AuPS/ASB Meeting - Newcastle 2007

Free communications: Skeletal muscle 1

Monday 3 December 2007 – Hunter Room

Chair: Nicole Beard

Properties of the tubular system network in twitch muscle

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The tubular (t-) system is an invagination of the surface membrane in skeletal muscle that enters the cell in a uniform fashion at the level of the sarcomere. The t-system membrane is closely apposed to the terminal cisternae of the sarcoplasmic reticulum (SR) throughout the cell. This allows the protein-protein contacts essential for excitation-contraction coupling. Also, the t-system acts as conduit for extracellular fluid deep inside the cell. The t-system appears to be a functional longitudinally connected network, as mechanically skinned fibres of fast-twitch rat muscle display spontaneous force responses (Posterino *et al.*, 2000). In the absence of a surface membrane, the t-system appears to remain connected by: (i) the realignment or "crossing-over" of regular t-tubules across misregistered sarcomeres; and (ii) longitudinal connections that run perpendicularly between regular t-tubules. Our aim was to examine the properties of the t-system network in more detail to help define its role in skeletal muscle.

All experiments were approved by the Animal Ethics Committee at The University of Queensland. Cane toads were stunned by a blow to the head and double pithed. The iliofibularis muscles were removed. Rats and mice were killed by asphyxiation and the soleus and extensor digitorum longus (EDL) muscles were removed. Intact fibre bundles were exposed to Na^+-based physiological solution containing either Cascade Blue or purified 500- or 2000-kDa fluorescein dextran and imaged on a 2-photon microscope. In other experiments, isolated intact fibres were exposed to either, or a combination of, 500-kDa fluorescein dextran, rhod-2 salt or fluo-5N salt. Single fibres were then mechanically skinned to trap fluorescent dye in the sealed t-system, and then transferred to a custom-built experimental chamber containing a K^+-based internal solution. Additionally skinned fibres were stimulated with platinum electrodes in a bath containing a K^+-repriming solution with 0.1 mM rhod-2 above an Olympus FV1000 confocal microscope. Cytoplasmic Ca^{2+} release was imaged in linescan mode, with the scanning line parallel to the longitudinal axis of the fibre. This maneouver allowed determination of whether action potentials were propagating radially or longitudinally by the apparent angle of the elicited SR Ca^{2+} release to the scanning line. All experiments were performed at 20 ± 2°C.

Field stimulation of skinned fibres from rat EDL showed an action potential-induced Ca^{2+} release from SR uniformly along the length of the scanning line, indicating stimulation elicited action potentials at each t-tubule, which in turn propagated radially across the fibre. Spontaneously activating Ca^{2+} release was imaged at a consistent angle to the scanning line, indicating action potentials propagated longitudinally along the fibre at a constant rate of 7.4 ± 1.3 mm/s ($n = 8$). To assess the diameter of the t-system lumen, fluorescein dextrans of 500- and 2000-kDa were employed. These dextrans were calculated to have diameters of 29 and 55 nm, respectively. Imaging of intact fibre bundles exposed to the dyes all showed a strong fluorescence band immediately outside the fibre. Only Cascade Blue and 500-kDa fluorescein dextran showed dye entry into the t-system, consistent with estimates of t-tubule diameter of ~30 nm (Luff & Atwood, 1971). To determine whether the longitudinal connections had a similar diameter as the regular t-tubule, we trapped the 500-kDa fluorescein dextran and rhod-2 (MW = 869) in the t-system of the same skinned fibre preparation. Confocal imaging showed that both the dextran and rhod-2 had access to the entire regular t-system. However, only rhod-2 had access to the perpendicular longitudinal connections between regular t-tubules. This indicated that these longitudinal connections have a smaller diameter than that of the regular t-tubules. Finally it was important to probe the mobility of molecules of different size through the lumen of the t-system network. To do this we measured fluorescence recovery after photobleaching (FRAP) of the 500-kDa dextran and rhod-2 or fluo-5N (MW = 958). The small molecular weight dyes showed full FRAP within 20-30 min in all three twitch fibre types examined. Only partial FRAP of the dextran was observed even after 1 h in the three fibre types examined ($n > 3$ in all cases).

In conclusion, we have shown that the t-system network is excitable and has a lumen that is fully connected that can evenly distribute small molecules. Furthermore, the regular t-tubules of the network have a significantly larger diameter than the longitudinal connections that run perpendicularly to the regular tubules.

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An action potential activated Ca^{2+} current in skeletal muscle

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Ca^{2+} entry into cells is a fundamental process that enables cells to regulate cytoplasmic [Ca^{2+}], [Ca^{2+}] in intracellular stores and many Ca^{2+}-dependent intracellular processes from gene expression to muscle contraction. Cardiac cells have an absolute requirement for Ca^{2+} entry via the L-type Ca^{2+} channel upon membrane excitation to induce Ca^{2+} release from the sarcoplasmic reticulum (SR) and consequently activate the contractile apparatus. The L-type Ca^{2+} channel also exists in skeletal muscle but the duration of action potentials (APs) in skeletal fibres is too brief (2-5 ms), compared to that in cardiomyocytes (100-200 ms), to activate the channel to any degree. Instead, the α-subunit of the L-type Ca^{2+} channel in skeletal muscle acts as a voltage sensor, which directly activates Ca^{2+} release from the SR. This is not to say that skeletal muscle L-type Ca^{2+} channels cannot pass Ca^{2+}, they simply require a relatively long period of depolarization that does not occur under normal physiological conditions. Yet, there is evidence for Ca^{2+} entry associated with periods of low frequency excitation of skeletal muscle (Gissel & Clausen, 1999), but the pathway of Ca^{2+} entry during normal excitation in skeletal muscle fibres has not been identified due to inherent limitations in the techniques used to record very small Ca^{2+} fluxes during normal excitation. Our aim was to use a recently developed fluorescence technique (Launikonis & Rìos, 2007) to identify whether there is a t-system Ca^{2+} current associated with normal excitation in skeletal muscle.

The Animal Ethics Committee at Rush Medical Centre approved the use of animals in this project. Male rats (3 months old) were killed by asphyxiation and the extensor digitorum longus (EDL) muscles were removed. Intact fibres were exposed to a Na^+-based physiological solution containing mag-indo-1 salt. Fibres were mechanically skinned, trapping the dye in the t-system, and transferred to a chamber containing a K^+-repriming solution with rhod-2. Net changes in the finite t-system [Ca^{2+}] ($[Ca^{2+}]_{t-sys}$) of the skinned fibre, $d[Ca^{2+}]_{t-sys}/dt$, could be equated to t-system Ca^{2+} current (Launikonis & Rìos, 2007). The chamber was equipped with platinum electrodes that ran parallel to the mounted fibre. In other experiments, skinned fibres without dye in the t-system were bathed in a K^+-repriming solution with indo-5F and rhod-2. The indo analogues with rhod-2 were simultaneously imaged during field stimulation on a Leica SP-2 confocal microscope in linescan mode, with the scanning line positioned parallel to the long axis of the fibre. The group scanning speed of the three lasers used to excite mag-indo-1 (or indo-5F) and rhod-2 was 1.9 ms/line.

Imaging the cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyto}$) transient during field stimulation of skinned fibre preparations in the presence of rhod-2 and indo-5F in the bathing solution produced a uniform and rapid (~ 5 ms) increase in $[Ca^{2+}]_{cyto}$ as indicated by both dyes. This imaging technique allowed calibration of the Ca^{2+} transient in skinned fibres for the first time (with indo-5F) and the parallel imaging of rhod-2 provided a reference for EC coupling viability the next group of experiments. Under our imaging conditions, $\gamma K_{D,Ca}$ of indo-5F was 2.38 μM and indicated a peak $[Ca^{2+}]_{cyto}$ of 1.1 μM following excitation. Following correction of the raw $[Ca^{2+}]_{cyto}$ calibration for the slow off rate of indo-5F (75 s^{-1}), a peak $[Ca^{2+}]_{cyto}$ of 4 μM was estimated to be reached in about 2 ms. Thus skinned fibres release Ca^{2+} at a normal rate and magnitude in response to physiological excitation. Simultaneous imaging of cytoplasmic rhod-2 and t-system trapped mag-indo-1 showed that there was indeed an influx of Ca^{2+} into the cell following an AP when $[Ca^{2+}]_{t-sys}$ was 0.2 mM or greater. The current decayed exponentially and lasted approximately 70 ms. Subsequent APs produced no further t-system Ca^{2+} current in the following 200 ms, even though Ca^{2+} was released from sarcoplasmic reticulum, thus defining an inactivation period for this current. When $[Ca^{2+}]_{t-sys}$ was about 0.1 mM, a transient rise in $[Ca^{2+}]_{t-sys}$ was observed almost concurrently with the increase in $[Ca^{2+}]_{cyto}$ following the action potential. The change in direction of Ca^{2+} flux was consistent with changes in driving force for Ca^{2+}. This is the first direct demonstration of a marked Ca^{2+} flux that inactivates, associated with an AP in skeletal muscle.

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A slowing of relaxation in EDL muscle from the genetically obese mouse is associated with alterations in SR Ca^{2+} handling

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There is growing evidence to suggest that the isometric contractile properties of fast-twitch skeletal muscles from animal models of obesity are different from those of lean controls (Warmington *et al*., 2000; Bruton *et al*., 2002; Blazev *et al*., 2005). In the present study, we specifically investigated the relaxation kinetics of extensor digitorum longus (EDL) muscle isolated from the genetically obese (*ob/ob*) mouse and its lean counterpart. We also used a single fibre approach to more precisely address any observed differences in relaxation at the whole muscle level.

Male *ob/ob* and lean mice (18-22 weeks) were killed by halothane overdose in accordance with Victoria University AEEC procedures. EDL muscles were dissected and placed in carbogen bubbled Krebs solution at 25 °C, before being supramaximally stimulated to elicit tetanic (110 Hz) responses, as described previously (Blazev *et al*., 2005). EDL muscles not incubated in Krebs solution were used to obtain mechanically skinned fibre segments that were electrophoretically typed. Since the EDL muscles of both *ob/ob* and lean mice contain predominantly type IIB fibres (Blazev *et al*., 2005) their properties related to Ca^{2+}-sensitivity of the contractile apparatus and sarcoplasmic reticulum (SR) Ca^{2+} handling were investigated following the procedures routinely used in our laboratory (Bortolotto *et al*., 2000, 2001). All results are given as mean ± SEM.

The half relaxation time (1/2 RT) for tetanic responses was significantly slower ($p < 0.05$) in EDL muscles from *ob/ob* mouse (n = 8) as compared to lean controls (n = 8) (46.0 ± 2.3 *vs* 39.9 ± 1.3 ms). There was no difference in the Ca^{2+}-sensitivity of the contractile apparatus between IIB fibres isolated from EDL of *ob/ob* and lean mice, as determined from the pCa ($-\log_{10}[Ca^{2+}]$) giving 50% maximum force (*i.e.*, pCa_{50}: 5.77 ± 0.01, n = 14 *vs.* 5.74 ± 0.02, n = 12). A single fibre investigation of the ability of the SR to maximally sequester Ca^{2+} was carried out by normalising the area under the 30 mM caffeine-induced force response (in the presence of 0.5 mM EGTA and 0.05 mM free Mg^{2+}), following maximal SR Ca^{2+} loading at pCa 7.3, to the maximum Ca^{2+}-activated force response (F_{max}). This normalised value for EDL IIB fibres from *ob/ob* mice (175 ± 16 $\%F_{max}$.s, n = 8) was significantly smaller than that for fibres from lean mice (268 ± 37 $\%F_{max}$.s, n = 8), indicating a lower SR Ca^{2+} loading capacity at pCa 7.3 for *ob/ob* fibres. The lower SR Ca^{2+} content of these fibres was not due to differences in the rate of passive Ca^{2+} leak (% min^{-1}) from the SR between IIB *ob/ob* and lean fibres (59.6 ± 11.0, n = 5 *vs.* 59.3 ± 9.2, n = 6). Furthermore, investigation of slow/fast SR characteristics (Fryer and Stephenson, 1996; Bortolotto *et al*., 2001) by loading maximally at two different $[Ca^{2+}]$, revealed that the ratio ($R_{6.2/7.3}$) derived from the area under the force response to 30 mM caffeine following loading at pCa 6.2 versus pCa 7.3 was similar in IIB fibres from *ob/ob* and lean mice ($R_{6.2/7.3}$: 1.50 ± 0.13, n = 7 *vs.* 1.49 ± 0.11, n = 8). Thus, functional characteristics of the SR Ca^{2+} pumps were the same in these fibres.

Taken together, the above results suggest that the density of the SR Ca^{2+} pumps (expressed per fibre volume) was significantly lower in EDL IIB fibres from *ob/ob* mice than in fibres from lean mice. This reduces the ability of the SR to sequester Ca^{2+} and return the myoplasmic $[Ca^{2+}]$ back to ~ theoretical resting levels following muscular contraction in *ob/ob* mice as compared to the lean counterparts, and contributes to the slowing of the 1/2 RT observed in the present study for the tetanic response at the whole muscle level in *ob/ob* mice.

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This work is supported by the NHMRC and ARC (Australia).

The effects of acute and chronic central leptin infusion on metabolism in peripheral tissues

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Introduction. The adipocyte hormone leptin is known to regulate food intake and whole body energy expenditure. There is some evidence that adenosine monophosphate activated protein kinase (AMPK), a cellular energy sensor, may be involved. In rodents acute (6 h) peripheral administration of leptin activates skeletal muscle AMPK at its receptor (ObRb) and acute central administration activates skeletal muscle AMPK through sympathetic nervous system activation. Given that leptin is a long term hormonal regulator acute studies may not be physiologically relevant and investigation in species which are more closely related to humans are required. Sheep are not nocturnal and more closely resemble humans in terms of the hormonal regulation of metabolism and were therefore chosen for this study.

Aim. To examine the effects of acute (6 h) and chronic (7 days) central leptin administration on AMPK activation in skeletal muscle and adipose tissue of sheep.

Methods. Under general anaesthesia (induced by pentobarbitone and maintained with isofluorane and oxygen), Corriedale ewes underwent: 1) surgery for removal of the ovaries; and 2) A month later, surgery for the placement of an infusion cannula into the lateral ventricle (LV) of the brain. A month later ewes (n = 4 per group) received either continuous infusion of leptin (50 μg/h; 60 μl/h) or artificial cerebrospinal fluid (aCSF) (50 μg/h; 60 μl/h) into the lateral ventricle for 7 days. A second control group received aCSF and was pair-fed to match the leptin treated group. Muscle and fat biopsies were obtained under local anaesthesia (1% lignocaine without adrenaline) before, and at 6 hours (acute) and 7 days (chronic) into the infusion period. Tritiated glucose was infused on day 1 and 7 for calculation of whole body rate of glucose appearance (Ra) and disappearance (Rd). On day 8 all animals were killed using an intravenous overdose of pentobarbitone (Lethabarb 25ml/animal) and tissues were collected. Plasma was analysed for insulin, free fatty acids and catecholamines. Tissues were analysed by western blot for protein abundance and phosphorylation of AMPK and its downstream target acetyl CoA carboxylase β (ACCβ).

Results. Chronic leptin infusion reduced ($p < 0.05$) food intake and was associated with a decrease ($p < 0.05$) in body weight. Plasma adrenaline concentration was increased ($p < 0.05$) following 6 hours and 7 days of leptin treatment suggesting increased sympathetic nerve activity. Chronic leptin infusion reduced ($p < 0.05$) glucose Ra and Rd compared with the control group. Despite central leptin infusion having these peripheral metabolic effects, no activation of skeletal muscle or adipose AMPK was observed at either 6 h or 7 days of leptin infusion.

Conclusion. Both acute and chronic administration of central leptin in sheep alters physiological function as indicated by reduced food intake, increased plasma adrenaline and reduced whole body glucose turnover. Despite this, no effect on skeletal muscle AMPK activity was observed suggesting that differences may exist between the rodent and ovine models in this regard. Given that sheep more closely resemble humans in terms of the hormonal regulation of metabolism, the present findings suggest that results of previous rodent studies may not be relevant to humans.

Effects of the inflammatory agent carrageenan on contractile function in mammalian skeletal muscle

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Inflammation and muscle weakness are two very prominent phenomena that commonly accompany muscle damage and muscle diseases such as Duchenne muscular dystrophy. Whether the inflammatory component of these conditions plays an active role in skeletal muscle weakness is yet to be identified. This question is difficult to address as the effects of inflammation may be confounded by the underlying damage or disease state of the muscle. Lambda carrageenan, an algal-derived polysaccharide, has previously been shown to increase intramuscular levels the pro-inflammatory cytokines IL-1β and IL-6 (Loram *et al.*, 2007). When injected intramuscularly, carrageenan forms a bolus which is localised to the site of injection, thereby providing a model of local muscle inflammation that enables the investigation of the role of inflammatory cytokines in muscle weakness without any confounding influences of preceding muscle damage. This study aimed to determine if the cytokine-mediated inflammatory response directly affected skeletal muscle force production.

All experimental procedures and methods undertaken in this study were approved by the University of Western Australia Animal Ethics Committee. Experiments were conducted on 12 six week old female ARC mice. Each animal received a single 50 µl injection of a lambda-carrageenan solution (2 mg/100 µl saline, lambda carrageenan; Fluka) subcutaneously into the belly of the right tibialis anterior (TA) muscle while anaesthetised (intraperitoneal injection of sodium pentobarbitone, 40 mg/kg body weight). A control injection of 50 µl saline was administered to the contralateral TA muscle. Animals were left for 24 hours to allow carrageenan time to promote an inflammatory response (Loram *et al.*, 2007).

All tissue samples were removed from mice under deep anaesthesia. Mice were anaesthetized *via* an intraperitoneal injection of sodium pentobarbitone (40 mg/kg body weight) such that they were unresponsive to tactile stimuli. Each TA muscle was surgically removed and mounted in an in vitro muscle test system (1200A, Aurora Scientific Inc. Ontario, Canada), bubbled with 95% O_2 and 5% CO_2, whilst bathed in mammalian Ringer solution at 25°C (in mM: 137 NaCl, 24 $NaHCO_3$, 11 glucose, 5 KCl, 2 $CaCl_2$, 1 NaH_2PO_4, 1 $MgSO_4$ and 0.025 d-tubocurarine chloride). The preparation was manually adjusted for optimum muscle length stimulated with supramaximal square-wave pulses by platinum wire electrodes. After removal of the muscles animals were euthanized with an overdose of anaesthetic.

Carrageenan significantly reduced maximum twitch force (~30% reduction in peak twitch tension; $p < 0.05$) and maximal tetanic force (˜20% reduction in peak tetanic tension; $p < 0.05$) when compared to control, saline injected muscles. Furthermore, the force frequency curve was shifted to the right with, carrageenan treated muscles producing significantly less tetanic force compared to saline-treated muscles at 40, 60 and 80 Hz ($p < 0.05$). These results indicate that, in the absence of direct muscle damage, the presence of the inflammatory mediator carrageenan, directly reduces skeletal muscle force production. The effect on the force frequency relationship suggests that the presence of carrageenan may lead to a decrease in net sarcoplasmic reticulum Ca^{2+} release.

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LDH isozyme profile of striated muscles and electrophoretically-typed single fibres from cane toad (*Bufo marinus*)

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In mammalian tissues (other than gonads), lactate dehydrogenase (LDH) is a tetrameric enzyme (Tsuji *et al*., 1994) produced by five random combinations of two different subunits, A and B [LDH1 (B_4)-lowest activity; LDH2 (A_1B_3); LDH3 (A_2B_2), LDH4 (A_3B_1); LDH5 (A_4)-highest activity], which are encoded by different genes (LDH-A and LDH-B). The expression of these genes and therefore the distribution of LDH isozymes (LDH_{isoz}) are tissue-specific. Using histochemically typed single fibre segments from rabbit skeletal muscles, Leberer & Pette (1984) found a predominance of B-type LDH_{isoz} in type I fibres and of A-type LDH_{isoz} in type II fibres, but found no difference in the LDH_{isoz} profile between type IIA and IIB fibres. The authors concluded that the expression of the LDH subunits is not related to specific MHC isoforms (MHC_i). Recently we reported that rectus abdominis muscle of the cane toad contains a wide range of MHC-based fibre types that include pure twitch, hybrid twitch (*i.e.*, comprising one or several twitch MHC_i, respectively) and hybrid tonic-twitch fibres (Nguyen & Stephenson, 2002) of sizes that allow both LDH_{isoz} and MHC_i analyses. Currently there is no information on the number of LDH_{isoz} in cane toad (*Bufo marinus*) skeletal muscles or on their distribution among different muscles and fibre types. In this study we aimed to address this cognitive gap using whole muscle homogenates and single muscle fibres from adult cane toads.

Male and female toads (79-149g) were killed by double pithing in accordance with procedures approved by Victoria University. Crude extracts from several skeletal [*rectus abdominis* (n = 9), *hyoglossus* (n = 4), *iliofibularis* (n = 4), *semimembranosus* (n = 6), and *sartorius* (n = 5)] and cardiac (n = 7) muscles of the toad and from mouse *soleus* muscle (n = 4; used as a mammalian LDH_{isoz} marker) were prepared in 0.9% NaCl and then further diluted in BPB-S solution [0.5 mg/ml bromphenol blue; 40% (w/v) sucrose; 0.1M Tris-HCl; pH 7.0] to a final concentration (w/v) of 0.45% (toad skeletal muscle), 1.2% (toad cardiac muscle), and 10.0% (mouse *soleus* muscle). Single fibre segments (1.8-85.3 nl), isolated from *rectus abdominis* muscles (n = 6), were placed in 10.5 µl BPB-S solution and frozen at -84°C for later analysis of LDH_{isoz} and MHC_i. LDH_{isoz} (in tissue extracts and single fibres) and MHC_i (in single fibres) were resolved by non-denaturing polyacrylamide gel electrophoresis (LDH_{isoz}) and SDS-polyacrylamide gel electrophoresis (MHC_i) using the protocol of Leberer & Pette (1984), with modifications, and the method described by O'Connell *et al*. (2006), respectively. LDH_{isoz} were visualized by an activity-based gel staining procedure using a system of coupled reactions which link lactate oxidation to reduction of nitroblue tetrazolium to formazan. MHC_i bands were visualized by silver staining and quantified densitometrically (Molecular Dynamics). Each fibre segment was examined first for LDH_{isoz} and then for MHC_i composition.

Five LDH_{isoz} were detected in both toad cardiac muscle and mouse *soleus* muscle, but their electrophoretic mobilities were different. These isozymes are presumably formed by the tetrameric combination of species-specific A and B subunits. With the exception of the toad *semimembranosus* muscle samples, which displayed the toad equivalent of mammalian LDH4 and LDH5 ($LDH5_{toad}$ and $LDH4_{toad}$) isozymes, all the toad skeletal muscle samples examined in this study displayed only one LDH_{isoz}, *viz.* $LDH5_{toad}$. The population of 100 single *rectus abdominis* fibres comprised 31 tonic-twitch hybrids, of which 14 fibres contained predominantly (> 80% MHC_{total}) the tonic MHC_i, and 69 pure and hybrid twitch fibres. Of the 14 predominantly tonic fibres, 13 fibres (93%) displayed only $LDH5_{toad}$, regardless of the identity or the number of the twitch MHC_i co-expressed with the tonic isoform, and one fibre contained both $LDH5_{toad}$ and $LDH4_{toad}$. Similarly, 99% (68/69) of the pure and hybrid twitch fibres and 17 tonic-twitch hybrid fibres displayed only $LDH5_{toad}$, with one hybrid twitch fibre containing both $LDH5_{toad}$ and $LDH4_{toad}$. The LDH_{isoz} composition of the two fibres containing both $LDH5_{toad}$ and $LDH4_{toad}$ was not unique to their MHC classification as other fibres of the same type showed only the $LDH5_{toad}$ isozyme. These results indicate that in cane toad skeletal muscles, like in rabbit skeletal muscles, there is no simple and tight relationship between the molecular forms of LDH (key enzyme in anaerobic metabolism) and those of MHC (molecular motor in muscle contraction).

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This study was supported by the Australian Research Council.

MuRF1 and Nedd4 are differentially expressed in denervated rat fast- and slow-twitch muscles

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Denervation atrophy of skeletal muscle is predominantly mediated by ATP-dependent ubiquitination (Furuno *et al*. 1990), where target proteins are marked for degradation by the 26S proteasome. The last step of this process involves the transfer of ubiquitin from an E3 ubiquitin ligase to a specific group of target proteins. Three major families of E3 ligases have been identified in skeletal muscle, including the RING finger proteins, the F-box proteins (which form part of the Skp1–Cul1–F-box (SCF) protein complex) and the homologous to E6AP carboxyl terminus domain (HECT) proteins (Powell, 2006). However, very little is known about the regulation and temporal pattern of expression of these three families of E3 ligases in skeletal muscle. The aim of this study was to characterise the expression pattern of two of these E3 ligases during denervation-induced atrophy; a RING finger protein (Muscle RING finger, MuRF1) and a HECT protein (Nedd4). MuRF1 is known to target myofibrillar and related structural proteins, including titin, nebulin, myosin light chains and myosin heavy chains (Fielitz *et al.*, 2007), while Nedd4 has been found to target signalling proteins such as Notch1 (Koncarevic *et al.*, 2007). We tested the hypothesis that the expression of these two proteins would differ temporally and spatially in denervated *extensor digitorum longus* (EDL, fast-twitch) or *soleus* (slow-twitch) muscles.

Adult male Sprague-Dawley rats (300-350g, n = 5 per timepoint) were anaesthetised (100 mg/kg ketamine and 10 mg/kg xylazine) and the sciatic nerve severed, taking care not to damage adjacent muscles and ensuring the resected nerve ends were not in contact. The 'nerve intact' muscles of the contralateral limb served as controls. After 1, 3, 7 or 14 days denervation, rats were anaesthetised deeply with pentobarbital sodium (60 mg/kg) and the EDL and soleus muscles surgically excised, trimmed of their tendons and any adhering connective tissue, weighed on an analytical balance and frozen for western immunoblotting of MuRF1 and Nedd4.

EDL muscle mass was increased by 11% compared with contralateral control muscles after 1 day of denervation, indicative of oedema. However, after 7 and 14 days of denervation EDL muscle mass was decreased by 24% and 46%, respectively ($p < 0.05$). In contrast, soleus muscle mass was unchanged after 1 and 3 days of denervation, and reduced by 46% and 54% after 7 and 14 days of denervation, respectively, compared with contralateral control muscles ($p < 0.05$). EDL muscles exhibited a significant increase in MuRF1 protein after 3, 7 and 14 days of denervation, with a maximal 3-fold increase observed at 7 days ($p < 0.05$). Nedd4 protein levels were increased after 7 and 14 days, with a maximal 4-fold increase after 7 days. In denervated *soleus* muscles, MuRF1 protein levels were increased significantly after 1, 3, 7 and 14 days, with a maximal 3-fold increase after 7 days. Nedd4 protein levels were increased after 3, 7 and 14 days with a maximal 2-fold increase after 14 days ($p < 0.05$).

These results indicate that the E3 ligases MuRF1 and Nedd4 and differentially regulated in denervated fast- and slow-twitch skeletal muscles undergoing muscle atrophy. In addition, the temporal expression of these E3 ligases during the early stages of denervation has been identified. This study demonstrates a previously unidentified role for Nedd4 in denervated EDL muscles, and provides important information about the timing of structural (MuRF1) and signalling (Nedd4) protein ubiquitination during denervation atrophy.

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This work was supported by grants from the Australian Research Council and the National Health & Medical Research Council.

Local insulin-like growth factor binding proteins are required for successful skeletal muscle regeneration after injury

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Although exogenous administration of insulin-like growth factor I (IGF-I) to mice can hasten muscle fibre regeneration after injury (Schertzer & Lynch, 2006), the levels and actions of IGF-I are modulated tightly by IGF binding proteins (IGFBPs) at both the systemic and local tissue level (Duan & Xu, 2005). Administration of an IGF aptamer to mice inhibits the systemic actions of IGFBPs, elevating 'free' endogenous IGF-I, a strategy which was also found to hasten muscle fibre regeneration (Schertzer *et al*., 2007). We tested the hypothesis that local IGFBPs are required for successful skeletal muscle repair after injury, and that specific inhibition of IGFBP-2 would compromise skeletal muscle fibre regeneration.

Twelve-week old C57BL/6 mice were anaesthetised (100 mg/kg ketamine/ 10 mg/kg xylazine), and the tibialis anterior (TA) muscle of the right hindlimb was injected with the myotoxin, notexin, to cause complete degeneration of all fibres and thus initiate spontaneous muscle fibre regeneration. Muscles were harvested at 3, 5, 7, 10, 14, 21 and 28 days post-injury to examine transcript expression of the six IGFBPs. In separate groups of mice, the TA muscles were injected with an IGF aptamer (100μg in DMSO, NBI-31772; Calbiochem) or IGFBP-2 antibody (200 μg/ml; R & D Systems) 3 days post-notexin injection. Muscle structure and function were evaluated at 10, 14 and 21 days post-injury. To examine muscle function, mice were anaesthetised (60 mg/kg, sodium pentobarbital), and the right TA muscle was surgically exposed and the distal tendon attached to the lever arm of a force transducer, with the knee and foot immobilised. The TA muscle and surrounding limb was immersed in mineral oil at 37°C and maximal isometric force (P_o) determined at optimal muscle length *in situ*.

The various IGFBP transcripts were differentially expressed during muscle regeneration, indicating that IGFBPs have different roles during the various phases of muscle regeneration. Inhibiting IGFBPs with the IGF aptamer inhibited functional recovery (P_o), and reduced viable muscle tissue at 10 and 14 days post-injury ($p < 0.05$). Inhibiting IGFBP-2 during the early stages of regeneration, with an IGFBP-2 antibody, affected the regenerative process. Structural and functional recovery during regeneration were compromised ($p < 0.05$, main effect) after IGFBP-2 antibody injection. These data indicate an inadequate functional redundancy where other IGFBPs were unable to compensate for the lack of IGFBP-2.

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Supported by the Muscular Dystrophy Association (USA)