup>]<sub>i</sub> in *mdx* fibres following stretched contractions was significantly inhibited (*p* < 0.01) by 1 μM diphenyleneiodium (DPI), an NADPH oxidase inhibitor, and this was also accompanied by increased muscle force. In preliminary experiments, another NADPH oxidase inhibitor, apocynin, also decreased the [Ca<sup>2+</sup>]<sub>i</sub> after stretched contractions.

The results of these experiments show that ROS are an important cause of stretch-induced damage to *mdx* muscle. As well as deleterious effects on proteins and muscle membranes, ROS also activate SACs, causing an influx of Ca<sup>2+</sup> and the activation of Ca<sup>2+</sup>-dependent damage pathways, such as calpains. We also provide evidence that NADPH oxidase is a primary source of ROS in stretched *mdx* muscle. We now aim to investigate why SACs are more sensitive to ROS in *mdx* muscle and to determine the key proteins targeted by ROS, which impair muscle function and contribute to dystrophic muscle damage.

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