AuPS/ASB Meeting - Adelaide 2010

Free communications: Regulation of skeletal muscle growth and repair

Tuesday 30th November 2010 - Broughton Room - 11:00

Chair: Kate Murphy

The role of G-CSF in the growth and development of skeletal muscle cells *in vitro*

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Background: Granulocyte-Colony Stimulating Factor (G-CSF) is a cytokine which stimulates the production of hematopoietic stem cells from bone marrow. Since its discovery and approval for clinical use, various roles for G-CSF outside the hematopoietic system have emerged. Recently, G-CSF treatment has been shown to increase skeletal muscle mass, strength and regeneration in rodent models of muscle disease and damage (Stratos *et al.*, 2007; Pitzer *et al.*, 2008). However, the molecular mechanisms underlining these responses are poorly understood. In cells expressing the G-CSF Receptor (G-CSFR), ligand binding activates several intracellular signalling cascades such as JAK/STAT, Akt, and ERK1/2 (Liongue *et al.*, 2009). These signalling pathways are of vital importance in the regulation of skeletal muscle during hypertrophy, atrophy and regeneration. However, it is unknown whether the G-CSFR is expressed in skeletal muscle, or if these signalling pathways are activated in response to G-CSF treatment.

Methods: *RT-PCR:* mRNA expression for the G-CSFR was determined by RT-PCR. The resulting PCR fragment was separated and purified from a 2% Agarose gel and sequenced. *Western Blotting:* Protein was separated on a polyacrylamide gel and transferred to PVDF membrane. The membrane was probed for the proteins of interest. *Proliferation:* C_2C_{12} proliferation was measured by the BrdU Labelling and Detection Kit III (Roche), according the manufacturers instructions. *Protein Degradation / Synthesis:* Protein synthesis and degradation was determined by the amount of radio-labelled H^3-tyrosine incorporated and released from the cells, respectively.

Results: The expression of the G-CSFR was detected in C_2C_{12} cultures by RT-PCR and western blotting, as well as in mouse and human muscle by western blotting and immunofluorescence. 30 min G-CSF (4ng/ml, 40ng/ml) treatment in C_2C_{12} myotubes increased the phosphorylation of STAT3. Preliminary data showed Akt and ERK1/2 phosphorylation was also increased. However, the rate of proliferation, protein synthesis and protein degradation remained unchanged under basal and catabolic conditions.

Summary/Conclusion: The expression of the G-CSFR in skeletal muscle suggests that G-CSF/G-CSFR may be of importance to muscle physiology. Activation of STAT3 signalling, and the potential activation of Akt and ERK1/2 in C_2C_{12} myotubes, elicits potential signalling pathways for G-CSF/G-CSFR in skeletal muscle. However, a functional outcome remains elusive.

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β_2-adrenoceptors are the dominant subtype involved in early muscle regeneration after injury

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Skeletal muscles can be injured by a myriad of insults that can compromise their functional capacity. Regenerative processes are often slow and incomplete, and so developing novel therapeutic strategies to enhance muscle regeneration represents an important research area. We have shown previously that the β-adrenoceptor (AR) signalling pathway plays an important role in skeletal muscle regeneration after injury (Beitzel *et al.*, 2004, 2007), and that transgenic mice lacking both β_1- and β_2-ARs have delayed regeneration following myotoxic injury (Sheorey *et al.*, 2008). In the present study we investigated the contribution of β-AR signalling to early muscle regeneration, to determine the relative contribution of individual β-AR subtypes to muscle repair after injury.

Mice (8-9 weeks) lacking β_1-adrenoceptors (β_1-AR KO), β_2-adrenoceptors (β_2-AR KO), or both subtypes of β-adrenoceptors (β_1/β_2-AR KO), were obtained from The Jackson Laboratory (Bar Harbour, ME, USA). Littermate wildtype mice were used as controls for the β_1-AR KO and β_2-AR KO mice, while control mice for the β_1/β_2-AR KO mice were from a C57BL/6 background, as employed previously (Sheorey *et al.*, 2008). Muscle function was determined by assessing the contractile properties of the *tibialis anterior* (TA) muscle *in situ* (Gehrig *et al.*, 2010). Briefly, mice were anaesthetised (60 mg/kg, sodium pentobarbital, *i.p.*), the right TA muscle was surgically exposed, and the distal tendon was attached to the lever arm of a force transducer, with the knee and foot immobilised. At the conclusion of the experiment the mice were killed by cardiac excision while still anaesthetised deeply.

When muscle function was examined in uninjured TA muscles, both β_2-AR KO mice and β_1/β_2-AR KO mice produced significantly less force than their respective controls ($p < 0.05$), however, TA muscles from β_1-AR KO mice showed no significant deficit in force production. To determine the relative contribution of the individual β-AR subtypes to early muscle regeneration, mice were anaesthetised (ketamine 80 mg/kg and xylazine 10 mg/kg; *i.p.*) and the TA muscle of the right hindlimb was injected with the myotoxin, Notexin (1 μg/ml, *i.m.*) to cause complete muscle fibre degeneration. Mice were allowed to recover for 7, 10 or 14 days, after which TA function was assessed *in situ*. β_1/β_2-AR KO mice produced significantly less force than their controls at 7 days post-injury ($p < 0.05$) but force production had increased to similar levels as control at 10 and 14 days post-injury. Muscles from β_2-AR KO mice showed a similar pattern of force production during regeneration with significantly less force at 7 days but similar force production at 10 and 14 days post-injury, while muscles from β_1-AR KO mice did not exhibit force deficits at any stage during regeneration.

These results suggest that the β_2-adrenoceptor is the dominant β-AR subtype involved in early muscle fibre regeneration. Selective stimulation of β_2-adrenoceptors may therefore be a therapeutic strategy to improve the rate, extent and efficacy of the regenerative process, and may have important implications for other conditions where muscle wasting and weakness are indicated.

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Muscle-specific heat shock protein 72 (HSP72) overexpression improves muscle structure and function in dystrophic *mdx* mice

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Duchenne muscular dystrophy (DMD) is the most severe of the muscular dystrophies, affecting 1 in 3,500 live male births. Affected patients generally die in their twenties, with respiratory and/or cardiac failure ultimately causing death in most cases (Finsterer, 2006). Absence of the dystrophin protein results in muscle fibre fragility, whereby contractions result in membrane tears and Ca^{2+} influx. Coupled with abnormalities in intracellular Ca^{2+} handling, this results in an elevated cytosolic [Ca^{2+}], resulting in the subsequent activation of degenerative pathways. Chronic muscle fibre degeneration and increasingly ineffective regeneration results in fibrotic tissue infiltration leading to major functional impairments in DMD patients. Heat shock protein 72 (HSP72) has been shown to protect contractile function and improve calcium handling dynamics under conditions of stress in cardiac muscle (Kim *et al.*, 2006). We tested the hypothesis that HSP72 overexpression would ameliorate the dystrophic pathology and thus preserve muscle function in *mdx* dystrophic mice.

Female *mdx* mice were crossed with male mice expressing a rat inducible HSP72 transgene under the control of a chicken β-actin promoter, which limited transgene expression to skeletal and cardiac muscle (and brain) tissue (Marber *et al.*, 1995). F_1 generation males were mated with female *mdx* mice to yield an equal proportion of *mdx*^{HSP72} and *mdx* littermate controls. Mice (25-30 week old) were anaesthetised (60 mg/kg sodium pentobarbitone), and the functional properties of diaphragm muscle strips were measured *in vitro* as described previously (Lynch *et al.*, 1997). Mice were killed by diaphragm and cardiac excision while still anaesthetized deeply. Diaphragm muscle strips were also frozen for subsequent histological analysis. Blood was sampled to measure serum creatine kinase (CK) levels, a myoplasmic protein commonly used as a measure of whole body muscle breakdown. In a separate group of mice, Evans blue dye (EBD) was injected (1% w/v, 10μl/g BM, *i.p.*) for assessment of damaged and necrotic muscle fibres.

HSP72 protein expression was elevated significantly in the muscles of *mdx*^{HSP72} compared with *mdx* littermate control mice. HSP72 overexpression improved specific (normalised) force in isolated diaphragm muscle strips ($p < 0.05$), reduced collagen infiltration ($p < 0.05$) and reduced minimal Ferets variance coefficient (used as an index of the severity of the pathology; $p < 0.05$). Serum CK levels were significantly lower in *mdx*^{HSP72} compared with *mdx* littermate controls ($p < 0.05$), which was further supported by a reduction in EBD-positive fibres indicating fewer damaged and/or necrotic fibres ($p < 0.05$).

Overexpression of HSP72 improved the dystrophic skeletal muscle pathology in *mdx* mice, especially in the severely affected diaphragm muscle. Further research is required to determine the therapeutic potential of this novel approach for DMD and related conditions.

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Properties and proteolytic activity of m-calpain in rat skeletal muscle

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m-Calpain is a ubiquitously expressed Ca^{2+}-dependent protease with diverse functionality in skeletal muscle including, but not limited to, roles in cell migration, fusion and membrane repair. It is believed to require >100 μM free [Ca^{2+}] for activation (Cong *et al.*, 1989; Elce *et al.*, 1997), although this requirement may be dependent on phosphorylation status and/or phospholipid binding (Goll *et al.*, 2003). Given the peak tetanic [Ca^{2+}] within skeletal muscle fibres normally reaches only 2-20μM (Baylor & Hollingworth, 2003), this raises the question of how m-calpain fulfills its role as a protease in skeletal muscle.

EDL and *soleus* muscles were dissected from male Long-Evans hooded rats sacrificed by anaesthetic overdose (4% v: v halothane) with approval of the La Trobe University Animal Ethics Committee. Western blotting was used to quantify the absolute amount of m-calpain by comparing known concentrations of pure rat recombinant m-calpain to whole skeletal muscle homogenates. The total amount of m-calpain was found to be ~1.0 μmol/kg muscle mass in predominantly slow-twitch soleus muscle and ~0.3 μmol/kg muscle mass in fast-twitch *extensor digitorum longus* muscle. Experiments in which mechanically skinned fibre segments were washed in aqueous solutions for set times showed that ~75% of the total m-calpain is freely diffusible within a quiescent fibre.

The proteolytic activity of m-calpain was also assessed using mechanically-skinned single fibres. Once skinned, the fibre segment was stretched to approximately twice its resting length so that no force-producing cross-bridges could be formed, with the resulting passive force being due to extension of titin, a large elastic sarcomeric protein that is a known substrate for m-calpain. Proteolysis of titin was gauged from the decline in passive force when a stretched fibre segment was exposed to 1 μM rat recombinant m-calpain over a range of elevated free [Ca^{2+}]. Proteolytic activity of m-calpain was observed even with free [Ca^{2+}] as low as 4 μM, and the rate of decline of passive force reached ~17% / min at 20 μM free Ca^{2+}. The rate of passive force decline was even greater at higher free [Ca^{2+}], reaching ~250% / min at 500 μM Ca^{2+}. In the presence of 20 μM free [Ca^{2+}], porcine-derived native m-calpain added exogenously at 1 μM resulted in proteolysis of titin at 9% / min, approximately half the rate observed with the rat recombinant mcalpain under the same conditions. Passive force decline over the physiological range of free [Ca^{2+}] was also measured both with and without ATP present in the solution and proteolytic activity was found to be the same in both cases. With both native and recombinant m-calpain, proteolytic activity could always be rapidly stopped by lowering the free [Ca^{2+}] to <10 nM. Furthermore, the proteolytic activity of mcalpain at 2 μM free Ca^{2+} was unchanged irrespective of whether or not the m-calpain had been activated at higher [Ca^{2+}] beforehand.

In conclusion, these findings demonstrate that m-calpain displays considerable proteolytic activity at physiological Ca^{2+} conditions occurring in muscle fibres. Furthermore, the findings distinguish its regulation from that of the other ubiquitous calpain, μ-calpain, which becomes more Ca^{2+}-sensitive following exposure to elevated [Ca^{2+}], suggestive that the ubiquitous calpains likely have quite different roles in skeletal muscle.

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Properties of AMP kinase (AMPK) β isoforms and glycogen related proteins in segments of single fibres from rat skeletal muscle

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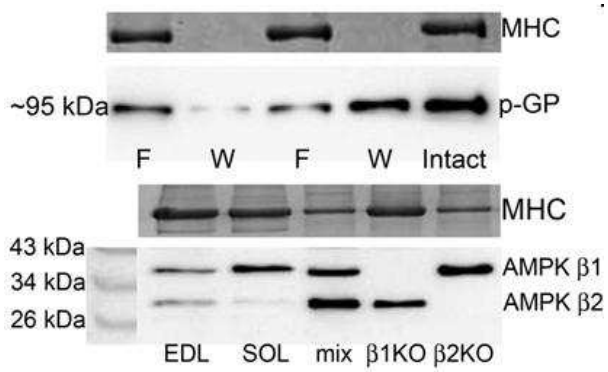
To understand function and regulation of proteins it is important to have knowledge about their properties in the physiological environment. In this study, we have used small segments of single skeletal muscle fibres dissected from rat skeletal muscle to examine diffusibility and fibre type expression of a number of glycogen related proteins as well as AMPK isoforms.

Male Long-Evans hooded rats (6-8 months old) were sacrificed using a lethal overdose of fluothane in accordance with the La Trobe University Animal Ethics Committee and the *extensor digitorum longus* (EDL) and *soleus* (SOL) muscles were excised. To compare fibre type differences EDL (exclusively type II) and SOL (predominantly type I) muscle fibres were analyzed for AMPK β1 and β2, glycogen branching enzyme (GBE), glycogen debranching enzyme (GDE), glycogen phosphorylase (GP), phospho-GP and glycogen synthase (GS). To measure protein diffusibility, individual fibres were dissected from muscles that had been immersed in paraffin oil and then mechanically-skinned and exposed to physiological K^+-based solution (pCa < 10) for 1 and 10 min. The wash solution (W) and their matched fibres (F) were analyzed side by side using Western blotting (Murphy *et al.*, 2006). For fibre type comparisons, the amount of each protein was normalized to the amount of an abundant muscle protein (*i.e.* actin or myosin) and then expressed relative to the amount of the given protein present in the SOL fibres. Identity of AMPK β isoforms was confirmed by their absence in muscle homogenates from respective knock-out mice.

Proteins related to glycogen breakdown (GDE, GP, p-GP) were present in higher amounts in fast- compared with slow-twitch muscle (Table). Proteins related to glycogen synthesis were similar (GS) or lower (GBE) in slow- compared with fast-twitch muscle (Table). The AMPK β isoforms had the opposite abundances in fast- and slow-twitch muscle. Some proteins were freely diffusible (GBE, AMPK β1 and β2), some appeared to be weakly bound (GP, p-GP), whilst GDE was much more tightly associated with a muscle structure and likely bound with glycogen which reportedly washes out <40% in 10 min (Goodman *et al.*, 2005). Given that ~80% of the GP appeared in the wash, it is likely that there is considerable excess of this enzyme compared with the amount of glycogen present .These findings strongly suggest that when glycogen related proteins or AMPK are examined in skeletal muscle, the fibre type dependence must be taken into account. This is particularly important in human skeletal muscle which is typically heterogeneous with respect to fibre types present and so these proteins should be examined in individual fibres.

Protein / enzyme	Approx amount in EDL compared with SOL	Amount appearing in wash after 10 min	
		EDL	SOL
AMPK β1	0.4	70%	70%
AMPK β2	4	70%	70%
GBE	0.2	80%	85%
GDE	5	20%	50%
GP	100	80%	40%
phospho-GP	10	60%	40%
GS	1	ND	ND



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