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Symposium: Exercise, free radicals and skeletal muscle

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Chair: Michael McKenna, Mark Hargreaves

Redox modulation of contractile function in skeletal muscle

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For the last half century, scientists have studied the biological importance of free radicals in respiratory and limb muscles. We now know that muscle fibers continually produce both reactive oxygen species (ROS) and nitric oxide (NO) and that both cascades play critical roles in contractile regulation. Under basal conditions, muscle-derived ROS and NO exert opposing effects. Low-level ROS activity is an essential part of the homeostatic milieu and is required for normal force production whereas NO derivatives function as a brake on the system, limiting force. The modulatory effects of ROS and NO are disrupted by conditions that exaggerate production including mechanical unloading, inflammatory disease, and strenuous exercise. Marked increases in ROS or NO levels cause contractile dysfunction, resulting in muscle weakness and fatigue. Loss of force may reflect alterations in intracellular calcium regulation or myofilament function, processes controlled by redox-sensitive regulatory proteins. These principles provide a foundation for ongoing research to identify the mechanisms of ROS and NO action and develop interventions that protect muscle function.

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Influence of skeletal muscle ROS production on excitation-contraction coupling at physiological temperatures

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There is a long-standing issue in skeletal muscle research, which effectively limits experimentation on isolated mammalian skeletal muscle to sub-physiological temperatures. This issue arises from the rapid and irreversible functional deterioration of isolated skeletal muscle preparations from mammals when incubated at temperatures in the “normal” physiological range. The effects of physiological temperatures on individual excitation-contraction coupling events in fast-twitch skeletal muscle were investigated using isolated intact muscle preparations and freshly mechanically skinned muscle fibres from the rat.

Extracellularly released reactive oxygen species (ROS) were measured in intact isolated rat *extensor digitorum longus* (EDL) muscles at 22°C, 32°C, and 37°C, and tetanic force was measured at 22°C and 37°C under the same conditions. The rate of ROS production showed a marginal increase between 22° to 32°C, but increased fivefold when the temperature was increased from 22°C to 37°C. The increase in ROS was accompanied by a marked decrease in tetanic force after 30 min incubation at 37°C (Edwards *et al.* 2007). Using mechanically skinned fibres we demonstrated that endogenously produced ROS acts on the contractile apparatus, reducing Ca²⁺-activated force (van der Poel & Stephenson, 2002), and on the sarcoplasmic reticulum (SR), increasing the Ca²⁺ leak rate from the SR that is not *via* the ryanodine receptor (van der Poel & Stephenson, 2007). The resting membrane and intracellular action potentials of isolated muscle preparations were also significantly influenced by temperature, which was associated with an increase in ROS (van der Poel *et al.*, 2007).

These results implicate the relatively high level of ROS production as a potential cause of the down-regulation of skeletal muscle function *in vitro* at physiological temperatures. The efficient perfusion of the muscle with blood *in vivo* would efficiently remove ROS from the muscle and prevent its accumulation to levels that cause depression in the force response at normal physiological temperature.

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Skeletal muscle Na⁺,K⁺-pump activity, reactive oxygen species and fatigue in exercising humans

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The Na⁺,K⁺-pump (Na⁺,K⁺-ATPase, NKA) plays a pivotal role in skeletal muscle function, regulating transmembrane Na⁺ and K⁺ concentration gradients, and membrane excitability. Increased NKA activity during contractions plays a vital modulatory role in these events, and thus also in minimising muscle fatigue. Despite this, substantial Na⁺ and K⁺ leaks occur during exhaustive exercise, with up to a 75% decline in the intracellular/extracellular [K⁺] gradient and near-doubling of the extracellular/intracellular [Na⁺] gradient. Furthermore, our laboratory has found that acute exercise also decreases the maximal NKA activity (Fraser *et al.*, 2002; McKenna *et al.*, 2006), which would both exacerbate Na⁺/K⁺ disturbances, and lessen the NKA electrogenic effects. Thus, we propose muscle Na⁺ and K⁺ ion disturbances together with NKA inactivation as important contributors to muscle fatigue.

Studies in recent years have highlighted a clear role of reactive oxygen species (ROS) in muscle force modulation and in fatigue, with enhanced ROS scavenging reducing muscle fatiguability (Reid, 2001; Smith & Reid, 2006). Support for a ROS-scavenging role in attenuating human muscle fatigue was gained from a landmark finding that intravenous infusion of the non-specific antioxidant N-acetylcysteine (NAC) attenuated fatigue during stimulation of tibialis anterior muscle (Reid *et al.*, 1994). Numerous target proteins for ROS effects have been identified, but whether skeletal muscle NKA is redox-sensitive, and whether ROS-sensitive mechanisms might be involved in exercise-induced NKA inactivation and fatigue were unknown. In two series of voluntary human exercise studies, we firstly investigated acute NKA inactivation and secondly, the role of ROS in fatigue. We developed a modified NAC infusion protocol to investigate whether enhanced ROS scavenging would attenuate muscle fatigue during intense intermittent exercise, and prolonged endurance exercise (Medved *et al.*, 2003; Medved *et al.*, 2004a). We have then fused these investigative lines together in a combined study into muscle redox status, ROS, NKA, K⁺ and fatigue (McKenna *et al.*, 2006).

Intense intermittent exercise performance was unchanged by NAC, despite evidence of improved blood redox status by elevated glutathione (GSH) and lower oxidised GSH (GSSG) (Medved *et al.*, 2003). NAC effects on prolonged exercise performance were related to aerobic power (Medved *et al.*, 2004a). Studies in endurance athletes confirmed an ergogenic effect of NAC during prolonged exercise, together with elevated muscle GSH, cysteine and NAC, indicating greater redox buffering (Medved *et al.*, 2004b). This confirms a role for ROS in muscle fatigue and suggests antioxidant capacity may be an important factor affecting muscle performance. The exercise-induced decline in muscle NKA activity was markedly attenuated by NAC, and the rise in plasma [K⁺] with exercise lowered, suggesting a strong link between ROS and NKA inactivation and further implicating ROS, NKA and K⁺ in fatigue (McKenna *et al.*, 2006).

Finally, we recently investigated whether increased NKA gene expression with exercise (Murphy *et al.*, 2004) involves ROS-linked mechanisms. The acute upregulation of the NKA $\alpha 2$ isoform mRNA by exercise was abolished by NAC, whereas changes in mRNA of other isoforms were not. As the $\alpha 2$ isoform is the most abundant NKA isoforms in muscle, this further indicates a link between ROS and gene expression and thus likely also of adaptability, of this key regulatory protein.

In summary, muscle NKA inactivation may be important in exacerbating fatigue in exercising humans. NAC infusion studies indicate ROS scavenging can enhance exercise performance and establishes a link between ROS, NKA inactivation and muscle fatigue.

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Role of reactive nitrogen species in skeletal muscle glucose uptake and mitochondrial biogenesis

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Nitric oxide / nNOS μ and contraction-stimulated glucose uptake. The uptake and metabolism of glucose by skeletal muscle is a major determinant of whole body glucose homeostasis. People with type 2 diabetes have reduced insulin-stimulated skeletal muscle glucose uptake, however, muscle glucose uptake during exercise is normal. The signalling pathways associated with contraction-stimulated glucose uptake are largely undefined, but known to differ from insulin pathways. There is some evidence that AMP-activated protein kinase (AMPK), Calcium-calmodulin dependent protein kinase II (CaMKII) and nitric oxide derived from neuronal nitric oxide synthase (nNOS, expressed in skeletal muscle) may be involved. Nitric oxide donors increase skeletal muscle glucose uptake and GLUT-4 translocation and NO production / NOS activity increases in skeletal muscle during exercise/contraction. We have found that infusion of a NOS inhibitor into the femoral artery of humans attenuates the increase in leg glucose uptake during cycling exercise in normal, healthy young individuals, people with type 2 diabetes and matched controls (Bradley *et al.*, 1999; Kingwell *et al.*, 2002). Importantly, the NOS inhibitor had no effect on leg blood flow, blood pressure, arterial insulin and glucose concentration. Furthermore, we recently found that local NOS inhibition attenuated the increase in skeletal muscle glucose uptake during *in situ* contraction in rats without influencing capillary blood flow (Ross *et al.*, 2007). Rat studies from other groups have yielded conflicting results, with some studies reporting a reduction in glucose uptake during contraction/exercise with NOS inhibition and others finding no effect (review in McConell & Kingwell, 2006). The reasons for these discrepancies are unclear but are likely to include methodological differences, especially as many studies have examined glucose uptake 20 or more minutes after contraction/exercise (McConell & Kingwell, 2006). We also have preliminary evidence that the activation of skeletal muscle glucose uptake is attenuated in nNOS KO mice. It will be important to confirm these findings and also to determine the factors downstream of NO/nNOS that are associated with GLUT-4 translocation.

NO / nNOS μ and the regulation of mitochondrial biogenesis. Defective small skeletal muscle mitochondria are now recognised as a major component of the metabolic abnormalities of diabetes. Exercise increases mitochondrial volume and improves mitochondrial function. Many research groups are attempting to determine how exercise increases mitochondrial biogenesis and it appears that the same signals that may be involved in regulating glucose uptake during exercise may also be activating gene expression after exercise (AMPK, CaMK and perhaps nitric oxide). It has been shown that NO donors and cGMP analogues increase mitochondrial biogenesis in muscle cells (Nisoli *et al.*, 2004). It was important to extend this line of research to examine whole animals and also to examine whether NO was involved in the increase in skeletal muscle mitochondrial biogenesis in response to exercise. We found that ingestion of the NOS inhibitor L-NAME for 2 days reduces basal skeletal muscle mitochondrial biogenesis but has no effect on the increase in mitochondrial biogenesis in response to an acute exercise bout (Wadley & McConell, 2007). Similarly, the increase in mitochondrial biogenesis markers in response to acute exercise and exercise training is intact in nNOS KO and eNOS KO mice (Wadley *et al.*, 2007). Interestingly, however, rather than mitochondrial biogenesis being lower in nNOS KO mice muscle it was actually higher (Wadley *et al.*, 2007). Although more studies are required, these studies suggest that NO plays a role in basal mitochondrial biogenesis but not in the increase in mitochondrial biogenesis in response to exercise.

Significance. Exercise is considered the best prevention and treatment option for diabetes, but unfortunately, many people with diabetes do not or can not exercise regularly. Alternative therapies are therefore critical to effectively manage diabetes. If skeletal muscle nNOS/nitric oxide is found to play an important role in regulating glucose uptake and/or mitochondrial biogenesis, pharmaceutical agents designed to mimic these exercise effects may improve glycaemic control.

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