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Free communications 8: Skeletal muscle, microcirculation, and reproduction

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Chair: Derek Laver

Role of the calcineurin signal transduction pathway in muscle regeneration in dystrophic *mdx* mice

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Whilst sharing the same genotype as in Duchene muscular dystrophy (DMD), *mdx* mice exhibit a more benign dystrophic phenotype. Only the diaphragm muscle of *mdx* mice shows a severe and progressive pathology. Limb muscles of *mdx* mice undergo a bout of severe muscle degeneration at 2 to 4 weeks of age, but a high regenerative capacity ensures almost complete functional and structural recovery (Lynch *et al.*, 2001). The cellular mechanisms responsible for the enhanced regenerative capacity of *mdx* hindlimb muscles are not well understood. Calcineurin, a phosphatase enzyme that regulates transcription by sensing changes in intracellular calcium, has been shown to regulate skeletal muscle regeneration (Sakuma *et al.*, 2003). We have recently shown that inhibiting the calcineurin signal transduction pathway interferes with successful muscle regeneration in young *mdx* mice (Stupka *et al.*, 2002).

When 18 day old *mdx* mice were treated for 16 days with cyclosporine A (CsA; 30 $mg \bullet kg^{-1} \bullet day^{-1}$), an inhibitor of calcineurin, muscle regeneration was severely impaired. EDL and soleus muscle mass was ~25% lower and maximum force producing capacity was 30-35% lower in CsA treated *mdx* mice compared with vehicle treated littermates (Stupka, *et al.*, 2002). In the present study, we performed histological and immunohistochemical analyses to confirm the inhibitory effects of CsA treatment on muscle regeneration in young *mdx* mice.

Muscle sections were stained with haematoxylin and eosin for analysis of general muscle architecture, Van Gieson's stain for collagen deposition, and reacted with antibodies against myogenin (marker of satellite cell differentiation), and macrophages, as markers of regeneration. In CsA treated *mdx* mice, EDL and soleus muscle fibre cross-sectional area was ~25-30% smaller, had fewer centrally nucleated fibres, and more collagen, connective tissue, and mononuclear cell infiltration, than vehicle treated littermates. CsA administration did not affect macrophage infiltration in EDL or soleus muscles from *mdx* mice. Despite having significantly fewer centrally nucleated fibres, EDL and soleus muscles from CsA treated *mdx* mice had two to four times more myogenin positive nuclei than control *mdx* mice. Even though satellite cells from CsA treated *mdx* mice expressed myogenin they did not undergo normal differentiation and myoblast fusion.

Given that the calcineurin signal transduction pathway is essential for successful regeneration of hindlimb muscles in young *mdx* mice, we hypothesise that the pathology of the diaphragm muscle in *mdx* mice may be due to impairment of the calcineurin signal transduction pathway. To test this hypothesis, both upstream and downstream markers of the calcineurin signal transduction pathway in soleus, tibialis anterior, and diaphragm muscles from adult *mdx* and wild type (C57BL/10) mice were examined using a variety of biochemical and immunohistochemical techniques. Preliminary data suggests that there are differences in phosphorylated (inactivated) and dephosphorylated (activated) NFATc1 protein content and calcineurin-A protein content. Understanding the cellular mechanisms responsible for the difference in pathology between *mdx* diaphragm and hind limb muscles may provide insights into the regenerative process of dystrophic muscle and potential novel treatment strategies for DMD.

Lynch, G.S., Hinkle, R.T., Chamberlain, J.S., Brooks, S.V. & Faulkner, J.A. (2001) *Journal of Physiology,* 535, 591-600.

Stupka, N., Gregorevic, P., Plant, D.R. & Lynch, G.S. (2002) *Proceedings of the Australian Health and Medical Research Congress,* A2404.

Sakuma, K., Nishikawa, J., Nakao, R., Watanabe, K., Totsuka, T., Nakano, H., Sano, M. and Yasuhara, M. (2003) Acta Neuropathologica 105, 271-280.

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Muscle damage in *mdx* mice is reduced after treatment with streptomycin

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Extensive research has been carried out on the cellular mechanisms underlying Duchenne Muscular Dystrophy (DMD) and a number of therapeutic options have been proposed for the treatment of this muscle disease. However, while much progress has been made, there are currently no effective therapies that significantly slow the progression of the disease. It has been hypothesised that dystrophin may be important in maintaining the normal function of certain membrane channels, in particular stretch-activated channels (SAC), and that calcium entry through these channels could initiate degradative pathways that leads to muscle fibre degeneration (Franco & Lansman, 1990). Recent research in our laboratory has focused on the role of SAC in muscle damage using *mdx* mice, an animal model of DMD. In single muscle fibres from *mdx* mice, it has been found that a component of the damage induced by a series of eccentric contractions can be prevented by two known SAC blockers, gadolinium and streptomycin (Yeung, Head & Allen, 2003; personal communication).

The current study aimed to investigate the role of SAC in muscle damage *in vivo* by using *mdx* mice that were given either normal drinking water (control) or water containing streptomycin (3mM). A previous study by McBride *et al*., (2000) showed that this concentration of streptomycin prevents the muscle fibre depolarisation caused by eccentric contractions, which has also been attributed to the entry of Na^+ through SAC. It is known that muscle damage in *mdx* mice begins at about 21 days after birth (McGeachie *et al*., 1993), and that the first signs of regenerating fibres, evident by the presence of centrally located nuclei, occurs at 24 days. Thus, the mice used in the current study began the streptomycin treatment at 18 days, that is, three days before the onset of any muscle damage. At various times after the onset of the treatment, mice were killed by cervical dislocation and the EDL muscles were dissected out and placed in a normal physiological solution. Each muscle was attached to steel frame on a cork pad and immersed in embedding medium (Tissue-Tek) before being frozen in liquid nitrogen. Muscle cross-sections (10μm thick) were stained with haematoxylin and eosin, and viewed under a light microscope, with digital images taken for analysis of the location (central or peripheral) of muscle fibre nuclei.

At 24 days, the number of fibres with nuclei that were centrally located was 25% for control *mdx* mice compared to 4% for the streptomycin treated *mdx* mice. Over the next three days, the number of fibres with central nuclei for the control mice remained fairly similar with a peak of 30%. Values for the streptomycin treated mice also increased, peaking at 20%, but always remained lower than those of their age-matched control mice. This difference was statistically significant ($P<0.05$; two-factor ANOVA). Other indicators of muscle damage, such as plasma creatine kinase levels and serum albumin localisation within muscle fibres, are currently being used in order to further investigate and quantify the effect of streptomycin in preventing damage in *mdx* muscle.

Franco, A. & Lansman, J.B. (1990) *Nature,* 344, 670-673.

McBride T.A., Stockert, B.W., Gorin, F.A. & Carlsen, R.C. (2000) *Journal of Applied Physiology,* 88, 91-101.

T.A. & Morgan, J.E. (1993) *Journal of the Neurological Sciences,* 119, 169-179.

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Influence of lowered $[Na^+]_o$ on single and trains of action potentials in soleus muscle fibres of the mouse

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A reduction of extracellular $[Na^+]$, ($[Na^+]_o$), diminishes peak force and exacerbates fatigue during continuous tetanic stimulation of isolated skeletal muscle (Bezanilla *et al.*, 1972; Bouclin *et al.*, 1995; Cairns & Dulhunty, 1995). The mechanism for this effect is not fully understood. The aim of this study was to determine whether changes to the action potential could explain the reduced force at lowered $[Na^+]_o$ in mammalian skeletal muscle.

Isometric contractions and action potentials were elicited by supramaximal electric field stimulation via wire electrodes (10 V, 0.3 ms pulses) in isolated soleus muscles from mice. Muscles were bathed in control Krebs solution containing 147 mM Na^+, and then at lowered $[Na^+]_o$ (100, 60, 40 or 30 mM; NaCl was replaced by N-methyl-D-glucamine) at 25°C. Intracellular recordings of action potentials were made using conventional glass microelectrodes. Trains of action potentials (50 or 125 Hz, for 2 s) were recorded in deep fibres after stretching the muscle, in order to prevent movement artifacts.

Single action potentials: Lowered $[Na^+]_o$ had no effect on the resting membrane potential but caused action potentials to become progressively smaller and broader. The overshoot fell from +32 mV at 147 mM Na^+, to -20 mV at 30 mM Na^+. All fibres were excitable at 147-60 mM Na^+, but 19% and 40% of the fibres were inexcitable at 40 and 30 mM Na^+, respectively.

Trains of action potentials: At 147 mM Na^+, the 2-s trains of stimuli triggered action potentials on every occasion (100 or 250 action potentials), in association with a decrease in resting potential (between action potentials) and overshoot during the trains. At 40 mM Na^+, complete trains of small action potentials were produced at 50 Hz, as observed in frog muscle fibres (2). However, at 125 Hz there was considerable skipping leading to a complete failure to generate action potentials, usually within 500 ms.

Action potentials and force: The peak twitch force - overshoot relationship (determined by combining twitch and single action potential responses) showed that force was well maintained until the overshoot disappeared. Trains of action potentials evoked at 125 Hz at 147 mM Na^+, often had the peak of the action potential between 0 and -30 mV and this occurred without any fade (decline of peak force within a tetanus). At 40 mM Na^+, the peak tetanic force fell to 53% of the control at 50 Hz, and to 19% of the control at 125 Hz; the difference was linked to the failure to generate action potentials during a train.

In summary, the decline of peak tetanic force at lowered $[Na^+]_o$ can be explained by (i) the presence of inexcitable fibres, and (ii) a failure to generate action potentials during trains when evoked at high frequency. There is a considerable safety margin for a decline of the overshoot before peak twitch force is impaired. Our data also suggests that (iii) smaller action potentials during a train may make a moderate contribution to the decline of peak tetanic force.

Bezanilla, F, Caputo, C, Gonzalez-Serratos, H. & Venosa, R.A. (1972) *Journal of Physiology,* 223: 507-523.

Bouclin, R, Charbonneau. E. & Renaud, J.M. (1995) *American Journal of Physiology,* 268: C1528-C1536.

Cairns, S.P. & Dulhunty, A.F. (1995) *Muscle and Nerve,* 18: 890-898.

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The effect of reactive oxygen species on muscle fatigue at room temperature compared to body temperature

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The production of reactive oxygen species (ROS), in particular superoxide anion radicals, plays a significant role in the modulation of muscle function (Clarkson & Urso, 2003). During intense exercise, there is an increased production of ROS, which leads to oxidative stress on muscle. Such stresses are thought to lead to the loss of contractile function, reduction in Ca^{2+} handling and contribution to fatigue (Reid, 2001). Also, it has been shown that increased temperature results in muscle dysfunction due to oxidative stress (van der Poel & Stephenson, 2002).

The present study is concerned with muscle performance during several bouts of fatigue at room temperature compared to body temperature. In particular, the influence of ROS at these temperatures is assessed in terms of onset of fatigue and maximum force.

Small muscle bundles (5 - 10 fibres per bundle) were dissected from the flexor brevis muscle of mice and were subjected to a fatigue protocol at room temperature (25°C) and at body temperature (37°C). Muscles were fatigued until force reached 50 % of the initial force. For each preparation, bundles were subjected to three fatigue runs (R1, R2, R3) allowing adequate time for muscles to recover between each run (45 minutes).

It was observed that there was no significant difference in the time taken for muscles to fatigue to 50% of the maximum force ($T_{1/2}$) at 25°C for R1, R2 and R3. Tiron (20mM), a free radical scavenger, was applied for 30 minutes between R1 and R2 and this treatment had no significant effect on the $T_{1/2}$ of R2 and R3 at 25°C. At 37°C however, $T_{1/2}$ was reduced to 65 ± 6 % for R2 and 26 ± 11 % for R3 compared to the $T_{1/2}$ for R1. When muscle preparations were treated with 20mM tiron, $T_{1/2}$ recovered to 106 ± 16 % for R2 and 103 ± 15 % for R3 compared to the value of R1. These results show that the ROS production has a profound effect on muscle fatigue at 37°C.

At 25°C there was no significant change in the fall of maximum force between each of the fatigue runs. At 37°C however, there was a significant decline in the maximum force when comparing R2 and R3 ($P<0.05$); and R1 and R3 ($P<0.01$). Treatment with tiron significantly reversed the decline of maximum force at 37°C. These results suggest that the production of free radicals at higher temperatures adversely affect the contractile properties, which are in some way responsible for the observed decline in maximum force.

The present study clearly shows that multiple bouts of fatigue at body temperature, in contrast to room temperature, progressively decreases muscle performance in terms of the onset of fatigue and maximum force development. Reduced muscle performance at 37°C appears to be partly caused by an increase in the production of ROS.

Reid, M.B. (2001) *Journal of Applied Physiology*, 90, 724-731
Urso, M.L. & Clarkson, P.M. (2003) *Toxicology*, 189, 41-54.
Van der Poel, C. & Stephenson, D.G. (2002) *Journal of Physiology*, 544.3, 765-776

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Nutritive and non-nutritive blood flow and oxygen consumption in active rat skeletal muscle

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The objective of this study was to investigate the relationship between muscle metabolism and vascular distribution in the rat hindlimb. Clark *et al.*, (1995) categorised vasoconstrictors into two groups using a perfused sacrificed hindlimb model. All increase perfusion pressure, with Type A (low dose noradrenaline (NAd), vasopressin, angiotensin II) increasing oxygen uptake ($\dot{V}O_2$) redirecting blood into nutritive capillary beds associated with the muscle tissue) and Type B (serotonin (5-HT), high dose noradrenaline) decreasing hindlimb oxygen consumption, redirecting blood into non-nutritive capillary beds (associated connective tissue, adipose and septum). We used the in vivo autoperfused rat hindlimb with maintained vascular resistance to test the hypothesis that nutritive/ non-nutritive blood flow distribution can be observed in metabolically active (contracting) muscle and can be differentiated by vasodilators.

Male Wistar rats were anaesthetised with sodium pentobarbital (6mg/100g body weight i.p.). Polyethylene cannulae were filled with 0.9% heparinised saline containing 6% w/v dissolved dextran70. Mean systemic blood pressure was recorded from the left common carotid artery. The right femoral artery was cannulated to supply blood to the left femoral artery (perfused) passed through a pump for constant flow. Perfused hindlimb pressure was recorded via a side arm pressure transducer distal to the pump. Passive venous return occurred from the left femoral vein to the right external jugular vein. The left sciatic nerve was stimulated via a bipolar electrode and tension development recorded in the gastrocnemius muscle bundle. Vasoactive drugs (2 constrictor, 8 dilator) were prepared with saline and 0.01% ascorbic acid, and injected into the arterial loop. Blood was sampled from the venous and arterial loops and $\dot{V}O_2$ determined using the Fick equation.

During basal conditions, NAd (100nM – 256μM) increased mean perfusion pressure by up to 260 ± 34% ($P < 0.001$, n = 6, mean ± SEM) and 5-HT (12.5μM – 100μM) by up to 225 ± 30% ($P < 0.005$, n = 6). The $\dot{V}O_2$ did not change during NAd infusion but decreased by up to 67 ± 7% during 5-HT infusion ($P < 0.005$). Mean perfusion pressure was decreased during the infusion of isoprenaline by 33 ± 2% ($P < 0.001$, n = 6) and histamine by 25 ± 2% ($P = 0.05$, n = 6) whilst $\dot{V}O_2$ did not change.

During muscle contraction, NAd increased mean hindlimb pressure by 96 ± 3% ($P < 0.001$) and 5-HT increased by 112 ± 12% ($P < 0.001$). $\dot{V}O_2$ by 46 ± 10% ($P < 0.05$). Isoprenaline and histamine decreased mean perfusion pressure by 24 ± 3%($P < 0.005$) and 9 ± 3% respectively ($P < 0.01$). Both vasodilators increased $\dot{V}O_2$, isoprenaline by 175 ± 40% ($P < 0.01$) and histamine by 96 ± 40% ($P < 0.05$).

These results show that the vasoconstrictors NAd and 5-HT have opposing effects on $\dot{V}O_2$ during both basal and twitch conditions. However we were unable to find a vasodilator that could decrease $\dot{V}O_2$ in a similar fashion to 5-HT. The reduced effect of 5-HT on $\dot{V}O_2$ during twitch maybe due to local effects of the twitch (such as vasoactive metabolites) on oxygen demand, hence overriding the vasoconstriction of the nutritive pathway.

Clark, M.G., Colquhoun, E.Q., Rattigan, S., Dora, K.A., Eldershaw, T.P.D., Hall, J.L. & Ye, J. (1995) *American Journal of Physiology: Endocrinology and Metabolism*, 31, E797-E812.

Glutathione synthesis in whole human red blood cells

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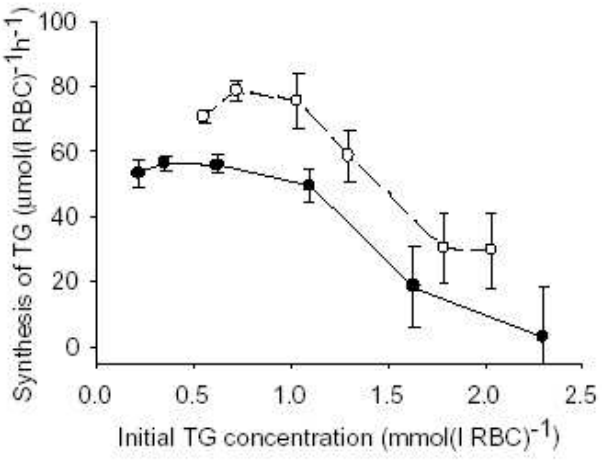
The maintenance of adequate levels of the cell antioxidant glutathione (GSH) is essential for defence against oxidative disruption of membranes, proteins and DNA. These vital cell constituents are protected by the preferential oxidisation of GSH to glutathione disulfide (GSSG) which is rapidly reduced back to GSH by NAPDH and glutathione reductase. Recycling of GSSG to GSH allows for slow replacement of total glutathione (GSH & GSSG; TG) with a turnover time of six days in red blood cells (RBCs). However, oxidative stress can cause accumulation of GSSG and its export from the cell. Under such circumstances, the rate of TG synthesis is thought to be increased by reduced product inhibition by GSH on the rate limiting enzyme γ-glutamyl-cysteine synthase.

GSH synthesis has been studied using lysates and purified enzymes, but little is known of the control of GSH production within whole RBC. Although sensitive and specific spectrophotometric methods have long been available for measuring TG (Tietze, 1969), its synthesis in RBC is too slow to measure in this way particularly against the high concentrations (2.3 mmol(l RBC)$^{-1}$) normally present.



GSH is also active in glutathione S-transferase catalysed conjugation with hydrophobic, electrophilic compounds including toxins and drugs. Glutathione S-conjugates are actively exported from RBCs. One such S-transferase substrate 1-chloro-2,4-dinitrobenzene (CDNB) has been used extensively for depleting cell TG content for experimental purposes. For RBCs suspended at a 10% haematocrit in buffer containing 2 mM dithiothreitol, I found that incubation for 40 minutes at 37°C with CDNB concentrations from 0.08 to 0.40 mM reduced the TG levels by 30 to 90%, respectively. When the TG depleted RBCs were washed free of the water soluble glutathione-CDNB conjugate and resuspended in a HEPES buffered solution containing 5 mM N-acetylcysteine and glucose, the TG levels in the RBC, measured by a modified version of Tietze's assay, increased in a linear fashion for several hours. The rate of TG production was dependent on the degree of depletion of TG with a maximum rate of synthesis of 72.6 ± 15.0 µmol(l RBC)$^{-1}$h^{-1} (mean ± SD) which is a five-fold increase on rates previously measured in RBCs with normal levels of GSH (Griffith, 1981). The results of 2 of 5 such experiments are presented in the Figure. The error bars are the SE of the rates of synthesis returned by linear regression. Buthionine sulfoximine a potent inhibitor of γ-glutamyl-cysteine synthase was shown to inhibit the rate of TG increase in RBC by 55% confirming that the accumulation of TG observed was due to synthesis. The controlling factors of TG synthesis within whole RBCs will be determined by measuring TG production under various experimental conditions. Once identified, manipulation of controlling factors may be used to prevent depletion or stimulate production of GSH in several disease states characterised by reduced cell TG.

Griffith, O.W. (1981) *Journal of Biological Chemistry* 256, 4900-4.
Tietze, F. (1969) *Analytical Biochemistry* 27, 502-22.

Circadian gene expression in mouse uterus and liver

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The circadian rhythm of the suprachiasmatic nucleus (SCN) of the brain is entrained by light. Upon input of light, the circadian rhythm is generated as follows: the transcription factors CLOCK and BMAL1 heterodimerise to upregulate transcription of the genes *Per1-3* and *Cry1-2*. PER and CRY proteins heterodimerise, then translocate to the nucleus where they down-regulate CLOCK/BMAL1 activity. This reduces *Per* and *Cry* transcription and PER and CRY decay, releasing the inhibition on BMAL1. Eventually BMAL1 levels increase, initiating the next 24 h cycle of transcription. The circadian clock in the SCN coordinates circadian clocks in peripheral tissues (Reppert & Weaver, 2002). In the female reproductive tract and embryo, timers control when developmental events occur (Johnson & Day, 2000). It is possible that the circadian clock acts as one such timer as we have shown that the circadian genes, *Per1-3, Cry1-2, Bmal1* and *Clock*, are expressed in the female reproductive tract (uteri and oviducts) and in preimplantation embryos of the mouse (Johnson *et al.*, 2002). To determine whether a circadian clock exists in the uterus, we have quantified mRNA using real-time PCR.

Female MF1 mice were housed on a 12/12 h light/dark cycle. Liver and uterine tissues were obtained from mice euthanised by cervical dislocation at circadian times 0, 4, 8, 12, 16 and 20 of the proestrous, oestrous and dioestrous periods of the oestrous cycle. Whole tissues were snap frozen in liquid nitrogen and total RNA was purified. The RNA concentration was determined by RiboGreen assay, then reverse transcribed to cDNA. Expression of the circadian genes *Per2, Cry1, Bmal1* in the cDNA was then quantified by real-time PCR using SYBR Green I. Standard curves were run for each gene (β-*actin, Per2, Cry1, Bmal1*) using cDNA. From each standard curve the amount of each gene expressed was calculated and then normalised to β-*actin*.

Timed liver cDNA samples showed changes in circadian gene expression similar to that in the literature (Lee *et al.*, 2001). This confirmed the use of the real time PCR protocol for use on uterine samples. In uterine cDNA, preliminary results suggest that *Per2* and *Bmal1* expression change with a circadian rhythm. Further research will determine whether a circadian clock exists in other periods of the oestrous cycle.

Johnson, M. H. & Day, M. L. (2000) *BioEssays*, **22:** 57-63.

Johnson, M. H., Lim, A., Fernando, D. & Day, M. L. (2002) *Reproductive BioMedicine Online,* **4:** 140-145.

Lee, C., Etchegaray, J.-P., Cagampang, F.R.A., Loudon, A.S.I. & Reppert, S M. (2001) *Cell*, **107:** 855-867.

Reppert, S.M. & Weaver, D.R. (2002) Coordination of circadian timing in mammals. *Nature,* **418:** 935-941.