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Ageing alters the inflammatory response in rat skeletal muscles after injury

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Some of the most serious consequences of ageing are its effects on skeletal muscle, characterized by wasting and weakness (Ryall *et al.* 2008). Although the mechanisms responsible for these changes have yet to be elucidated fully, an age-related impairment in regenerative capacity is a contributing factor (Carlson and Conboy, 2007). Successful muscle regeneration after injury requires a carefully regulated inflammatory response that removes damaged cells and initiates satellite cell activation (Charge & Rudnicki, 2004). However, the impact of ageing on the inflammatory/cytokine response of skeletal muscle after injury remains poorly understood. We investigated whether ageing affected the expression of inflammatory markers in injured/regenerating muscles and tested the hypothesis that the inflammatory process in injured muscles of old rats would be prolonged.

Male Fischer 344 rats aged 3 months (young, n = 20), 12 months (adult, n = 20) and 24 months (old, n = 20) were anaesthetised (100 mg/kg ketamine and 10 mg/kg xylazine i.p, 2 ml/kg). The extensor digitorum longus (EDL) muscles of the right hindlimb were injected with bupivacaine hydrochloride in the distal, proximal, and midbelly regions to ensure complete degeneration of all muscle fibres. The EDL muscle of the left hindlimb served as the non-injected uninjured control. EDL muscles were excised at either 12-, 24-, 36-, or 72-hours after bupivacaine injury. RT-PCR was used to determine mRNA expression levels of the inflammatory markers: TNFα, IFNγ, IL1β, IL18, IL6, and CD18. Gene expression was quantified using the cycle threshold (CT) method, before being normalised to the concentration of input cDNA. Relative gene expression was determined by comparisons with uninjured controls.

At 12 and 24 hours post-injury, all inflammatory cytokines were increased in regenerating muscles, but there was no significant difference between age groups. At 36 hours post-injury, there was a 2-3 fold increase in all cytokines examined in the muscles of old compared with young and adult rats. At 72 hours post-injury cytokine levels in regenerating muscles of young and adult rats were decreased but remained elevated (~10-fold higher) in regenerating muscles of old rats. These findings indicate that ageing is associated with an altered muscle inflammatory response after injury, which contributes to the age-associated decrease in muscle regenerative capacity.

Ryall JG, Schertzer JD, Lynch GS. (2008) *Biogerontology*, **9:** 213-28.
Carlson ME, Conboy IM. (2007) *Ageing Cell*, **6:** 371-82.
Charge SB & Rudnicki MA. (2004). *Cell*, **113:** 422-3.

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Influence of caveolin-3 upon membrane raft lipids and its implications for trafficking in muscle

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Lipid rafts are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids. Some members of the transient receptor potential canonical (TRPC) channel family have been shown to be present in lipid rafts. Caveolae are a subtype of lipid rafts present in some cells expressing proteins of the caveolin family. We recently reported that the muscle caveolae-forming protein, caveolin-3, binds to TRPC1 facilitating plasma membrane targeting and channel activity (Gervásio *et al*., 2008). By what mechanism might caveolin-3 influence TRPC1 targeting and function? Since caveolins are associated with cholesterol/sphingolipid-enriched membrane microdomains, we investigated the effect of caveolin-3 expression upon the mobility and density of lipid rafts using Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Resonance Energy Transfer (FRET) methods respectively. Cholera toxin subunit B (CT-B) was used to label the raft domain-associated ganglioside GM1, on C2 myoblasts.

FRAP measurements confirmed that in the absence of transfected caveolin-3, the raft marker was highly mobile on the plasma membrane (mobile fraction 0.77). However, when caveolin-3-YFP was transfected into the cells, the mobility of the lipid raft marker was decreased compared to non-transfected control cells, particularly in regions of the plasma membrane rich in caveolin-3-YFP (mobile fraction 0.27; $p < 0.05$). Next we developed a new FRET method to measure the assembly of the raft marker into molecular lattices (using CT-B-Alexa555 and CT-B-Alexa647 as FRET donor and acceptor respectively). Interestingly, cavaeolin-YFP-rich regions of the plasma membrane showed the tightest CT-B packing density (highest FRET) ($p < 0.05$) compared to non-transfected cells. We propose that the convex shape of the caveolae may contribute to higher FRET efficiency in those microdomains, as a result of closer proximity of neighboring CT-Bs. Together this suggests that cavaeolin-3 drives the packing and immobilization of raft marker GM1 into stable membrane domains (presumably clusters of cavaeolae). Treatment of cells with cytochalasin D, an agent that disrupts microfilaments, was able to rescue the mobility of lipid rafts in caveolin-3-rich regions of the plasma membrane (mobile fraction 0.74; $p < 0.05$). Double labeling revealed a sub-compartment of caveolin-3 under the plasma membrane, which stained positively for the raft marker GM1. Using 4 dimensional laser scanning confocal microscopy analysis (Z stack plus time), we observed that mechanical stretch was able to induce trafficking of caveolin-3 to the plasma membrane in some cells. The ability of mechanical stretch to induce remodelling of such microdomains suggests a mechanism whereby TRPC1 channel, and other caveolin/lipid rafts-associated proteins, might be shuttled to the plasma membrane in response to stretch.

Gervásio O.L., Whitehead N.P., Yeung E.W., Phillips W.D., Allen D.G. (2008) *Journal of Cell Science* **121**, 2246-2255.

Myoplasmic and sarcoplasmic reticulum calcium in intact mouse muscle during fatigue

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Studies on isolated single muscle fibres at room temperature have established that tetanic calcium declines during repeated tetani and that this decline is one contributor to the reduced force in fatigued muscles (Allen *et al.*, 2008). The aim of the present experiments was to determine whether similar changes occur in intact muscles perfused with blood and operating at body temperature. For this purpose we used genetically encoded Ca^{2+} sensors, cameleons (Miyawaki *et al.*, 1997), targeted to the myoplasm or the sarcoplasmic reticulum (SR). Plasmids for the sensors were transfected into the tibialis anterior 2 weeks before the experiments under anaesthesia (Zoletil + Rompun i.p.). On the day of the experiment, mice were anaesthetised, the tibialis anterior exposed and the distal tendon detached and connected to a force transducer. The muscles were stimulated directly by near-maximal pulses through platinum electrodes touching the upper surface of the muscle. Ca^{2+} signals were obtained from those fibres which expressed the sensors using FRET and a Leica multiphoton microscope (Rudolf *et al.*, 2006). This is an upright microscope and the objective was optically coupled to the muscle with artificial tears (a viscous salt-containing solution).

Many fibres expressed the indicators but movement of the muscle during tetani prevented optical measurements of the same fibre at rest and during tetani. Thus tetanic measurements were only possible irregularly. Tetanic force (1 s tetani at 100 Hz repeated every 4 s) generally fell monotonically to 49 ± 5 % of the control and was then usually stable for some minutes. Recovery, measured by tetani at 2 min intervals, was to 86 ± 4% after 4 min. Peak tetanic myoplasmic [Ca^{2+}] declined during fatigue from 1.36 ± 0.08 (mean ± SE, n = 5) to 1.27 ± 0.09 ratio units (p < 0.01, paired t test) and in two experiments, in which it could be measured, the peak tetanic [Ca^{2+}] recovered by 0.075 units. The free calcium in the SR ($[Ca^{2+}]_{SR}$) declined during fatigue from 1.42 ± 0.08 (n = 9) to 1.12 ± 0.11 (p < 0.02, paired t test) and showed a partial recovery to 1.26 ± 0.11 (p < 0.02).

These data show for the first time that calcium handling changes during fatigue in intact mammalian muscle at body temperature. The fact that $[Ca^{2+}]_{SR}$ declined during fatigue suggests that precipitation of calcium phosphate in the SR might make a contribution to this process.

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Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, & Tsien RY. (1997) *Nature*, **388:** 882-7.
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Effect of S-nitrosoglutathione (GSNO) on excitation-contraction coupling in mechanically-skinned muscle fibres of the rat

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S-nitrosoglutathione (GSNO) is a naturally-occurring compound found in fast-twitch muscle fibres, which is able to S-glutathionate and/or S-nitrosylate various target proteins involved in excitation-contraction (EC) coupling (Aracena *et al.*, 2003). S-glutathionation or S-nitrosylation-induced changes to EC coupling proteins such as the Na^+/K^+ and SR Ca^{2+}-ATPases might alter their efficacy and contribute to muscle fatigue under certain conditions. We sought to identify which EC coupling steps are more susceptible than others to GSNO-modulated changes and whether these changes might be important in understanding of muscle fatigue.

Male Long-Evans hooded rats were killed under deep anaesthesia (2% v:v isoflurane) and the extensor digitorum longus and soleus muscles swiftly excised and immersed in paraffin oil at resting length. Single fibres were mechanically-skinned with forceps and a segment connected to a force transducer (stretched to 120% resting length) and immersed in either a standard K-HDTA solution with weak Ca^{2+} buffering (50 μM EGTA, pCa 7) to examine transverse tubular (T-) system depolarization-induced force responses or into heavily Ca^{2+}-buffered (50 mM EGTA) solutions to examine the contractile apparatus properties. All solutions contained as follows: 1 mM free Mg^{2+}, 8 mM ATP; 10 mM creatine phosphate, at pH 7.1, and were equilibrated to room temperature (~23°C) before use. GSNO was dissolved directly into solution immediately (~1 min) before use.

Single fibres were electrically stimulated (75 V cm^{-1}, 1 ms pulse) to produce twitch or tetanic (50 and 100 Hz) force responses before and after GSNO exposure (2 mM for 2 min in all cases). Additionally, paired pulses with differing intervals (0-20 ms) were applied to determine the repriming period of sodium channels in the T-system membrane (an indirect but sensitive measure of T-system polarization, see Dutka & Lamb, 2007). The Ca^{2+}-sensitivity of the contractile apparatus was determined by transferring the single skinned fibre segment from the 50 mM EGTA-based solution to solutions with progressively higher free [Ca^{2+}] (made by mixing 50 mM EGTA solution with 50 mM Ca-EGTA solution as appropriate, pCa range ~10-4.6) before and after GSNO treatment (2 mM, 2 min in 50 mM EGTA solution). In some other cases, 10 mM DTT (added from a 1M stock) was added to the solution. Maximum Ca^{2+}-activated force produced by the contractile apparatus was virtually unchanged by exposure to GSNO. The Ca^{2+}-sensitivity of the contractile apparatus was substantially potentiated (~0.12 pCa) after exposure to fresh GSNO (n = 7), and this effect was fully reversed by 10 mM DTT. In two slow-twitch SOL fibres GSNO exposure virtually had no effect. Interesting in electrically-stimulated fast-twitch fibres, the equivalent exposure to GSNO initially caused a moderate decrease of 35% and 20% in peak twitch and tetanic force responses respectively (compared to the pre-exposure level, n = 11). Furthermore, subsequent twitch and tetanic force responses became progressively smaller and this was not reversed fully by 10 mM DTT but was substantially ameliorated by a small additional Ca^{2+} load (10 s at pCa 6.7 or equivalent to ≤50% of the endogenous SR Ca^{2+} level), suggesting that the cause of the decline in force involved loss of SR Ca^{2+}. The progressive decrease in depolarization-induced force was not attributable to changes in T-system excitability because the repriming period for T-system Na^+ channels was not significantly altered (4.3 ± 0.2 ms and 4.0 ± 0.3 ms before and after GSNO). These data indicate that the GSNO exposure glutathionated the contractile apparatus, causing a substantial increase in Ca^{2+}-sensitivity (~0.12 pCa). GSNO treatment had little effect on T-system excitability, thus implying Na^+ channels, voltage-sensors, and Na^+/K^+ pumps remained functional unaltered. The progressive loss of depolarization-induced force after GSNO treatment was seemingly due to a loss of Ca^{2+} from the SR either through the SR Ca^{2+} pump itself or through the RyR. Alternatively, the uptake by SR Ca^{2+} pumps might be reduced at the resting Ca^{2+} level. Taken together, glutathionation in intact fibres might aid in maintaining force output when it might otherwise decline.

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