AuPS/ASB Meeting - Adelaide 2010

Free communications: Regulation of protein expression in skeletal muscle

Wednesday 1st December 2010 - Broughton Room - 11:30

Chair: Matthew Watt

Atrogin-1 regulation in human and mouse skeletal myotubes

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Atrogin-1, an E3 ubiquitin ligase, is increased in numerous models of muscle atrophy and is seen as a potential therapeutic target to combat muscle wasting. While previous rodent studies have consistently shown that under catabolic conditions, Atrogin-1 is regulated by FoXO transcription factors, studies in atrophic human skeletal muscle do not support a dominant role of FoXO. Our aim was to identify potential transcriptional regulators of Atrogin-1 in human and mouse myotubes. Human primary and C2C12 myotubes were infected with a c-MyC, C/EBPa or PPARd adenovirus for 48 h. Atrogin-1 mRNA levels were increased by 72% and decreased by 52% with PPARd and C/EBPa over-expression, respectively. mRNA analysis in human myotubes is in progress. At the protein level there was a 74% and 46% increase in Atrogin-1 with C/EBPa over-expression in mouse and human myotubes, respectively. c-MyC and PPAR**d** over-expression increased Atrogin-1 protein by 46% and 62% in mouse myotubes respectively, while in human myotubes infection with c-MyC and PPARd decreased Atrogin-1 protein levels by 23% and 26% respectively. These preliminary results suggest that Atrogin-1 may be transcriptionally regulated by factors other than FoXO, and further highlight that Atrogin-1 regulation is species dependent. Future studies will determine direct transcriptional regulators of Atrogin-1 *via* luciferase assays.

NDRG2, a novel player in the control of skeletal muscle mass?

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The N-myc downstream-regulated genes (NDRG1-4) represent a family of molecules linked to cell growth, differentiation and stress (Melotte *et al*,, 2010); however, how they function and their protein partners are poorly described. Recently, we identified that the knockdown of NDRG2 affected myoblast proliferation and differentiation (Foletta *et al.*, 2009). In addition, we identified that NDRG2 expression increased markedly with muscle differentiation and that its gene expression increased also following treatment with catabolic agents, and conversely, decreased under hypertrophic conditions in myotubes. Furthermore, the profile of NDRG2 gene expression closely matched the mRNA profiles of the E3 ligases atrogin-1/MAFbx and MuRF1, key regulators of the ubiquitin proteasome pathway and skeletal muscle mass. This outcome suggests that these three genes are regulated by related factors and that they may have connected roles during changes in muscle mass. Here, we sought to characterize further the potential relationship of these molecules in differentiated muscle cells.

Protein synthesis and degradation as measured by ^{3}H-tyrosine incorporation and release, respectively, was assessed in mouse C2C12 myotubes following the knockdown of NDRG2 protein levels by siRNA under basal, 10 nM insulin and 1 μM dexamethasone treatments. Co-immunoprecipitation analyses of overexpressed NDRG2, atrogin-1 and MuRF1 proteins in C2C12 myoblasts in the presence or absence of the proteasome inhibitor MG132 also were performed.

A 20% increase in insulin-mediated protein synthesis ($p<0.01$) was found in myotubes lacking NDRG2 although no effect on protein degradation was measured. Co-immunoprecipitation analyses also revealed an ability of NDRG2 to interact with both atrogin-1 and MuRF1. Moreover, the interaction between NDRG2 and atrogin-1 was enhanced by 20 μM MG132, but not for the NDRG2 and MuRF1 interaction, suggesting that the inhibition of atrogin-1 activity may promote NDRG2-atrogin-1 binding.

These data provide corroborative evidence of a relationship between NDRG2 and the ubiquitin proteasome regulators, atrogin-1 and MuRF1, and that NDRG2 may also impact on the control of skeletal muscle mass. Currently, we are characterising the signaling pathways through which NDRG2 may affect protein synthesis. These studies will help provide greater insight into the complex molecular mechanisms governing muscle mass regulation.

Melotte V, Qu X, Ongenaert M, van Criekinge W, de Bruine AP, Baldwin HS and van Engeland M. (2010). *FASEB Journal* **24**:1-14

Foletta VC, Prior M, Stupka N, Carey K, Segal DH, Jones, S, Swinton C, Martin S, Cameron-Smith D and Walder KR. (2009). *Journal of Physiology* **587**: 1619-34.

Multiple cell types express myokines following intense resistance exercise

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Optimal repair of skeletal muscle following injury requires a significant and well orchestrated inflammatory response. The infiltration of leukocytes, and particularly monocytes/macrophages, in the hours/days following injury is a critical component in the repair of skeletal muscle (Chazaud *et al.*, 2009; Koh & Pizza, 2009). These cells are not only responsible for the clearance of cellular debris, but also the release of factors that help to control the myogenic program of stem cells (Chazaud *et al.*, 2009). While the appearance and functions of these cells have been widely investigated, the factors that are responsible for the recruitment and chemotaxis of leukocytes into skeletal muscle are still somewhat unknown. Our aim was to investigate the effect of a single bout of resistance exercise on the expression and localization of 2 major chemoattractive factors, monocyte chemoattractant protein 1 (MCP-1) and interleukin 8 (IL-8).

Eight young males (22.1±0.2yr) completed three sets of resistance exercise for the leg muscles (leg press, leg extension and squat). Two sets consisted of 8-12 repetitions at 80% 1-RM, whereas in the final set the subjects exercised until exhaustion. Muscle biopsies were obtained before exercise, and 2, 4 and 24 h after exercise. Expression of MCP-1 and IL-8 was analyzed *via* Multiplex analysis (protein) and PCR (gene). Immunohistochemistry was used to establish localization.

Large increases in both gene and protein expression of MCP-1 and IL-8 were evident 2 h following exercise completion, returning to resting levels by 24 h. Neither factor was prevalent within the cytoplasm of myofibres following exercise. MCP-1 was localized predominately to Pax7 and CD68 positive mononucleated cells, but not strictly confined to these cell types. The distribution of IL-8 immunoreactivity was different to that of MCP-1 and seemed to be in close proximity to collagen IV expressing cells.

Both MCP-1 and IL-8 have been identified as major regulators of muscle mediated leukocyte recruitment *in vitro* (Chazaud *et al.*, 2003; Peterson & Pizza, 2009). The present study indicated that both factors increased dramatically in response to a single bout of resistance exercise, which is in accordance with previous literature (Nieman *et al.*, 2004; Hubal *et al.*, 2008). The localization of these factors within a variety of cell types, and the contrasting pattern of expression, suggest a complex and multifaceted response occurs within the muscular microenvironment to regulate inflammation and muscular repair in response to resistance exercise.

Chazaud B, Brigitte M, Yacoub-Youssef H, Arnold L, Gherardi R, Sonnet C, Lafuste P & Chretien F. (2009). Dual and beneficial roles of macrophages during skeletal muscle regeneration. *Exercise and Sport Sciences Reviews* **37,** 18-22.

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Koh TJ & Pizza FX. (2009). Do inflammatory cells influence skeletal muscle hypertrophy? *Frontiers in Bioscience (Elite Ed)* **1,** 60-71.

Nieman DC, Davis JM, Brown VA, Henson DA, Dumke CL, Utter AC, Vinci DM, Downs MF, Smith JC, Carson J, Brown A, McAnulty SR & McAnulty LS. (2004). Influence of carbohydrate ingestion on immune changes after 2 h of intensive resistance training. *Journal of Applied Physiology* **96,** 1292-1298.

Peterson JM & Pizza FX. (2009). Cytokines derived from cultured skeletal muscle cells after mechanical strain promote neutrophil chemotaxis in vitro. *Journal of Applied Physiology* **106,** 130-137.

PGC-1α and PGC-1β regulate protein synthesis in C2C12 myotubes

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Skeletal muscle atrophy is characterised by increased rates of protein degradation and/or decreased rates of protein synthesis. Overexpression of peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) or PGC-1β can attenuate muscle atrophy, and this has been attributed to a decrease in protein degradation (Brault, Jespersen & Goldberg, 2010; Sandri *et al.*, 2006).

This study investigated the role of PGC-1α and PGC-1β in protein synthesis in C2C12 myotubes. Myotubes were infected with GFP, PGC-1α, or PGC-1β adenoviruses, and protein synthesis was measured at basal levels and with dexamethasone treatment, by the uptake of [^{3}H]-tyrosine.

PGC-1α or PGC-1β overexpression resulted in a 25-28% increase in protein synthesis. Dexamethasone decreased protein synthesis by 15% in the GFP-infected myotubes. However, overexpression of PGC-1α or PGC-1β was able to prevent the dexamethasone-induced decrease. Treatment with LY294, an inhibitor of PI3K/Akt, did not prevent the PGC-1α or PGC-1β driven increase in protein synthesis. This effect was therefore independent of Akt, a major kinase involved in muscle growth.

Another potential mechanism for the PGC-1α and PGC-1β driven increase in protein synthesis may be *via* their regulation of microRNAs (miRNAs). The expression of miR-1 and miR-133a, two miRNAs that are thought to play a role in muscle hypertrophy, were downregulated by PGC-1α or PGC-1β overexpression. Further studies will determine if these two miRNAs are involved in the regulation of protein synthesis with PGC-1α and PGC-1β overexpression.

Brault JJ, Jespersen JG & Goldberg AL. (2010) Peroxisome proliferator-activated receptor γ coactivator 1α or 1β overexpression inhibits muscle protein degradation, induction of ubiquitin ligases, and disuse atrophy. *Journal of Biological Chemistry* **285,** 19460-71.

Sandri M, Lin J, Handschin C, Yang W, Arany ZP, Lecker SH, Goldberg AL & Spiegelman BM. (2006). PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. *Proceedings of the National Academy of Sciences USA* **103,** 16260-16265.

Properties and amounts of heat shock proteins in skeletal muscle

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Heat shock proteins (HSP) are considered to be important in protecting and maintaining cellular homeostasis by binding to partially denatured proteins and acting as molecular chaperones. αB-crystallin, HSP25 and HSP72 are thought to protect key components in skeletal muscle such as SERCA pumps or actin. In the present study, we have measured the amounts, diffusibility and activation characteristics of these proteins in fast-twitch (*extensor digitorum longus*) and predominantly slow-twitch (*soleus*) fibres from rat skeletal muscle.

Male Long-Evans hooded rats (6-8 months old) were sacrificed using a lethal overdose of isoflurane with approval of the La Trobe University Animal Ethics Committee, and the *extensor digitorum longus* (EDL) and *soleus* (SOL) muscles were excised. Muscles were either used for obtaining skinned fibre segments or homogenized and the entire muscle homogenate analysed using a quantitative Western blotting technique. To determine the absolute amounts of αB-crystallin, HSP25 and HSP72 in unstressed muscle, known amounts of pure HSP25, HSP72 and αB-crystallin were run on Western blots alongside or with the muscle homogenates samples (Murphy *et al.*, 2009) (see Table). From these measurements αB-crystallin is almost 29 times more expressed compared to HSP72 and ~13 time more than HSP25 in SOL muscle. These measurements of the absolute amounts of HSPs present give insight into the importance of αB-crystallin as well as the binding limitations and physiological function of Hsps in skeletal muscle.

To measure diffusibility, individual fibre segments were mechanically skinned, removing the surface membrane and allowing proteins to diffuse out of the fibre and into the bathing solution. The skinned fibre segment and the matched bathing solution were run on Western Blots and the diffusibility of αB-crystallin, HSP25 and HSP72 could be determined.

In unstressed muscle between 50 – 90% of HSP25, HSP72 and αB-crystallin appeared in the bathing solution within 10 min, indicating these proteins are broadly in rapid equilibrium with the cytoplasm in quiescent fibres. When a muscle was exposed to a potent oxidative stress of 10 mM H_2O_2 whilst pinned at room temperature, or bubbled with 95% O_2 at an elevated temperature (~31°C) for more than 1 hour, the diffusibility of αB-crystallin, HSP25 and HSP72 remained the same as that of an unstressed muscle.

Diffusibility was also investigated after a muscle was heated to 40°C for 30 min whilst pinned under paraffin oil. HSP25 and αB-crystallin became almost completely bound within the fibre, whereas HSP72 showed no change in diffusibility from that of an unstressed muscle. However when the temperature was raised to 45°C, HSP72 was no longer diffusible and became bound within the fibre. When fibre segments from a 40°C heated muscle were washed in the presence of 10 mM DTT, HSP25 and αB-crystallin remained bound and did not became diffusible, indicating the bonds between HSP25 or αB-crystallin and the target site was not a simple disulfide bond.

	Amount (μmol/kg muscle ± SEM)	
	EDL	SOL
HSP72	1.0 ± 0.0	4.3 ± 0.1
HSP25	2.8 ± 0.3	8.9 ± 1.3
αB-crystallin	3.3 ± 0.8	123.9 ± 16.7

Murphy RM, Larkins NT, Mollica JP, Beard NA, Lamb GD. (2009) *Journal of Physiology* **587(2):** 443-60.

Knockdown of STARS alters protein synthesis and degradation

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Background and Aim: Striated muscle activator of Rho signalling (STARS) is a muscle specific actin-binding protein (Arai *et al.*, 2002). We have recently shown that STARS is up-regulated in hypertrophied human skeletal muscle following resistance exercise and is decreased following atrophy-stimulating detraining (Lamon *et al.*, 2009). STARS mRNA is also reduced in sarcopenic mice (Sakuma *et al.*, 2008). These studies suggest that STARS may be involved in skeletal muscle protein synthesis and/or degradation; however this has not been determined. Therefore, the aim of this study was to establish the role of STARS in protein synthesis and degradation in C2C12 myotubes.

Methods: STARS over-expression and knockdown in C2C12 myotubes was achieved using adenoviral infection and siRNA transfection, respectively. Myotubes were also treated with insulin (100nM) to promote protein synthesis or dexamethasone (DEX) (10μM) to promote protein degradation. Protein synthesis and degradation was determined by the amount of radio-labelled ^{3}H-tyrosine incorporation into and release from the myotubes, respectively.

Results: STARS over-expression did not influence basal protein synthesis or degradation, nor did it influence insulin stimulated or dexamethasone attenuated protein synthesis. However, knockdown of STARS significantly reduced basal and insulin stimulated protein synthesis by 25%. Additionally, knockdown of STARS significantly increased basal and dexamethasone-induced protein degradation by 20% and 50%, respectively.

Conclusion: These observations show that STARS is necessary to maintain the fine balance between basal protein synthesis and degradation. Furthermore, a reduction in STARS reduces the influence of anabolic stimuli and enhances the effect of catabolic stimuli. A minimum amount of STARS may be required to sustain a healthy level of protein turnover.

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Sakuma K, Akiho M, Nakashima H, Akima H, Yasuhara M. (2008). Age-related reductions in expression of serum response factor and myocardin-related transcription factor A in mouse skeletal muscles. *Biochimica et biophysica Acta*. **1782(7-8):** 453-61.