AuPS/ASB Meeting - Canberra 2005

Free Communications 7: Muscle physiology

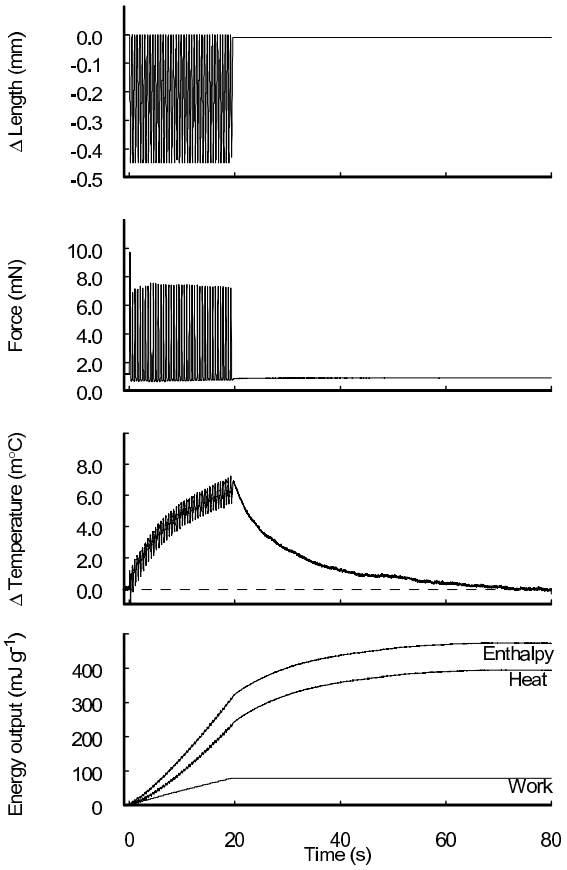
Thursday 29 September 2005

Chair: Derek Laver

Active metabolism of mouse papillary muscle

C. Widén and C.J. Barclay, Muscle Energetics Laboratory, Heart Foundation Research Centre, School of Physiotherapy & Exercise Science, Griffith University, PMB50 Gold Coast Mail Centre, Gold Coast, QLD 9726, Australia.

With the development of genetically modified mice, there is need for a cardiac muscle model for determining the physiological and functional consequences of the various genetic manipulations. There have been no measurements of energy use or work capacity of the isolated mouse papillary muscles and the aim of this study was to characterise the mechanical and energetic properties of these preparations.



Papillary muscles were dissected from the left ventricle of hearts from 6- to 12-week old male Swiss mice. The mice were rendered unconscious by inhalation of 80% CO_2-20% O_2 gas mixture and killed by cervical dislocation. All animal-handling procedures were approved by the Griffith University Animal Ethics Committee. Active metabolism of left ventricular papillary muscles was measured *in vitro* (27°C) using the myothermic technique (see Figure). Muscles were bathed in aerated (95% O_2-5% CO_2) Krebs solution with glucose provided as metabolic substrate.

The energy output of the mouse papillary muscles performing isometric contractions was measured at contraction frequencies 1 – 4 Hz. The mean absolute heat output was 6.8 ± 1.1 mJ g^{-1} $twitch^{-1}$ (mean ± SEM; n = 11) at 1 Hz and decreased with increasing contraction frequency. Tension-independent heat, an index of metabolism primarily associated with calcium cycling, was also measured. The tension-independent heat accounted for 18.9 ± 2.6 % (n = 6) of the total metabolism. In a more realistic contraction protocol (Mellors & Barclay, 2001), designed to closely simulate the reported changes in muscle shortening (Semafuko & Bowie, 1975) work output and enthalpy output were measured and resulted in a maximum net mechanical efficiency of 17 % (n = 10).

The model is now well established and will be used to study energetic aspects of cardiac pathologies and heart-focussed genetic changes.

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Functional and electrophoretic identification of two Troponin C isoforms in toad skeletal muscle fibres

B. O'Connell, R. Blazev and G.M.M. Stephenson, School of Biomedical Sciences, Victoria University, Melbourne, VIC 3011, Australia.

Activation of contraction in striated muscle of vertebrates is regulated by the binding of Ca^{2+} to the myofibrillar protein Troponin C (TnC). In mammals, TnC is known to exist as two isoforms, one found in fast-twitch skeletal muscle (TnC-f), the other found in both slow-twitch skeletal and in cardiac muscle (TnC-s/c) (Gomes *et al*., 2002). These isoforms confer to fibres in which they are expressed different contractile activation characteristics with respect to Ca^{2+} and Sr^{2+} (for example, see O'Connell *et al*., 2004b).

So far only one TnC isoform from anuran muscle, similar in structure and Ca^{2+} -binding properties to the rabbit TnC-f, has been purified and sequenced. However, single fibre studies have shown inter-fibre differences with respect to contractile activation characteristics, which suggests that anuran striated muscle expresses more than one TnC isoform. Thus, the main aims of the present study were (i) to definitively establish whether anuran striated muscle expresses more than one TnC isoform, and if so (ii) to examine the relationship between the myosin heavy chain (MHC) and TnC isoform expression in anuran muscle fibres and (iii) to characterise the anuran TnC isoforms according to the Sr^{2+}- and Ca^{2+}-activation properties conferred to the single fibres in which they are found.

Adult (body weight 250-380 g) cane toads (*Bufo marinus*) were killed by double pithing in accordance with procedures approved by Victoria University AEEC. The TnC isoform composition of cardiac muscle and of 198 single fibres from the rectus abdominis muscle was investigated using a recently developed method for the unequivocal identification of TnC isoforms on SDS-polyacrylamide gels (O'Connell *et al*., 2004a). The same single fibres were also analysed for their MHC isoform content using the alanine-SDS-polyacrylamide gel electrophoresis protocol of Goodman *et al*. (2003). For a subpopulation of 15 fibres, the Sr^{2+} - and Ca^{2+} -activation characteristics were measured and related to the TnC isoform present.

Our results show that like mammalian striated muscle, the anuran striated muscle expresses two TnC isoforms which can be distinguished electrophoretically. The slowest migrating TnC isoform (TnC-t) was detected in all fibres displaying only twitch MHC isoforms, regardless of their number or identity; the other (TnC-T/c) was detected in fibres displaying the slow-tonic MHC isoform and in cardiac muscle. Fibres containing the TnC-T/c isoform were found to be ~47 times more sensitive to Sr^{2+} and ~3 times more sensitive to Ca^{2+} than fibres containing the TnC-t isoform. From these data we conclude that both anuran and mammalian striated muscle contain two TnC isoforms that play an important role in determining the contractile activation characteristics of the fibres in which they are expressed.

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This work is supported by the Australian Research Council.

X-ray diffraction analysis of the effects of myosin chain-2 phosphorylation on the structure of fast skeletal muscle fibres

Joseph F.Y. Hoh[1], Maki Yamaguchi[2], Masako Kimura[2], Shigeru Takemori[2] and Naoto Yagi[3], [1]Department of Physiology and Institute for Biomedical Research, The University of Sydney, NSW 2006, Australia, [2]Department of Physiology, The Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan and [3]Japan Synchrotron Radiation Research Institute (JASRI), Sayo-gun 679-5198, Japan.

The isometric twitch tension of a fast skeletal muscle is enhanced by a factor of about 2 following a brief tetanic stimulation (Close & Hoh, 1968). This phenomenon, known as post-tetanic potentiation (PTP), is currently thought to be due to the phosphorylation of the fast myosin light chain-2 (MLC2) by the enzyme myosin light chain kinase (MLCK), which is activated by Ca/calmodulin during the tetanus. Phosphorylation of MLC2 in permeabilized fibres enhances their Ca sensitivity, producing more force during submaximal Ca activation. Phosphorylation of MLC2 in isolated thick filaments causes the loss of the regular helical arrangement of myosin heads characteristic of normal relaxed filaments (Levine *et al.*, 1996). It was postulated that MLC2 phosphorylation increases the mobility of myosin heads, which spend more time in proximity to thin filaments, leading to force enhancement. In this work, we test this hypothesis by using X-ray diffraction to detect structural changes in muscle fibres following MLC2 phosphorylation.

The experiments were done on glycerinated rabbit psoas fibres. Muscle bundles were isolated from animals killed by stunning and exsanguination. Bundles containing 10 glycerinated fibres were prepared for X-ray diffraction after exposure to: 1) relaxing solution containing 10 mM 2,3-butanedione monoxime to dephosphorylate endogenously phosphorylated MLC2, 2) subthreshold Ca solution (pCa 6.8), 3) phosphorylating solution containing 2mM calmodulin, 0.15mM MLCK (pCa 6.8) and 10mM tautomycin to inhibit endogenous phosphatase, 4) calmodulin/MLCK solution without Ca. X-ray diffraction analyses were carried out on beam line BL45XU at the SPring-8 synchrotron facility.

Equatorial reflections 1,1 and 1,0 are due to longitudinally oriented planes in the muscle filament lattice that pass through thick and thin filaments (1,1) and thick filaments only (1,0). The 1,1/1,0 intensity ratio gives information about distribution of mass around the filaments. In the presence of relaxing solution, 1,1/1,0 ratio was low, indicating that myosin heads were mostly located near thick filaments. When fibres were exposed to pCa 6.8, the ratio was nearly doubled, indicating a movement of the myosin heads towards thin filaments even with no force development. After exposing fibres to phosphorylating solution for 20 minutes, the ratio significantly increased further. At 2 minutes after the enzyme was washed out in low Ca solution, the ratio decreased to control level. Prolonging the wash out time did not change the ratio significantly. Incubating fibres in enzyme without Ca produced no change in ratio. MLC2 phosphorylation and dephosphorylation under our experimental conditions were verified using two-dimensional polyacrylamide gel electrophoresis. Lattice spacings decreased slightly on exposure to low Ca, but no significant change was observed following phosphorylation. However, reducing the lattice spacing by increasing sarcomere length dramatically reduced the change in 1,1/1,0 ratio with phosphorylation.

The present results provide structural evidence for a movement of cross-bridges towards the thin filaments following MLC2 phosphorylation, thereby strongly supporting this as the molecular mechanism for PTP. Sarcomere length dependence of the effects of phosphorylation correlates well with earlier work showing that the phosphorylation-induced increase in Ca sensitivity was similarly reduced by increased sarcomere length, as well as by osmotic compression (Levine *et al.*, 1996). These procedures enhance Ca sensitivity in their own right by bringing cross-bridges closer to thin filaments. Thus, at long sarcomere lengths, the cross-bridges are already close to thin filaments, and phosphorylation has little further effect. We predict that in intact fibres, post-tetanic potentiation should decrease with sarcomere length. The increased 1,1/1,0 ratio at pCa 6.8 suggests that elevation of baseline Ca following a tetanus may contribute to twitch potentiation early in PTP.

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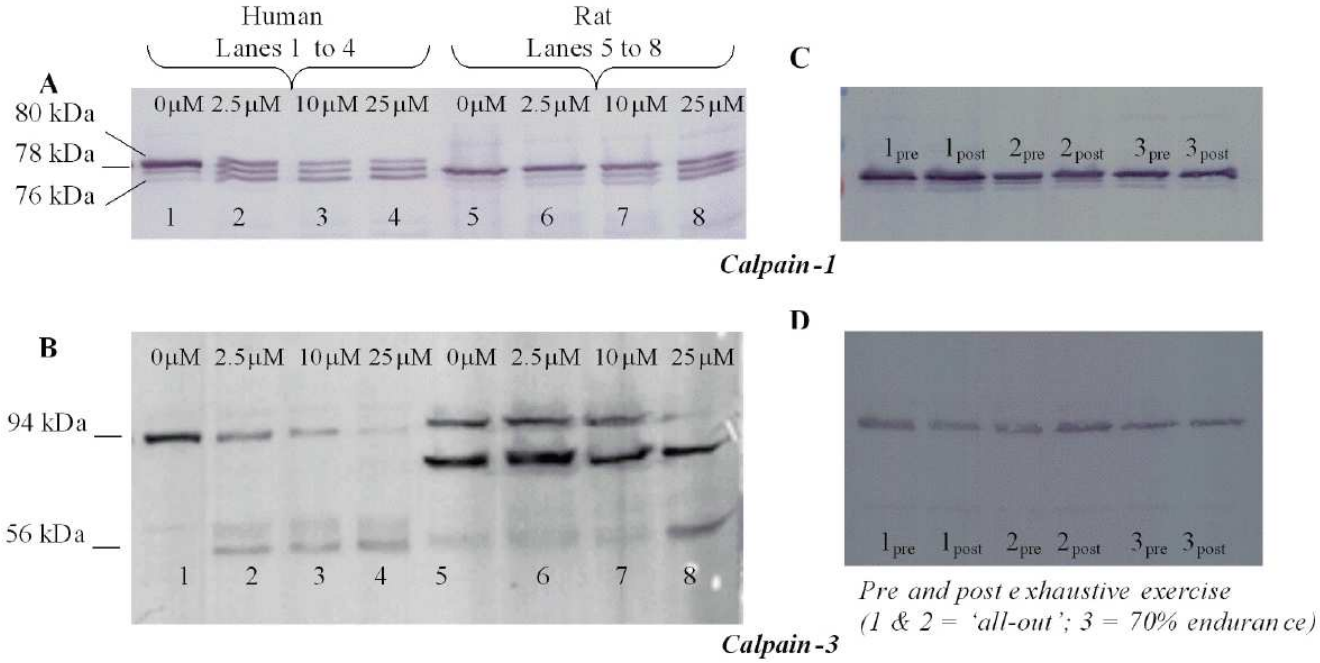
Calpain-1 and calpain-3 are not autolysed with exhaustive exercise in humans

R.M. Murphy[1], R.J. Snow[2] and G.D. Lamb[1], [1]Department of Zoology, La Trobe University, VIC 3086, Australia and [2]School of Exercise and Nutrition Sciences, Deakin University, Burwood, VIC 3125, Australia.

Calpain-1 and calpain-3 are Ca^{2+}-dependent proteases found in skeletal muscle. Autolysis of the calpains is observed by Western blotting as the cleaving of the full-length proteins to shorter products (see the Figure, A and B), which results in their activation. Biochemical assays suggest that calpain-1 becomes proteolytically active in the presence of 3-200 µM Ca^{2+}. Although calpain-3 is poorly understood, its activation is proposed to be much more Ca^{2+}-sensitive (~1 µM) than calpain-1. Adult Long Evans hooded rats were killed by an overdose of halothane, as approved by the Animal Ethics Committee at La Trobe University and the extensor digitorum longus (EDL) muscles were removed. Human muscle samples were obtained from the vastus lateralis using the needle biopsy technique. These samples were left over from a completed study which was approved by the Deakin University Human Ethics Committee. As shown in the Figure (A and B), we characterised the Ca^{2+}-dependence of autolysis of the calpains in human muscle samples and rat EDL muscle samples homogenised in solutions mimicking the intracellular environment at various [Ca^{2+}] (0, 2.5, 10 and 25 µM).



Autolysis of calpain-3 was found to occur over a similar [Ca^{2+}] range as that for calpain-1, and both calpains displayed a seemingly higher Ca^{2+}-sensitivity in human compared to rat muscle homogenates, with ~15 % autolysis observed following 1 min exposure to 2.5 µM Ca^{2+} in human muscle and almost none following 1-2 min exposure to the same [Ca^{2+}] in rat muscle. Since intracellular [Ca^{2+}] may transiently peak in the range found to activate calpain-1 and calpain-3, we examined the effect of two types of exhaustive cycling exercise (30 s "all-out", n=8 and 70 % VO2 peak until fatigue, n=3) on the amount of autolyzed calpain-1 or calpain-3 in human muscle. Following the sprint exercise, the percent decline in peak power was 45 ± 11 % (mean ± sd). In the endurance exercise trials, subjects cycled for 107 ± 27 min. Despite the exhaustive nature of the exercise, autolysis of calpain-1 or calpain-3 did not occur due to the exercise (Figure, C and D). These findings show that the time- and concentration-dependent changes in cytoplasmic [Ca^{2+}] occurring during concentric exercise fall near, but below that necessary to activate calpains *in vivo*.

Increased fatigue resistance in EDL muscle of the obese mouse is associated with an increase in the proportion of hybrid IIB+IID fibres

R. Blazev[1], J.G. Kemp[1,2], D.G. Stephenson[3] and G.M.M. Stephenson[1], [1]School of Biomedical Sciences, Victoria University, VIC 3011, Australia, [2]School of Exercise Science, Australian Catholic University, VIC 3065, Australia and [3]Department of Zoology, La Trobe University, VIC 3083, Australia.

Fatigue resistance is an important indicator of the functional status of a muscle. Current data on the fatigue characteristics of the extensor digitorum longus (EDL) muscle from the genetically obese (*ob/ob*) mouse, a commonly used animal model of type 2 diabetes, are limited and inconsistent. Of the two studies carried out to date on this muscle, one shows an increased fatigue resistance in the obese animal (Warmington *et al.*, 2000) while the other shows no difference between the obese animal and its lean control (Bruton *et al.*, 2002). Therefore, in the present study we re-examined the fatigue characteristics of EDL muscles from *ob/ob* and lean mice. We also determined, using a single fibre approach, the fibre type composition of the two muscles as this parameter is closely related to muscle fatigability.

Male *ob/ob* and lean mice (18-22 weeks, C57BL strain) were killed by halothane overdose in accordance with Victoria University AEEC procedures, and muscle dissection was carried out as described in Bortolotto *et al.* (2000). Isometric contractions in EDL muscle were elicited at optimal length *via* supramaximal pulses (13 V cm^{-1}; 0.2 ms duration) in carbogen bubbled Krebs solution (Pedersen *et al.*, 2003) with 10 mmol l^{-1} glucose and 10 μmol l^{-1} tubocurarin, at 25 ± 1°C. Force-frequency responses were determined using stimulation trains of 500 ms and train frequencies of 1-110 Hz, with a 3 min rest period between stimuli. Fatigue resistance was evaluated using a fatigue protocol similar to that described in Chin & Allen (1997), and consisted of repeated maximum tetanic stimulation (110 Hz, 350 ms train duration) at decreasing time intervals (4 s, 3 s, 2.5 s; each for total 2 min) until the force declined to 30% of the initial force (P_0). This protocol was repeated following a 60 min rest period. Contralateral EDL muscles were employed for electrophoretic analyses of myosin heavy chain isoform (MHC_i) composition in whole muscle homogenates and single muscle fibres using a modified version of the Talmadge & Roy (1993) SDS-PAGE protocol.

In comparison to EDL muscle from lean mice (n=8), EDL muscle from *ob/ob* mice (n=8) displayed an increased resistance to the first fatigue bout (time to 30% P_0: 164.4 ± 6.2 s *vs* 146.1 ± 2.8 s; P<0.05) and greater recovery of peak force between fatigue bouts. Type IIB was the predominant fibre type in randomly dissected single fibres from EDL muscle of *ob/ob* (78.9%, n=57) and lean (95.1%, n=61) mice. However, the fibre population from *ob/ob* mice contained a greater proportion of hybrid fibres (21.1% *vs* 4.9%) co-expressing MHCIIb and MHCIId isoforms (i.e. hybrid IIB+IID fibres). Consistent with this result, EDL muscle (n=6) from *ob/ob* mice contained a smaller proportion of MHCIIb (52.4% *vs* 65.7%) and larger proportions of MHCIId (31.9% *vs* 25.7%) and MHCIIa (15.7% *vs* 8.6%) isoforms. This shift in the MHC_i composition of EDL muscle from *ob/ob* mice towards a slower profile was also reflected in the force-frequency relationship at suboptimal frequencies (greater % force relative to maximum force at 30 Hz and 50 Hz in obese muscle) and a prolonged twitch half-relaxation rate (72.4 ± 6.0 ms in obese *vs* 49.2 ± 3.4 ms in lean; P<0.05).

The shift towards slower fibre types and the increased fatigue resistance observed in the present study for EDL muscle from the *ob/ob* mouse may be part of an adaptive response to the obese/diabetic condition, whereby the physiological role of the EDL muscle changes from a muscle enabling rapid movement to a muscle enabling better maintenance of posture under conditions of increased body weight.

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This work is supported by the NHMRC (Australia).

Insulin-like growth factor-I gene transfer by electroporation enhances skeletal muscle regeneration and function after injury

J.D. Schertzer and G.S. Lynch, Basic and Clinical Myology Laboratory, Department of Physiology, The University of Melbourne, Victoria 3010, Australia.

Although skeletal muscle has the ability to regenerate after injury, functional repair can be slow, inefficient, and is often incomplete. In addition to the tightly controlled induction of myogenic regulatory factors and other muscle specific genes, muscle damage and subsequent repair processes induce the release of various biologically active molecules which are critical for regeneration. Insulin-like growth factor-I (IGF-I) is particularly relevant given that levels are elevated after injury during the formation of new fibres or the growth of existing fibres. Given that several studies have demonstrated that IGF-I enhances various aspects of skeletal muscle regeneration, a basis exists for the administration of IGF-I to enhance muscle regeneration and to promote functional recovery after injury (Rabinovsky *et al.*, 2003; Takahashi *et al.*, 2003). However, a comparison of various delivery methods on the efficacy of IGF-I during skeletal muscle regeneration has not been performed.

The purpose of this study was to compare the time course of muscle regeneration following delivery of IGF-I to injured muscles *via* non-viral gene transfer or systemic protein administration. We assessed the time course of functional recovery during muscle regeneration following systemic administration of IGF-I protein *via* mini-osmotic pump (1 mg/kg/day) or electroporation-assisted plasmid-based gene transfer.

Twelve to fourteen-week-old male C57/BL10 mice were anaesthetised deeply (pentobarbitone sodium, 60 mg/kg) and tibialis anterior (TA) muscles were injured by an intramuscular injection of the myotoxic agent, notexin, which causes complete destruction of injected muscle fibres but does not damage muscle precursor cells that are activated for subsequent regeneration. Contractile properties of the TA muscle were measured *in situ* (with an intact nerve and blood supply) at 7, 14, 21 and 28 days post injury and the mice were killed by cardiac excision whilst anaesthetised. At 14 days post injury, tetanic force was 36% greater following electroporation-assisted IGF-I gene transfer compared to control ($P < 0.05$), whereas systemic IGF-I protein administration had no effect on tetanic force at this time. At 21 days post injury, tetanic force was 31% greater following electroporation-assisted IGF-I gene transfer and 35% greater following IGF-I protein delivery compared to controls ($P < 0.05$).

Our results show that IGF-I enhanced muscle regeneration and functional restoration after injury, regardless of the route of administration. However, electroporation-assisted plasmid delivery promoted functional recovery earlier than systemic IGF-I protein administration. The findings highlight the potential of IGF-I to minimise functional disability after injury and demonstrate that non-viral plasmid based gene transfer can be superior to continuous systemic protein administration.

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Supported by the Muscular Dystrophy Association (USA) and the National Health and Medical Research Council. JDS was supported by Melbourne International Research and Fee Remission Scholarships.