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Reactive oxygen species and redox-regulation of skeletal muscle adaptations to exercise

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Skeletal muscle has been shown to generate a complex set of reactive oxygen and nitrogen species (ROS) both at rest and during contractile activity. The primary ROS generated are superoxide and nitric oxide and the pattern and magnitude of their generation is influenced by the nature of the contractile activity. It is increasingly clear that the ROS generated by skeletal muscle play an important role in influencing redox-regulated processes that control, at least some of, the adaptive responses to contractile activity. These processes are also recognized to be modified during ageing and in some disease states, providing the potential that interventions affecting ROS activity may influence muscle function or viability in these situations.

Keywords: contractile activity; myotube; nitric oxide; superoxide

1. SKELETAL MUSCLE ADAPTS RAPIDLY TO CHANGES IN STRETCH OR CONTRACTILE ACTIVITY

Numerous studies indicate that skeletal muscle adapts rapidly to the type of work it is required to perform and/or the pattern of contractile activity undertaken such that specific responses in composition occur following changes in the pattern of contractile activity or external forces such as passive stretching. These adaptations lead to an improved ability of the tissue to deal with the increased stress. There are many comprehensive descriptions of the pattern of these responses and increasing amounts of published information on the signalling pathways involved (Widegren *et al.* 1998; Nader & Esser 2001; Hawley 2002). Manipulation or correction of aberrant adaptive responses requires a comprehensive understanding of the molecular and biochemical processes involved, but currently little is known about the factors that stimulate adaptive responses to stretch or contractile activity (Goldspink 1994; Nader & Esser 2001; Hawley 2002).

2. SKELETAL MUSCLE GENERATES A NUMBER OF REACTIVE OXYGEN SPECIES THAT ARE INCREASED DURING CONTRACTILE ACTIVITY

Many studies have demonstrated an increase in end-point indicators of the reactions of reactive oxygen species (ROS) in tissues during and following exercise (e.g. Dillard *et al.* 1978; Davies *et al.* 1982; Jackson *et al.* 1985). This increase in ROS activity appears to be in major part due to generation by contracting skeletal muscle. The primary ROS generated by skeletal muscle during activity are NO and superoxide and the superoxide dismutates rapidly to form hydrogen peroxide (McArdle & Jackson 2000). There are a number of potential sites for NO and superoxide generation in skeletal muscle (Jackson 2000). This tissue also has a well-developed system to regulate these ROS and prevent potentially deleterious effects. These protective systems include both mitochondrial and cytosolic isoforms of superoxide dismutase (MnSOD and CuZnSOD, respectively), catalase and glutathione peroxidase enzymes and a number of direct scavengers of ROS including glutathione, vitamin E and ascorbate. In general, slow twitch, mitochondria-rich (type I) fibres have an increased content of protective systems in comparison with fast (type II) fibres.

A clear understanding of the mechanisms of ROS generation in contracting skeletal muscle has not been obtained, in part due to a lack of suitable analytical techniques. We have developed complimentary approaches to this problem: (i) microdialysis techniques to study ROS in muscle interstitial fluid (McArdle *et al.* 2001; Pattwell *et al.* 2001) and (ii) the release of ROS from contracting myotubes in culture (McArdle *et al.* 2001; Pattwell *et al.* 2004), that have allowed us to obtain a greater understanding of the release and generation of specific ROS by muscle.

(a) *Nitric oxide is generated by skeletal muscle during activity*

NO is generated continuously by skeletal muscle, a production that is increased by contractions (Balon & Nadler 1994). Skeletal muscle normally expresses the neuronal (type I or nNOS) and the endothelial (type III or eNOS) isoforms of nitric oxide synthase (NOS). nNOS is strongly expressed in fast-twitch muscle fibres and localized to the muscle sacrolemma, where it is associated with the dystrophin–glycoprotein complex (DGC). eNOS is localized to the muscle mitochondria (Kobzik *et al.* 1995). iNOS (type II) is also expressed in skeletal muscle in some inflammatory conditions, but it does not play a significant role in normal muscle (Stamler & Meissner 2001). Release of NO was originally demonstrated from isolated muscles *in vitro*

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One contribution of 18 to a Theme Issue 'Reactive oxygen species in health and disease'.

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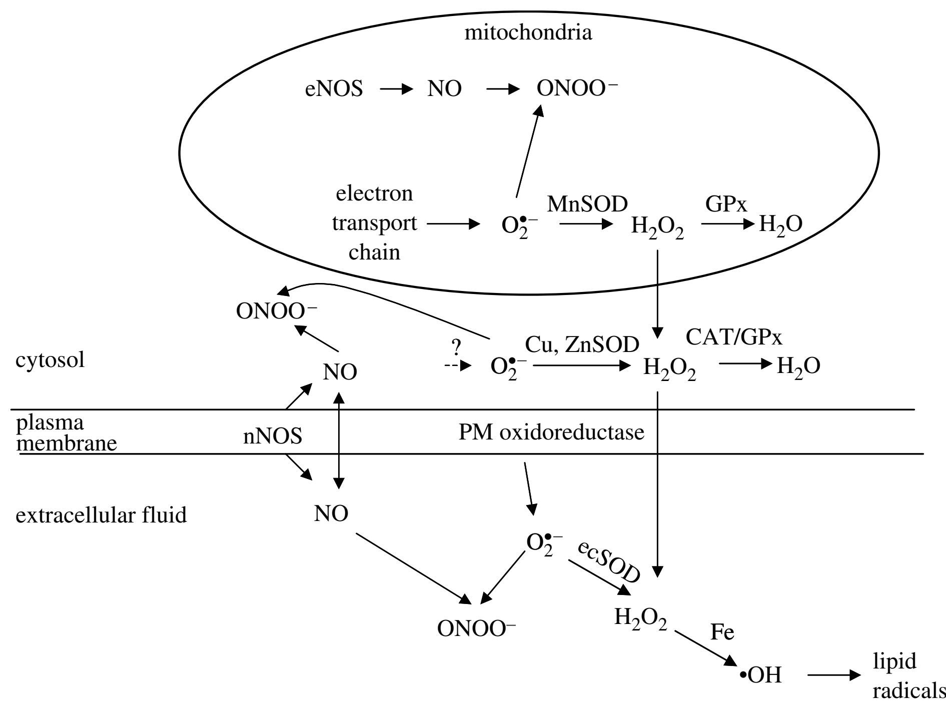

Figure 1. Schematic diagram depicting the different sites and interactions of ROS and NO generated by skeletal muscle cells. GPx, glutathione peroxidase. Modified from Pattwell & Jackson (2004).

(Balon & Nadler 1994), although the cellular source of the NO released was unclear. Our recent analysis of myotubes in culture has confirmed that skeletal muscle cells *per se* release increased amounts of NO during contractile activity, (Pattwell *et al.* 2004), a release that was greatly reduced by the NOS inhibitor, L-NAME. nNOS appears to be the prime source of the NO released from skeletal muscle (Hirschfield *et al.* 2000). Passive stretching of muscle has also been shown to increase NO release from rat skeletal muscle *in vitro* (Tidball *et al.* 1998), and nNOS expression is increased by repeated exposure of muscle to contractile activity or passive stretching (Roberts *et al.* 1999; Tidball *et al.* 1998).

(b) *Superoxide anion is released by skeletal muscle during activity*

Studies with isolated strips of diaphragm (Reid *et al.* 1992) and *in vivo* microdialysis studies (McArdle *et al.* 2001) indicate that increased amounts of superoxide anion are released into the muscle extracellular fluid during contractile activity. Complementary studies of myotubes in culture indicated that a major source of this superoxide is skeletal muscle cells (McArdle *et al.* 2001). Although standard texts cite mitochondria as the major site of superoxide generation (Halliwell & Gutteridge 1989), a number of observations suggest that this extracellular superoxide does not originate from mitochondrial generation. Superoxide is charged and reactive and does not readily cross membranes and although myotubes in culture contain few mitochondria, these cells release relatively large amounts of superoxide during contractile activity (McArdle *et al.* 2001). *In vivo* microdialysis studies with knockout mice also support this. Mice with reduced mitochondrial MnSOD activity (*Sod2*$^{+/-}$) have evidence of increased mitochondrial superoxide (Van Remmen *et al.* 1999), but baseline levels of extracellular superoxide and the increase during contractile activity were unchanged in the *Sod2*$^{+/-}$ compared with wild type mice (McArdle *et al.* 2004*c*). Potential alternative sources for extracellular superoxide release include plasma membrane oxidoreductases and our inhibitor studies indicate that superoxide release from stimulated myotubes was reduced by treatment with diphenylene iodonium, a non-specific inhibitor of NAD(P)H oxidases and other flavoproteins (Pattwell *et al.* 2004). NAD(P)H oxidase is expressed in diaphragm muscle (Javesghani *et al.* 2002) and this, and other, muscle plasma membrane oxidoreductases may contribute to extracellular superoxide (de Grey 2000; Morre *et al.* 2000).

Chronic contractile activity also appears to influence the ability of muscle to detoxify superoxide with increases in skeletal muscle total SOD (McArdle *et al.* 2001), MnSOD (Ji 1993) and extracellular SOD (Fukai *et al.* 2000) activities reported following single or repeated periods of contractile activity. Our preliminary microdialysis studies also indicate that mouse skeletal muscle releases hydrogen peroxide into the interstitial fluid during contractile activity (data not shown in detail). Although a portion of this hydrogen peroxide is likely to derive from the superoxide released into the interstitial fluid, our data obtained from animals with reduced muscle MnSOD activity (*Sod2*$^{+/-}$) indicate that the majority of the increase in extracellular hydrogen peroxide during contractile activity may originate in the muscle mitochondria and diffuse across intracellular and plasma membranes (McArdle *et al.* 2004*c*).

As a result of these and other data we have proposed the scheme for skeletal muscle ROS generation shown in figure 1. Current data indicate that the plasma membrane superoxide and NO generating systems can be activated by either mechanical distention or contractile activity. In contrast, a relatively large rise in hydrogen peroxide release is only seen following an

increase in muscle mitochondrial activity such as occurs with aerobic contractile activity.

3. FUNCTIONS OF ROS IN SKELETAL MUSCLE

Much previous work has examined the possibility that ROS are mediators of contraction-induced damage to skeletal muscle (see Reznick *et al.* 1998 for reviews). It is clear that ROS can exert such effects in situations where antioxidant defences are compromised (e.g. Jackson *et al.* 1983) or where ROS production is grossly excessive (e.g. McArdle *et al.* 1999), but evidence that ROS have these effects under physiological conditions *in vivo* is sparse. In contrast, it has recently become clear that ROS interact with multiple cell signalling and regulatory pathways to modulate changes in gene expression (see Dröge 2001; Haddad 2002; Jackson *et al.* 2002 for reviews).

(a) *Roles of NO in skeletal muscle*

NO within, and released from, skeletal muscle cells has a number of general functions (Grozdanovic 2001). The functions of many cellular proteins are modified by NO, including soluble guanyl cyclase and mitochondrial respiratory chain complexes. In skeletal muscle, NO has been implicated in the regulation of contractility, mitochondrial oxygen metabolism, glucose homeostasis and blood flow (Stamler & Meissner 2001). Of particular interest is the role of NO in signalling adaptive responses to contractile activity or stretch. Tidball and co-workers demonstrated that NOS activity mediated sarcomere addition during remobilization of muscle (Koh & Tidball 1999) and that NOS activity mediated increased expression of the structural proteins talin and vinculin during cyclic stretching of skeletal muscle (Tidball *et al.* 1999). In addition both NO and peroxynitrite have effects on redox sensitive regulating elements in signalling changes in gene expression and can induce changes in gene expression in many systems (see Dröge 2001; Jackson *et al.* 2002 for reviews).

(b) *Roles of superoxide/hydrogen peroxide in skeletal muscle*

There is relatively little information on the roles of superoxide in regulation of skeletal muscle, but increasing evidence that the dismutation product, hydrogen peroxide, can play important roles. These species are reported to modulate skeletal muscle force generation and fatigue (see Reid 2001 for a review) although these are not universal findings. Additionally hydrogen peroxide may exert effects on vasodilation (Miura *et al.* 2003). There has been considerable interest in the potential roles of superoxide/hydrogen peroxide in signalling adaptive responses in many cell types (see Jackson *et al.* 2002 for a review) and some changes in cell signalling following contractile activity in skeletal muscle are reported to be mediated by ROS (Wretman *et al.* 2001). We have obtained a number of pieces of evidence that contraction-induced ROS modulate at least some of the adaptive and stress responses that occur in skeletal muscle following contractile activity. A single period of contractile activity in mouse muscle was found to increase muscle SOD and catalase activity together with HSP60 and HSP70 content (McArdle *et al.* 2001), changes which were replicated in human muscle (Khassaf *et al.* 2001). Pre-supplementation with vitamin C reduced these responses, supporting the possibility that they are regulated by ROS (Khassaf *et al.* 2003).

The possibility that ROS can directly modulate stress responses to contraction in skeletal muscle has been examined by using *Clontech* cDNA expression arrays to determine whether hydrogen peroxide treatment could induce changes in stress gene expression in skeletal muscle in culture and comparison of these changes with those seen in skeletal muscle *in vivo* at the same time points following a period of non-damaging contractile activity known to induce a substantial generation of oxidants (McArdle *et al.* 2001). Treatment of myotubes with hydrogen peroxide induced a significant change in the expression of 19 of the 140 genes on the array at 4 h post-exposure. These mainly showed a reduction in expression. Following contractile activity in skeletal muscle, 23 mRNA species showed a significant change in expression at 4 h. A comparison of the pattern of changes in the mRNA from the two models identified six genes showing the same pattern with both stresses (McArdle *et al.* 2004*a*). It can be argued that changes in the expression of these six genes may represent part of an oxidant-mediated response to contractile activity. These are likely to be only a small portion of the genes whose expression is modulated by ROS because of the limited number of genes on these arrays. The six genes identified all respond to hydrogen peroxide in muscle cells and are modulated in a similar manner by contractile activity, but only one of these mRNAs showed upregulation following contractile activity: haem oxygenase-1. The validity of these array data is supported by data showing that haem oxygenase-1 protein is increased in skeletal muscle following contractile activity (Pilegaard *et al.* 2000) and in myotubes following hydrogen peroxide treatment (McArdle *et al.* 2004*a*).

One mechanism by which cells respond to ROS is through activation of certain transcription factors (Jackson *et al.* 2002), and supportive evidence for a role of superoxide in mediating adaptive responses to contractions also comes from recent studies of transcription factor activation. Data indicate that NFκB binding to DNA was increased following contractile activity (Ji *et al.* 2004).

Overall, therefore, it now appears that both NO and superoxide play roles in muscle regulation and adaptations to activity although the overall extent and nature of these roles remains to be fully evaluated.

4. INTERACTIONS OF NO AND SUPEROXIDE

NO reacts with superoxide to generate peroxynitrite ($ONOO^-$), a reaction that is three times more efficient than SOD in scavenging superoxide. Hence, peroxynitrite formation is preferred, where both radical species are present (Halliwell & Gutteridge 1989). The presence of one of these species can, therefore, affect the 'bioavailability' of the other: NO can reduce superoxide toxicity and conversely superoxide can decrease NO availability and inhibit effects such as

vasodilation. Such effects have been studied in endothelial cells (Cosentino & Luscher 1999; Hwang *et al.* 2003), but not in skeletal muscle. There is also evidence for a co-ordinated regulation of pathways influencing superoxide and NO levels. Exercise increases muscle NO and superoxide generation and leads to increases in muscle nNOS and intracellular and extracellular SOD activities (Roberts *et al.* 1999; McArdle *et al.* 2001; Fukai *et al.* 2000). NO or superoxide also appear to influence the regulatory pathways for the other compound: an increase in hydrogen peroxide content is reported to stimulate NOS expression in endothelial cells (Drummond *et al.* 2000), while elevated levels of NO decrease extracellular SOD activity in smooth muscle cells (Strålin *et al.* 2003). We tested the hypothesis that NO and superoxide react in the muscle extracellular space following increased release from contracting myotubes by measuring superoxide release from wild type cells when NOS was inhibited by L-NAME treatment. NOS inhibition increased the superoxide anions detected (Pattwell *et al.* 2004), demonstrating indirectly that peroxynitrite was formed, where both species were present. We have also examined the possibility that peroxynitrite is formed following contractile activity in myotubes by western blotting analysis of nitrotyrosine residues in muscle proteins. Nitrotyrosines were increased following a single period of contractile activity (data not shown in detail). We conclude that peroxynitrite may be formed from NO and superoxide generated by muscle cells, reducing the effective bioavailability of both. This appears to provide a system whereby relatively small changes in generation of one of the primary ROS (i.e. NO or superoxide) leads to sensitive changes in several other substances that potentially influence cellular responses. Thus, for example, a decrease in NO generation will lead to a decrease in peroxynitrite formation and an increase in superoxide and hydrogen peroxide content. In other cell types an imbalance of NO and superoxide has been recognized to cause functional changes (Cosentino *et al.* 2001), but this possibility does not appear to have been examined in skeletal muscle.

5. NOS ENZYMES ARE A POTENTIAL SOURCE OF BOTH NO AND SUPEROXIDE

NOS enzymes contain both reductase and oxygenase domains. Formation of NO involves an electron transfer from NADPH bound at the reductase domain to the haem centre of the oxygenase domain. Calmodulin binding appears to facilitate this transfer. NOS enzymes have tetrahydrobiopterin as a cofactor that mediates coupling of oxygen reduction to haem catalysed L-arginine oxidation to form NO and citrulline. In recent years in has become apparent that when NOS enzymes lack substrate (arginine), cofactor (tetrahydrobiopterin) or specific protein–protein interactions (Xia *et al.* 1996; Cosentino *et al.* 1998; Bender *et al.* 1999; Pou *et al.* 1999; Song *et al.* 2002; Ou *et al.* 2003), the oxygen reduction and arginine oxidation become 'uncoupled' leading to generation of superoxide by the enzymes. In endothelial cells this uncoupling leads to superoxide-mediated endothelial dysfunction (Cosentino & Lüscher 1999; Cosentino *et al.* 2001). The potential for uncoupling appears to apply to all three NOS isoforms but, again, this does not appear to have been examined in skeletal muscle.

6. SUPEROXIDE/NITRIC OXIDE INTERACTIONS IN MUSCLE DEGENERATION

Aberrant production or regulation of NO and/or superoxide have been claimed to play a role in some degenerative disorders of muscle (see Reznick *et al.* 1998 for a review). Of potential importance, but poorly understood, are roles in the muscular dystrophies and in muscle dysfunction associated with ageing.

(a) *Muscular dystrophies*

The muscular dystrophies are a group of inherited disorders of muscle characterized by significant muscle degeneration and weakness (Partridge 1993). Dystrophin, the defective protein in the most common form, Duchenne muscular dystrophy (DMD), is a component of a complex set of proteins and glycoproteins associated with the cell membrane, several of which may cause other forms of muscular dystrophy if defective or absent (Durbeej & Campbell 2002). The functions of many components of the DGC complex are unclear. The skeletal muscle isoform of nNOS, termed nNOSμ (or muNOS), is associated with the complex (Brenman *et al.* 1995). Loss of dystrophin protein (as occurs in DMD patients or in the *mdx* mouse model) leads to complete loss of the DGC and nNOS from the sarcolemma (Durbeej & Campbell 2002). nNOS is also lost from the sarcolemma in mice deficient in other components of the DGC (Crosbie *et al.* 2002). Loss of nNOS from the sarcolemma leads to redistribution of nNOS to the cytosol. Estimates of the residual nNOS present in the cytosol in dystrophin-deficient muscle vary greatly from an overall decrease to a 75% increase (Brennan *et al.* 1995; Chang *et al.* 1996). There is evidence that reversal of this loss of nNOS by overexpression of the protein in *mdx* mice partially ameliorates the degeneration in this model (Wehling *et al.* 2001), providing strong support that changes in redox regulation may be important in this disorder.

(b) *ROS generation in muscle of ageing mice*

There is evidence that abnormalities in muscle NO/superoxide interactions play a role in ageing-related muscle dysfunction. Loss of muscle strength and muscle wasting are characteristic of ageing and are caused by both a loss of muscle fibres and atrophy of the remaining fibres (Porter *et al.* 1995). All tissues of aged organisms contain products of oxidative damage to biomolecules such as phospholipid, DNA and proteins (Nohl 1993; Schoneich 1999). Aged mitochondria contain significant amounts of oxidative damage associated with a marked increase in the number of rearrangements of mitochondrial DNA (Melov *et al.* 1995). Sohal and co-workers (Sohal *et al.* 1994; Lass *et al.* 1998) suggest that these changes are due to an increase in mitochondrial superoxide and hydrogen peroxide production with increasing age although there is little direct evidence to support this

hypothesis. Lass *et al.* (1998) examined the superoxide generation from sub-mitochondrial particles from skeletal muscle and reported an increased release in aged animals while Bejma & Ji (1999) reported that homogenates of skeletal muscle from old animals induced greater oxidation of 2′, 7′-dichlorodihydrofluorescein (DCFH). Unfortunately, there may be alternative interpretations for these data, since the techniques used in both studies involved disruption of normal cellular structure. The hypothesis that an increased generation of oxidants *in vivo* plays a role in age-related tissue dysfunction is supported by studies in non-mammalian systems, where overexpression of CuZnSOD and catalase caused an extension of lifespan in *Drosophila melanogaster* (Orr & Sohal 1994) and by the description of extended lifespan in *Caenorhabditis elegans* treated with a MnSOD and catalase mimetic (Melov *et al.* 2000).

The apparent inconsistency between the large increase in ROS generation during exercise and the lack of a deleterious effect of muscle activity on muscle ageing appears to be due to the ability of skeletal muscle to adapt to the ROS generated during exercise by increasing the expression of protective proteins (McArdle & Jackson 2000). An increase in these proteins helps protect the tissue against subsequent exposure to exercise-induced increases in ROS generation (McArdle *et al.* 2004*a*). This ability to respond to oxidative stress does not appear to be maintained in ageing animals and humans (Rao *et al.* 1999; Vasilaki *et al.* 2002), but surprisingly is associated with an increase in the resting SOD, catalase, glutathione peroxidase (GPx) and glutathione reductase activities in the muscle of aged compared with adult mice (Leeuwenburgh *et al.* 1994). Muscle nNOS activities have been reported to be either elevated (Capanni *et al.* 1998) or decreased (Richmonds *et al.* 1999) in aged animals. Whether these changes are associated with any aberrant NOS activity in old muscle is also unclear although there is some evidence for a relocation of nNOS to the muscle cytosol in aged animals (Cappani *et al.* 1998).

The net effect of these changes in ROS activity in aged muscle is unclear as is the potential for any amelioration of age-related muscle dysfunction by reduction or modification of ROS levels. Despite this, our recent data indicate that transgenic approaches to express high levels of a protein that protects against oxidative damage (HSP70) can improve muscle function in old mice (McArdle *et al.* 2004*b*).

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