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Free communications 10: Skeletal Muscle 1

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Chair: Robyn Murphy

Low dose formoterol treatment reverses sarcopenia and improves muscle function in fast- but not slow-twitch skeletal muscles of aged rats

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Ageing is associated with progressive muscle wasting (sarcopenia) and weakness and in the frail elderly, the loss of muscle mass can be so severe it impacts on the ability to perform the tasks of everyday living (Lynch, 2004). There is a profound need for strategies to ameliorate sarcopenia and improve quality of life. One strategy is treatment with β_2-adrenoceptor agonists (β_2-agonists). Although traditionally administered at low doses for treating asthma, at higher doses, β_2-agonists have potent muscle anabolic effects. We have shown previously that treatment with the β_2-agonist fenoterol can reverse muscle wasting and weakness in old rats (Ryall *et al.*, 2004a). However, fenoterol was also associated with impaired cardiac function, likely mediated through actions at the β_1-adrenoceptor (Gregorevic *et al.*, 2005).

The β_2-agonist formoterol has a greater duration of action than the most widely used asthma drugs, and has an increased selectivity for the β_2-adrenoceptor (Anderson, 1993). Having found formoterol to be more potent and efficacious than fenoterol, with respect to its effects on skeletal muscle mass, (Ryall *et al.*, 2004b), we tested the hypothesis that low dose formoterol treatment would reverse the atrophy and weakness in skeletal muscles of old Fischer 344 rats, whilst having minimal unwanted effects on the heart (due to reduced actions at the 1-adrenoceptor). Young (3 months/age, n = 8), adult (16 months/age, n = 8) and old (28 months/age, n = 6) rats were treated daily with either formoterol (25 μg/kg/day, i.p ~0.5 mL total volume) or saline vehicle for 4 weeks. Following treatment, rats were anaesthetised with sodium pentobarbitone and the fast-twitch extensor digitorum longus (EDL) and predominantly slow-twitch soleus muscles were surgically excised from the hindlimb for determination of isometric contractile properties *in vitro*. Following completion of the functional measurements the deeply anaesthetised rats were killed by surgical excision of the heart.

Muscle mass was greater in adult than young rats, indicative of normal growth, whilst old rats exhibited a significant reduction in muscle mass compared to both young and adult rats (EDL (in mg): young 125 ± 3 *vs* adult 142 ± 2 *vs* old 80 ± 7, $P < 0.05$; soleus (in mg): young 113 ± 4 *vs* adult 129 ± 2 *vs* old 94 ± 9, $P < 0.05$). Similarly, maximum force of EDL and soleus muscles was greatest in adult rats, and significantly reduced in old rats (EDL (in mN): young 2737 ± 80 *vs* adult 3019 ± 40 *vs* old 1902 ± 210, $P < 0.05$; soleus (in mN): young 1373 ± 95 *vs* adult 1576 ± 35 *vs* old 1009 ± 159, $P < 0.05$). Treatment increased EDL muscle mass in young, adult and old rats by 23%, 23% and 40% respectively, with a concomitant increase in maximum force producing capacity. Treatment increased soleus muscle mass and maximum force producing capacity in young, but not adult or old rats.Treatment was associated with a significant increase in heart mass in young rats (743 ± 31 *vs* 868 ± 50 mg, $P < 0.05$), but not in adult or old rats.

Our findings indicate a muscle specific decrease in β-adrenergic responsiveness with age, with fast- but not slow-twitch skeletal muscle responding to low-dose administration of formoterol. We conclude that formoterol can restore muscle mass and strength of fast-twitch skeletal muscles in old rats without cardiac hypertrophy.

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β-adrenergic signalling in skeletal muscle regeneration after myotoxic injury

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β-adrenoceptor agonists (β-agonists) have therapeutic potential for skeletal muscle wasting disorders due to their potent muscle anabolic effects. β-agonist administration promotes skeletal muscle hypertrophy *via* cAMP-mediated increases in protein accretion (Navegantes *et al.*, 2001; Ryall *et al.*, 2002). We have shown previously that β-agonist administration can enhance functional repair of rat skeletal muscle after injury (Beitzel *et al.*, 2004). It has been suggested that adrenoceptor desensitisation may limit the therapeutic efficacy of β-agonists in skeletal muscle (Claing *et al.*, 2002), but little is known about β-adrenergic signalling during muscle regeneration. The aim of this study was to examine aspects of β-adrenergic signalling in skeletal muscle and test the hypothesis that during regeneration, β-agonist administration does not cause β-adrenoceptor desensitisation. Male rats (275-300g) were deeply anaesthetised (ketamine 100 mg/kg and xylazine 10 mg/kg, *i.p.*), and the extensor digitorum longus (EDL) and soleus muscles of the right hindlimb were surgically exposed and injected with a maximal volume of the myotoxic agent, bupivacaine hydrochloride, to cause complete destruction of all muscle fibres (Beitzel *et al.*, 2004). The EDL and soleus muscles of the contralateral hindlimb served as uninjured controls. Rats then received either the β-agonist, fenoterol (1.4 mg/kg/day, *i.p.*), or an equivalent volume of saline for 7 days post-injury. Following treatment, rats were anaesthetised deeply and the EDL and soleus muscles were excised for analysis. All rats were killed by cardiac excision whilst anaesthetised. β-adrenoceptor density was measured using radioligand binding assays on isolated muscle membranes (Beitzel *et al.*, 2004). In regenerating EDL muscles there was a ~2-fold increase in β-adrenoceptor density compared to control values. β-adrenoceptor density in regenerating EDL muscles from fenoterol treated rats was only 57% that for saline treated rats. In regenerating soleus muscles, β-adrenoceptor density was restored to control levels. Fenoterol treatment reduced β-adrenoceptor density during regeneration to 58% that for saline treated rats. Adenylate cyclase (AC) activity assays were performed on fresh isolated muscle membranes. Despite the marked reduction in β-adrenoceptor density in both regenerating EDL and soleus muscles with fenoterol treatment, receptor desensitisation did not occur, since AC activity was maintained during isoproterenol stimulation. Various AC stimulants (NaF, forskolin and Mn^{2+}) which act at different points in the AC signalling pathway were used to examine the underlying mechanisms responsible for these observations. The findings indicated compensation for homologous downregulation of the β-adrenoceptors by the heterologous sensitisation at the level of AC. These results highlight the unique β-adrenergic signalling responses of injured/regenerating muscles compared with uninjured muscles, so as to maximise functional recovery.

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Streptomycin reduces stretch-induced membrane permeability in isolated muscles from mdx (dystrophic) mice

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Duchenne muscular dystrophy (DMD) is a genetic disease, which causes severe muscle degeneration, leading to profound muscle weakness and early death. DMD is caused by the absence of a protein, dystrophin, which is attached to the surface membrane of muscle fibres.

The recent focus of our laboratory has been to investigate whether a component of the damage is caused by entry of Ca^{2+} through stretch-activated channels (SACs) in the surface membrane. We have recently shown in single muscle fibres from *mdx* (dystrophic) mice that following stretched (eccentric) contractions, Ca^{2+} influx can be prevented and isometric force improved by the addition of three known SAC blockers, one being the antibiotic streptomycin (Yeung *et al*., 2005). It is known that stretched contractions cause greater membrane permeability of *mdx* muscles compared to wild-type (Petrof *et al.*, 1993). In the present study we investigated whether this stretch-induced membrane permeability was due to Ca^{2+}-dependant membrane damage as a result of Ca^{2+} entry through SACs.

Extensor digitorum longus (EDL) muscles were dissected from 8-10 week old wild-type and *mdx* mice. Muscles with clips attached to the tendons were mounted in a chamber between a force transducer and the lever of a motor. In some experiments, streptomycin (200μM) was added to the perfusate 60 min before the stretched contractions. Muscles were set to the length that produced maximal isometric force (optimum length, L_0). Procion orange, a membrane impermeable fluorescent dye, was added to the perfusate in order to detect fibres with increased membrane permeability. Muscle damage was induced by 10 stretched contractions, where the muscle was stretched by 30% of its length from L_0, during a 400 ms tetanus. Following the stretched contractions, isometric force was measured at 30 and 60 min and the muscle was frozen in isopentane cooled in liquid nitrogen at either 0, 30 or 60 min. Muscle cross-sections (10μm) were viewed with a fluorescent microscope and the area of procion orange positive muscle fibres was calculated as a percentage of the entire muscle cross-sectional area.

Following the stretched contractions, isometric force measured from control *mdx* muscles (n=5) fell to 33.4% ± 3.3. In experiments on *mdx* muscles where streptomycin was added to the perfusate before the stretched contractions (n=8), the reduction in force was significantly less, reaching 44.6% ± 1.6 ($p<0.05$, t-test). Wild-type muscles had a smaller decrease in force than *mdx* muscles following the stretched contractions (60.3% ± 1.6, n=3) and there was no effect of streptomycin (60.3% ± 2.2, n=3). Procion orange uptake for control *mdx* muscles was 5.0% ± 0.9 (n=3) immediately after the stretched contractions and then increased to 10.3% ± 0.8 (n=4) at 30 min and 15.1% ± 2.5 (n=4) at 60 min. At all times, streptomycin significantly reduced procion orange uptake ($p<0.05$, t-test), with values of 1.6% ± 0.7 (n=3), 5.3% ± 1.4 (n=3), and 4.9% ± 1.4 (n=5) at 0, 30, and 60 min, respectively. Wild-type muscles had very little procion orange uptake, with mean values of 0.7% (without streptomycin, n=2) and 1.1% (with streptomycin, n=2).

This study showed that following stretched contractions, membrane permeability of *mdx* muscles increased progressively over 60 min, and importantly, most of this permeability could be prevented by the SAC blocker, streptomycin. Taken together, these results suggest that the increased membrane permeability is mainly due to Ca^{2+} entry through SACs and not the result of transient mechanical tears of the membrane during the stretched contractions (Petrof *et al.*, 1993). The mechanism by which increased intracellular Ca^{2+} causes muscle damage to dystrophic muscle is unclear but might be attributable to an increased production of reactive oxygen species and/or the activation of calcium-dependent proteases or phospholipase A_2. These damage pathways are now being explored in our current series of experiments.

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Muscle weakness in a mouse model of nemaline myopathy can be reversed with exercise and reveals a novel myofibre repair mechanism

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Nemaline myopathy (NM) is an inherited muscular disorder characterised by muscle weakness and the presence of distinct rod-shaped accumulations of sarcomeric proteins (nemaline rods) in muscle fibres. We previously generated a transgenic mouse model for nemaline myopathy that expresses in all skeletal muscles a mutant α-tropomyosin-slow (Met9Arg) protein that causes NM in humans (Laing *et al.*, 1995). This mouse shows all of the features of the human disease including late-onset muscle weakness (4-6 mo of age) and nemaline rods, the defining feature of the disease (Corbett *et al.*, 2001). A debilitating feature of NM in humans is prolonged muscle weakness after periods of inactivity. In the present study, we have examined endurance exercise as means of improving recovery following muscle inactivity in the transgenic NM mouse model. Physical inactivity was induced by bilateral hind limb immobilisation (using surgical tape) in a maximal dorsoflexed position that stretches the ventral muscles (e.g. soleus) and shortens the dorsal muscles (e.g. extensor digitorum longus, EDL). Mice were fully anaesthetised during the immobilisation procedure with ketamine/xylazine (100 and 10 mg/kg body weight, respectively). The mice were then subjected to one of three, 4 week recovery regimens: 1) minimal physical activity (cage rest), 2) low intensity voluntary free-wheel exercise, or 3) high intensity treadmill exercise (1.5h/day; 5 days/week; 20 m/min; 5% incline). Fours weeks of immobilisation resulted in muscle fibre atrophy and severe muscle weakness in both wild-type (WT) and NM mice. However, NM mice were weaker than the WT mice after immobilisation, and exercise, not cage-rest, was required to regain whole body strength. Immobilisation of the EDL in the shortened position, led to an increase in the number of nemaline rods in the NM mice and surprisingly these rods that were formed with immobilisation appeared to be resolved with endurance exercise. Together these results suggest that nemaline rods may have a role in muscle weakness in NM. Chronic stretch-immobilisation of the soleus muscle for 10 days resulted in myonecrosis and continued stretch-immobilisation for a further 18 days resulted in complete regeneration of the damaged fibres. Although muscle regeneration did occur in NM mice during immobilisation it occurred without the classical features of regeneration (centrally located myonuclei) indicating an alteration in the normal repair process of muscle in NM. In conclusion, exercise is effective at attenuating disuse-induced muscle weakness in the NM mouse model. The novel muscle repair process in the NM maybe a response to primary myofibrillar damage that occurs in NM and maybe distinct from the classical repair observed in muscular dystrophies.

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Contraction-mediated damage in *mdx* dystrophic mouse tibialis anterior muscles is not affected by the membrane sealant poloxamer

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Dystrophin deficiency causes Duchenne muscular dystrophy (DMD), a severe inherited and progressive disease of striated muscle in humans. Dystrophin is a subsarcolemmal protein responsible for linking the cytoskeleton to the extracellular matrix, and it is postulated to play a mechanical role in stabilising the muscle fibre membrane (sarcolemma) during contraction. The muscles of the *mdx* dystrophic mouse, an animal model for DMD, also lack dystrophin, which makes them more susceptible to contraction-induced injury (Dellorusso *et al.*, 2002). The increased susceptibility to stretch-mediated Ca^{2+} overload, leading to cell contracture and death, is prevented by treatment with the membrane sealant poloxamer 188 (P-188; Yasuda *et al.*, 2005). P-188 can incorporate into damaged membranes and effectively 'plug' holes caused by lengthening contractions. We tested the hypothesis that treatment with P-188 would reduce damage and promote membrane integrity in muscles from *mdx* mice following contraction-induced injury.

On the day prior to experimentation, 4-6 month old *mdx* and wild type (C57BL/10 ScSn) mice were injected with Evans blue dye (EBD; 100 mg/kg). Mice were anaesthetised by intraperitoneal injection with pentobarbitone sodium (60 mg/kg), the right external jugular vein exposed, and a bolus dose of P-188 (460 mg/kg body mass; dissolved in 200 µL sterile saline), or vehicle only, infused intravenously. The right tibialis anterior (TA) muscle was surgically exposed and the distal tendon firmly attached to the lever arm of a servomotor/transducer with the knee immobilised by a secure clamp. The right sciatic nerve was also exposed to deliver supramaximal square wave pulses *via* a needle electrode. The TA muscle was immersed in warmed paraffin oil to maintain temperature at 37°C and maximum isometric tetanic tension (P_o) recorded at the muscles optimum length *in situ* (intact nerve and blood supply). The muscle was subjected to two stretches of 40% strain (relative to muscle fibre length; initiated from the plateau of isometric contractions, Consolino & Brooks 2004). The magnitude of damage was assessed 5, 10 and 15 minutes later by the deficit in P_o (force deficit = $(P_{o\ (initial)} - P_{o\ (post\ strain)})/P_{o\ (initial)}$%). The TA muscle was then carefully dissected free and rapidly frozen for later cryosectioning. At the conclusion of experimentation mice were killed by cervical dislocation whilst deeply anaesthetised. Muscle cross sections (8µm) were analysed using a fluorescence microscope for quantification of intracellular infiltration of EBD.

Preliminary findings indicate that force deficit was greater in *mdx* than wild type mice (43 ± 9% vs 25 ± 9%, $P < 0.05$), but was unaffected by P-188 treatment. The proportion of EBD positive fibres was greater in *mdx* than wild type mice (15 ± 7% vs. 2 ± 1%, $P < 0.05$), and was reduced in *mdx* mice treated with P-188 (4 ± 2%, $P < 0.05$), irrespective of injury. The proportion of EBD positive fibres was not affected by the injury protocol in either wild type or *mdx* mice. The results indicate that P-188 does not affect the force deficit following contraction- induced injury but may play a role in maintaining sarcolemmal integrity in muscles from *mdx* mice, which might prevent Ca^{2+} overload and promote cell survival.

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Aberrant splicing of ryanodine receptor reduces Ca^{2+} release *via* an inter-domain interaction in myotonic dystrophy type 1

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Myotonic dystrophy type 1 (DM1) is a multisystem disorder with autosomal dominant inheritance. Expansion of CTG repeats in the 3' untranslated region of a putative protein kinase gene occurs in DM1. Downstream, it was reported that several mRNAs were aberrantly spliced in muscles from DM1 patients, but the cause of muscle weakness is unknown. We investigated splicing of two major proteins of the sarcoplasmic reticulum, the ryanodine receptor 1 (RyR1) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase. The fetal variants, ASI(-) of RyR1 which lacks residue 3481-3485, and SERCA1b which differs at the C-terminal were significantly increased in skeletal muscles from DM1 patients and the transgenic mouse model of DM1 (HSA^{LR}).

To examine the functional difference between the ASI(+) and the ASI(-) RyR1 isoforms, we characterized [^{3}H]ryanodine binding to microsomal vesicles of HEK293T cells transfected with ASI(+) and ASI(-) RyR constructs. [^{3}H]ryanodine binding is a standard technique for assessing the open probability of RyR channels, because ryanodine binds solely to open channels and the binding is proportional to open probability. Channel open probability was also measured form RyRs incorporated into using artificial lipid bilayers. Finally Ca^{2+} release was examined using Ca^{2+} imaging techniques in dypedic myotubes (lacking RyR1) transfected with ASI(+) and ASI(-) RyR1 cDNA.

The affinity of [^{3}H]ryanodine binding to ASI(+) was higher than that to ASI(-). Channel open probability was significantly decreased and mean open time was significantly shorter in ASI(-) than in ASI(+). Consistent with the lower activity of ASI(-) channels, the RyR1-knockout myotubes expressing ASI(-) exhibited a decreased incidence of Ca^{2+} oscillations during caffeine exposure compared with that observed form myotubes expressing ASI(+) RyR (Kimura *et al.*, 2005). To determine how this aberrant splicing affects the activity of RyR channels, we tested whether the splicing region is involved in inter-domain interaction using synthetic peptides (Yamamoto *et al.*, 2000). Both peptides corresponding to the Thr(3471)-Gly(3500) around the ASI region in the presence (ASI(+)) and the absence (ASI(-)) of exon ASI activated native RyRs. However, peptide ASI(-) activated the channels more than peptide ASI(+).

The results suggest that ASI(-) peptide interrupts an inhibitory interdomain interaction in the native RyR more strongly than the ASI(+) peptide. We therefore suggest that ASI(-) region may interact more tightly with other domains and produce stronger inhibition of ASI(-) RyR, resulting in reduced activity of the ASI(-) RyR.

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