

SYMPOSIUM REVIEW

Age-related changes in skeletal muscle reactive oxygen species generation and adaptive responses to reactive oxygen species

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Abstract Skeletal muscle generates superoxide and nitric oxide at rest and this generation is increased by contractile activity. In young and adult animals and man, an increase in activities of these species and the secondary products derived from them (reactive oxygen species, ROS) stimulate redox-sensitive signalling pathways to modify the cellular content of cytoprotective regulatory proteins such as the superoxide dismutases, catalase and heat shock proteins that prevent oxidative damage to tissues. The mechanisms underlying these adaptive responses to contraction include activation of redox-sensitive transcription factors such as nuclear factor κ B (NF κ B), activator protein-1 (AP1) and heat shock factor 1 (HSF1). During ageing all tissues, including skeletal muscle, demonstrate an accumulation of oxidative damage that may contribute to loss of tissue homeostasis. The causes of this increased oxidative damage are uncertain, but substantial data now indicate that the ability of skeletal muscle from aged organisms to respond to an increase in ROS generation by increased expression of cytoprotective proteins through activation of redox-sensitive transcription factors is severely attenuated. This age-related lack of physiological adaptations to the ROS induced by contractile activity appears to contribute to a loss of ROS homeostasis and increased oxidative damage in skeletal muscle.

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Abbreviations AP, activator protein; EAA, essential amino acid; FDB, flexor digitorum brevis; HSF, heat shock factor; NF κ B, nuclear factor κ B; ROS, reactive oxygen species; TF, transcription factor.

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The generation and potential roles of free radicals, reactive oxygen species (ROS) and reactive nitrogen species in skeletal muscle has been studied since the late 1970s (Dillard *et al.* 1978; Davies *et al.* 1982), but despite extensive investigation there remains substantial controversy about the nature and sources of the specific species that are generated, the factors influencing their generation and their potential functions and effects in muscle and other cells. In the majority of studies to date there has been an assumption that these species are deleterious to cells and tissues, and even the earliest studies report attempts to scavenge the ROS generated and look for potential functional benefits of this intervention (Dillard *et al.* 1978). In part, this emphasis on the potential damaging effects of free radicals and ROS has derived from the techniques generally available to detect these species which have predominantly relied on monitoring markers of the oxidative modifications to lipids, DNA and proteins that are collectively described as oxidative damage (Halliwell & Gutteridge, 1989).

There is now increasing recognition that ROS also mediate many physiological processes. ROS are important signalling molecules with regulatory functions that modulate changes in cell and tissue homeostasis and gene expression (Droge, 2002; Jackson *et al.* 2002). Signalling by these reactive molecules is mainly achieved by targeted modifications of specific residues in proteins (Janssen-Heininger *et al.* 2008). The main physiological mechanism by which cells regulate ROS activities (and hence protect against oxidative damage) is modification of the expression and activities of the regulatory enzymes such as superoxide dismutases MnSOD and CuZnSOD, catalase, glutathione peroxidases (GPx), haem oxygenase-1 (HO-1) and by increases in other cytoprotective proteins, such as heat shock proteins (HSPs). Changes in the expression of these proteins are mediated by activation of signalling pathways by ROS (Powers & Jackson, 2008).

Nature of the reactive oxygen and nitrogen species that are generated by skeletal muscle

Skeletal muscle fibres respond to contractile activity by an increase in the intracellular generation of superoxide and nitric oxide (NO) with the formation of secondary ROS and reactive nitrogen species (Pye *et al.* 2007; Palomero *et al.* 2008; Powers & Jackson, 2008). In addition to the intracellular generation of ROS and NO by skeletal muscle, superoxide (Reid *et al.* 1992; McArdle *et al.* 2001), hydrogen peroxide (Vasilaki *et al.* 2006b) and NO (Balon & Nadler, 1994; Kobzik *et al.* 1994) are released into the interstitial space from muscle fibres (or generated on the extracellular side of the muscle plasma membrane). These species are increased in the interstitial space by contra-

ctile activity, but there are few data on the function(s) of these extracellular ROS derived from skeletal muscle fibres. The sources of the free radicals and ROS generated by muscle during contractions have been studied since the 1980s (Davies *et al.* 1982). Most authors have assumed that the ROS generated by contractions are predominantly generated by mitochondria, but recent data argue against this possibility (for discussion see Powers & Jackson, 2008). Non-mitochondrial sources for the generation of ROS within skeletal muscle have not been extensively studied, but NAD(P)H oxidase(s) have been described in skeletal muscle and localised to the plasma membrane (Javesghani *et al.* 2002), sarcoplasmic reticulum (Xia *et al.* 2003) and T-tubules (Espinosa *et al.* 2006). The activity of this enzyme has also been reported to be activated by contractions (Espinosa *et al.* 2006). These and other potential sources for ROS generation in skeletal muscle have recently been reviewed by Powers & Jackson (2008).

In order to evaluate the relative magnitude of the increase in ROS activity that occurs in skeletal muscle fibres in response to contractions, Palomero *et al.* (2008) applied a protocol of electrically stimulated, isometric contractions to single isolated fibres from the mouse flexor digitorum brevis (FDB) muscle. This contraction protocol has been extensively utilised by our research group and has been shown (i) to induce release of superoxide and nitric oxide from muscle cells in culture and muscles of mice *in vivo* (McArdle *et al.* 2001), (ii) to lead to a fall in muscle glutathione and protein thiol content (Vasilaki *et al.* 2006a) and (iii) to stimulate redox-regulated adaptive responses (Vasilaki *et al.* 2006b) when applied to intact muscles *in vivo*. The increase in intracellular 2',7'-dichlorofluorescein (DCF) fluorescence induced by the contraction protocol was less than that following exposure of the fibres to 1 μM hydrogen peroxide. Palomero *et al.* (2008) calculated that the likely change in intracellular hydrogen peroxide following addition of 1 μM to the extracellular medium was $\sim 0.1 \mu\text{M}$. Thus, it can be inferred that the absolute increase in cytosolic ROS activity in muscle fibres that was achieved following contractile activity was potentially equivalent to $\sim 0.1 \mu\text{M}$ hydrogen peroxide. Transient changes of this magnitude in hydrogen peroxide have generally been associated with a signalling role for the oxidant rather than with oxidative damage to tissues.

Adaptive responses of skeletal muscle to increased ROS activities

We and others have obtained evidence that changes in ROS activities modulate a number of physiological responses in skeletal muscle. A single period of contractile activity in mouse muscle was found to increase the activity of muscle antioxidant defense enzymes such as superoxide dismutase (SOD) and catalase together with HSP60 and

HSP70 content (McArdle *et al.* 2001), changes that were replicated in human muscle studies (Khassaf *et al.* 2001). We also characterized the changes in gene expression that occur following an acute period of contractile activity in comparison with those induced by exposure of skeletal muscle cells to hydrogen peroxide and this identified a number of changes in gene expression that may be regulated directly by the hydrogen peroxide produced during contractile activity *in vivo* (McArdle *et al.* 2004b). In addition, other studies have implicated redox signalling in diverse processes in muscle such as maintenance of force production during contractions, glucose uptake and insulin signalling (Jackson, 2009).

ROS have become increasingly recognised as mediators of some adaptive responses of skeletal muscle to contractile activity through the activation of redox-sensitive transcription factors (TFs) (Jackson *et al.* 2002; Ji *et al.* 2004; Ristow *et al.* 2009). NF κ B is one such factor, along with activator protein-1 (AP-1) and heat shock factor 1 (Cotto & Morimoto, 1999). NF κ B is a redox-regulated factor and ROS have been proposed to be principal regulators of NF κ B activation in many situations (Moran *et al.* 2001). NF κ B family members expressed in skeletal muscle play critical roles in modulating the specificity of NF κ B and NF κ B modulates expression of a number of genes associated with myogenesis (Bakkar *et al.* 2008), catabolism-related genes (Van Gammeren *et al.* 2009) and cyto-protective proteins during adaptation to contractile activity (Vasilaki *et al.* 2006a). Moreover, skeletal muscle has been identified as an endocrine organ producing cytokines via NF κ B activation following a number of stresses including systemic inflammation or physical strain (Lee *et al.* 2007).

Reactive oxygen species play a role in the fundamental processes of ageing

An increased activity of reactive oxygen species (ROS) has been implicated in the processes underlying ageing and in all species, tissues (including skeletal muscle) of aged organisms contain increased amounts of oxidative damage to lipids, DNA and proteins (Drew *et al.* 2003; Vasilaki *et al.* 2006b). The hypothesis that this increased oxidative damage plays a key role in age-related tissue dysfunction has been examined in a number of transgenic studies that have examined whether life span is altered, which is thought to be the gold standard for assessing an effect of manipulations on ageing (Salmon *et al.* 2010). In non-mammalian models, some interventions designed to reduce the activities of ROS, such as overexpression of CuZn, superoxide dismutase (CuZnSOD), catalase or both in *Drosophila* (Orr *et al.* 2003) or treatment with a MnSOD and catalase mimetic in *C. elegans* (Melov *et al.* 2000) extended lifespan and thus support the hypothesis,

but these effects are not universally observed and are controversial (Gems and Doonan 2009). In mammals, only few genetic manipulations designed to reduce ROS activities have resulted in increased lifespan (Schriner *et al.* 2005; Yoshida *et al.* 2005). Salmon *et al.* (2010) conclude that oxidative stress is likely to play a limited role in ageing but that reduced oxidative stress retards pathology.

Much of the recent interest in ROS and ageing has concerned the potential role of mitochondria as a source and target for ROS (for recent reviews see Jang & van Remmen, 2009; Salmon *et al.* 2010). Miquel and co-workers (Miquel *et al.* 1980) originally suggested that accumulation of somatic mutations in mitochondrial DNA due to oxidative stress is the major contributor to ageing. Their theory indicates that ROS generated from the mitochondrial respiratory chain damages macromolecules, particularly mitochondrial DNA. This is thought to lead to defective mitochondrial respiration and a further increase in ROS generation (Jang & Van Remmen, 2009). There is considerable evidence that mitochondria isolated from tissues of aged individuals from different species do produce relatively increased amounts of ROS and that this occurs in association with impaired mitochondrial function and oxidative damage to mitochondrial components with ageing (e.g. see Vasilaki *et al.* 2006b). However, recent studies with transgenic and knockout mouse models have produced inconsistent results (Jang & van Remmen, 2009) and generally do not support the theory, although transgenic mice with catalase targeted to mitochondria have increased lifespan compared with control non-transgenic mice (Schriner *et al.* 2005). In summary therefore, the role of ROS, and particularly of mitochondria-derived ROS, in fundamental mechanisms of ageing remains controversial, but ROS generation and metabolism are clearly disrupted with increasing age. There is evidence that these changes in ROS contribute to the loss of muscle mass and function that occur with increasing age, but whether dysregulation of ROS is the prime cause of ageing, or a consequence of it, remains an open question.

Failure of oxidative signalling in muscle during ageing

The ability of cells and tissues from old mammals to respond to a variety of stresses by an increased content of HSPs and an increase in the activity of antioxidant defence enzymes is severely attenuated. The increase in HSP content and antioxidant defence enzyme activity that is evident in muscles of adult rodents following an acute period of isometric contractions was abolished in muscles of old rodents (Vasilaki *et al.* 2002, 2006) and this inability to adapt was shown to be due to the lack of complete activation of the appropriate transcription factors (Vasilaki *et al.* 2006a).

Further studies from our laboratory have demonstrated that this age-related inability to produce HSPs plays a critical role in the development of functional deficits that occur with ageing in skeletal muscle. Studies using transgenic mice overexpressing HSP70 in skeletal muscle demonstrated that increased muscle content of this protein provided protection against the fall in specific force associated with ageing, and facilitated rapid and successful regeneration following contraction-induced damage in muscles of old mice compared with the impaired regeneration and recovery normally observed in old non-transgenic mice (McArdle *et al.* 2004a). This protection was associated with maintenance of the ability of muscles of old HSP70 overexpressor mice to activate NF κ B following contractions (Broome *et al.* 2006). In recent studies, overexpression of a mitochondrial chaperone protein, HSP10, was also shown to preserve muscle function during ageing in mice (Kayani *et al.* 2010).

Activation of redox-responsive transcription factors is aberrant in muscles of old humans and mice and these muscles demonstrate both chronic constitutive activation of redox-sensitive TFs (Cuthbertson *et al.* 2005; Vasilaki *et al.* 2006a) and an inability to further activate these TFs following an acute non-damaging contraction protocol (Vasilaki *et al.* 2006a). The chronic activation of TFs such as NF κ B in muscles of old mice is associated with chronic increases in the expression of a number of genes. For example, increased content and activities of antioxidant defence enzymes, such as the superoxide dismutases and catalase (Broome *et al.* 2006), increased content of HSPs (Vasilaki *et al.* 2006; Kayani *et al.* 2008) and increased production of pro- and anti-inflammatory cytokines and chemokines by muscle cells (Febbraio & Pedersen, 2005). The inability to further activate NF κ B in response to an acute contraction protocol is associated with severe attenuation of normal changes in expression of cytoprotective genes (Demirel *et al.* 2003; Vasilaki *et al.* 2006a).

A diminished ability to respond to the stress of contractions has been reported to play an important role in other age-related defects in muscle function and adaptation. Ljubicic & Hood (2008) have reported a severe attenuation of the signalling pathways involved in mitochondrial biogenesis in fast muscle fibres of old rats following contractions compared with that seen in fibres from young rats. ROS are known to play an important role in the activation of signalling cascades (Irrcher *et al.* 2009). These authors suggest that ROS affect mitochondrial biogenesis via the up-regulation of transcriptional regulators such as peroxisome proliferator-activated receptor-gamma coactivator-1 protein-alpha (PGC-1 α), suggesting that an aberrant activation of ROS generation following contractions may be responsible for the diminished mitochondrial biogenesis in muscles of old

rats. This blunted or absent adaptation to stress in muscle of old humans and mice is not limited to the exercise response. Skeletal muscle of healthy elderly humans demonstrates a reduction in anabolic sensitivity and responsiveness of muscle protein synthesis pathways. Cuthbertson *et al.* (2005) demonstrated a reduction in the phosphorylation of mTOR and downstream translational regulators in response to essential amino acid (EAA) ingestion when compared with the young despite greater plasma EAA availability in elderly subjects. The authors concluded that the nutrient signal was not sensed or transduced as well by muscle in the elderly compared with muscle in the young, resulting in a lower protein synthesis response to the same nutrient stimulus.

Nature of the defect in redox signalling that occurs in skeletal muscle during ageing

The inability of skeletal muscle from old mice to activate redox-sensitive transcription factors such as NF κ B or AP-1 in response to stress is characterised by chronic activation of transcription factors at rest and an inability to further activate these factors following an acute non-damaging muscle contraction protocol (Vasilaki *et al.* 2006a). This lack of activation of redox-sensitive transcription factors with contractile activity is associated with an inability to increase the expression of various cytoprotective proteins (Vasilaki *et al.* 2006) and influences the susceptibility of skeletal muscle from old animals to oxidative damage. The lack of activation of HSF can be overcome with the use of HSF1 activating drugs, suggesting no inherent defect in this process but supporting the theory of a failed signal for activation (Kayani *et al.* 2008). ROS play an important role in the activation of several signalling cascades (Irrcher *et al.* 2009) and in skeletal muscle, the increase in intracellular ROS is a key activator of NF κ B and AP-1 in response to contractions, either through oxidation of upstream regulators or by oxidation of regulatory proteins for these transcription factors, such as thioredoxin 1 (Jackson, 2009). There are many potential sites at which the age-related defect in ROS-stimulated adaptive responses might occur (see Jackson, 2009 for a review), but paradoxically there is also evidence which indicates that attenuated transcriptional responses to contractions seen in aged mice are related to a lack of additional intracellular ROS generation in response to contractile activity. The contraction-induced increase in extracellular ROS derived from muscle is attenuated during ageing and the contraction-induced increase in extracellular superoxide seen in young mice is abolished in aged mice (Vasilaki *et al.* 2006b; Close *et al.* 2007). A consequence of this is that there is no transient increase in oxidation of both muscle and non-muscle tissues in old mice in response to muscle contractions (Vasilaki

et al. 2006b; Close *et al.* 2007) and we speculate this may underlie the inability to further activate redox-regulated transcription factors. A schematic diagram illustrating this age-related defect in redox signalling in skeletal muscle is shown in Fig. 1.

Conclusions

It is clear that the age-related loss of ability to adapt to free radicals and ROS induced in skeletal muscle by contractile activity is an important contributor to the loss of ROS

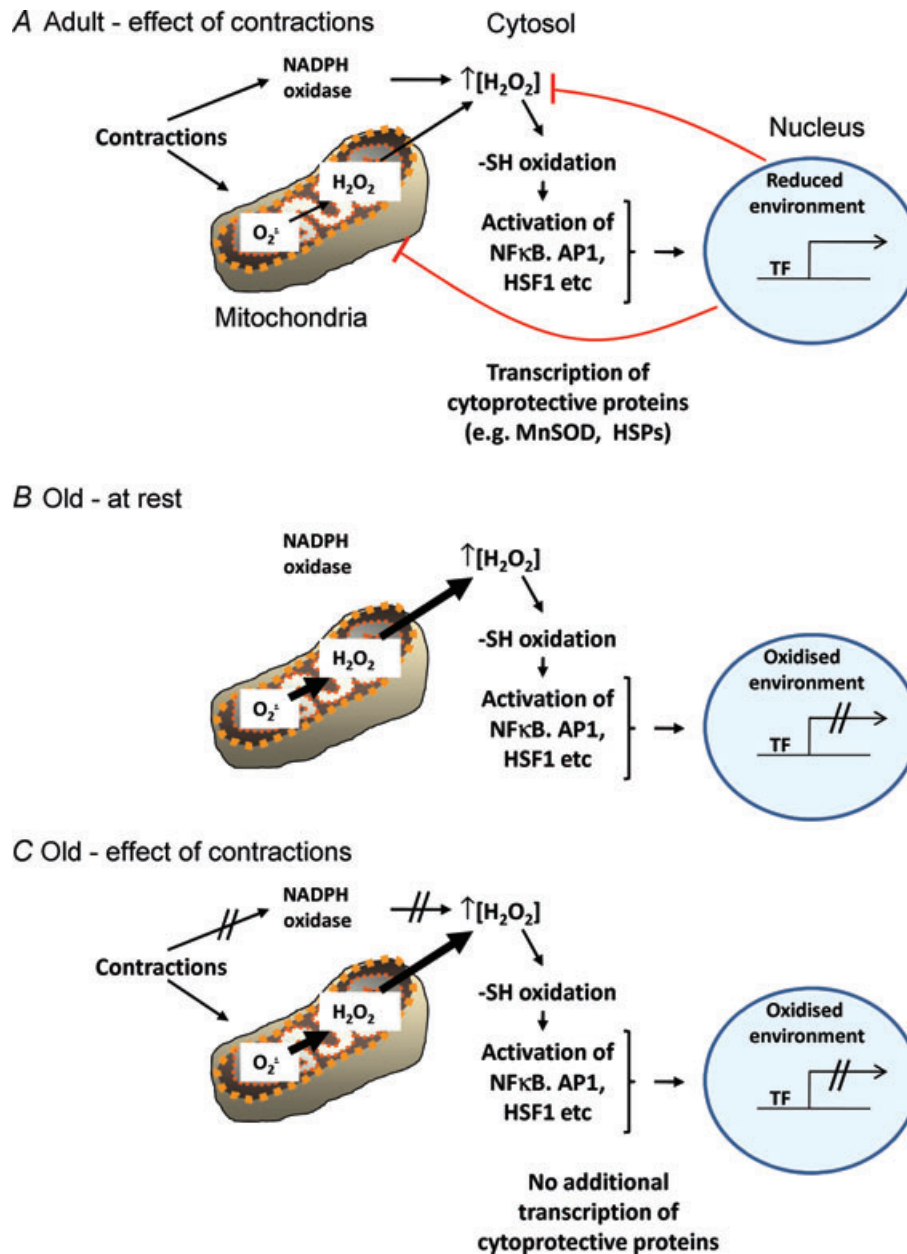


Figure 1. Schematic representation of the processes by which ROS are generated in skeletal muscle in response to contractions and lead to the local oxidation of thiol (-SH) groups to activate redox-sensitive transcription factors including NF κ B, AP1 and HSF1

A, the activated transcription factors migrate to the nucleus and bind to DNA in a locally reduced environment to stimulate transcription of cytoprotective proteins that act to restore ROS homeostasis. *B*, during ageing there is evidence for a chronic increase in release of hydrogen peroxide from mitochondria at rest and this is hypothesised to cause chronic oxidation of the redox-sensitive transcription factors at rest, but an oxidation of the nuclear environment that prevents DNA binding of the transcription factor. *C*, during contractile activity in the elderly there appears to be a failure of the ability to further activate transcription factors and increase the expression for cytoprotective proteins that may occur at several levels including a lack of activation of local ROS generation, a failure of further transcription factor activation and defective nuclear transcription factor binding.

homeostasis and oxidative damage that is seen in tissues from aged animals and man. Experimental manipulation of mice to overexpress specific protein products in muscle in order to by-pass the ageing-related block in adaptive responses has also demonstrated the importance of these responses in maintaining muscle function. Elucidation of the mechanisms by which ageing leads to defective adaptations to contractile activity may provide an insight into ways of reversing these defects in ageing humans and current data indicate this defect lies in a lack of signal for further activation of redox-sensitive transcription factors.

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