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**Reactive oxygen species in sarcopenia: should we focus on excess oxidative damage or defective redox signalling?**

**Malcolm J Jackson**

MRC-Arthritis Research UK Centre for Integrated research into Musculoskeletal Ageing (CIMA), Department of Musculoskeletal Biology

Institute of Ageing and Chronic Disease,

University of Liverpool,

Liverpool, L69 3GA. U.K.

Tel: +44(0)1517064072

Fax: +44(0)151706580

email: [mjj@liverpool.ac.uk](mailto:mjj@liverpool.ac.uk)

**Abstract**

Physical frailty in the elderly is driven by loss of muscle mass and function and hence preventing this is key to reduction in age-related physical frailty. Our current understanding of the key areas in which ROS contribute to age-related deficits in muscle is through increased oxidative damage to cell constituents and/or through induction of defective redox signalling. Recent data have argued against a primary role for ROS as a regulator of longevity, but studies have persistently indicated that aspects of the aging phenotype and age-related disorders may be mediated by ROS. There is increasing interest in the effects of defective redox signalling in aging and some studies now indicate that this process may be important in reducing the integrity of the aging neuromuscular system. Understanding how redox-signalling pathways are altered by aging and the causes of the defective redox homeostasis seen in aging muscle provides opportunities to identify targeted interventions with the potential to slow or prevent age-related neuromuscular decline with a consequent improvement in quality of life for older people.

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1. **References**

1. **Introduction**

Researchers have been interested in a potential role for free radicals or reactive oxygen species (ROS) in the process of aging for over 60 years (Harman, 1956) and in the potential physiological roles of reactive oxygen species in muscle for 35 years (Davies et al, 1982) and hence there is a great deal of background research in this area. Increased levels of oxidation products from lipids, DNA and proteins are general found in tissues from aged humans and animals but definitive transgenic approaches to determine whether such changes play an aetiological role in aging, or occur as a result of the aging process have not been available until relatively recently. Studies using these new approaches have argued against a primary role for ROS as a regulator of longevity, but some data persistently indicate that aspects of the aging phenotype and age-related disorders may be mediated by ROS (Muller et al, 2007b; Salmon et al, 2010). However despite the doubts over a primary role for ROS in aging, articles in the popular press and commercial product advertisements promote nutritional or nutraceutical antioxidants in anti-aging approaches to reduce the oxidative damage associated with aging and appear to ignore more recent studies of the beneficial physiological roles of ROS. In this review the roles of ROS in normal muscle physiological responses will be considered alongside the potential effects of increased oxidative damage and disruption of normal redox signalling that occurs in aging. The aim is to provide an overview of whether future research in this area should focus on excess oxidative damage or defective redox signalling as mediators of the reduced muscle mass and function that occurs during aging.

1. **Physiological redox signalling in skeletal muscle**

The increase in reactive oxygen and nitrogen species in contracting skeletal muscle was first described in the 1980’s (Davies et al, 1982; Jackson et al, 1985) and involves the generation superoxide and nitric oxide (NO) with the formation of secondary reactive oxygen species (ROS) and reactive nitrogen species (Powers & Jackson, 2008). Nitric oxide synthases generate NO, but the sites that generate superoxide during exercise have remained controversial. The mitochondrial electron transport chain was initially thought to be the predominant source of superoxide but a number of studies identified NADPH oxidase enzymes in the plasma membrane, T-tubules and mitochondria (Sakellariou et al, 2014). Recent studies that directly compared superoxide generation from mitochondrial and cytosolic sources in contracting skeletal muscle (Pearson et al, 2014; Sakellariou et al, 2013) indicate that NAD(P)H oxidases are the major source during a short period of contractions (Sakellariou et al, 2013; Pearson et al, 2014). Since the only function of NAD(P)H oxidases is to generate superoxide (or hydrogen peroxide) these data indicate that these species are not produced by chance, or as a by-product of metabolism during muscle contractions.

The ROS and NO generated during contractile activity in muscle appear to mediate the activation of a number of redox-regulated transcription factors, including NF-κB, AP-1, HSF-1 and nrf2 (Ji et al, 2004; Ristow et al, 2009; Vasilaki et al, 2006a) leading to an increased expression of regulatory enzymes and cytoprotective proteins (McArdle et al, 2001). The full extent to which redox-dependent systems regulate adaptations to contractions in muscle is still unclear, but appears to also include some catabolic processes and mitochondrial biogenesis (Powers and Jackson, 2008).

The precise mechanism by which ROS generated during contractions activate relevant signalling processes also remains unclear. Hydrogen peroxide (H2O2) is widely viewed as the only ROS likely to play a major role in signalling and H2O2 has been shown to activate NF-κB (Zhang et al, 2001), AP-1 (Aggeli et al, 2006) and other transcription factors (Marinho et al, 2014). Thus the concept has arisen that H2O2 interacts with activation pathways for these specific transcription factors leading to their activation. The in vitro studies that apparently demonstrate this possibility have utilised H2O2 concentrations typically in the range 10-4-10-3M and it is relevant to consider whether these concentrations have any *in vivo* relevance. Helmut Sies (Sies, 2014) has calculated the intracellular H2O2 concentration in cells to be 10-9-10-8M (Sies, 2014) and we have calculated that the increase in muscle during contractions appears to be to a maximum of 10-7M (Jackson, 2011). This latter figure is a factor of ~1000 below the concentrations reported to activate most transcription factors *in vitro*. The generally held concept of H2O2 generated during contractions diffusing through the cell to encounter redox-regulated proteins with which it reacts does not appear sustainable in the light of the calculated intracellular H2O2 concentrations.

An alternative pathway for the process of redox signalling involves the transfer of oxidative equivalents directly from a H2O2-sensitive thiol peroxidase to a specific target protein through direct protein-protein contact allowing conversion of the oxidising equivalent from H2O2 into a disulphide bond that can be subsequently transmitted to other substrates through formation of intermolecular disulphides. Thus thiol peroxidases transmit oxidising equivalents to a specific target protein to facilitate H2O2 signalling (Sobatta et al, 2015). This mechanism has been documented in yeast (Delaunay et al, 2002; Gutscher et al, 2009), but only recently been shown to account for activation of a transcription factor by H2O2 in animal cells (Sobatta et al, 2015). Key components of such signalling pathways are peroxiredoxins (Prx) and thioredoxins (Trx). Prx are a family of antioxidant enzymes which reduce hydroperoxides to water in the presence of electron donors and are generally considered to be important antioxidant enzymes in the cytosol (Prx1, Prx2, Prx5), mitochondria (Prx3, Prx5) and endoplasmic reticulum (Prx4). Importantly and in contrast to the relative poor reactivity of the proteins involved in activating transcription factors discussed previously, Prx are several order of magnitude more reactive with H2O2 (Sobatta et al, 2015) and act to scavenge H2O2 at the low concentrations found in muscle fibers. Studies in non-muscle cellsindicate that Prxs can function as a signal peroxidase to activate specific pathways. Prx1 has been shown to activate the transcription factor ASK1, (Jarvis et al, 2012) and Prx2 forms a redox relay with the transcription factor STAT3 such that oxidative equivalents flow from Prx2 to STAT3 generating disulphide-linked STAT3 oligomers with modified transcriptional activity (Sobatta et al, 2015).

1. **Age-related loss of skeletal muscle mass and function (sarcopenia)**

The term “sarcopenia” was coined over 20 years ago (Evans & Campbell, 1993), and the definition was recently revised as a “progressive age-related loss of muscle mass and associated muscle weakness” (Lynch, 2011). Between the ages of 50 and 80 years a 30-50% loss of muscle mass and decrease in strength occur that are major contributors to physical frailty which has a major negative effect on the quality of life of older people and contributes to loss of independence in older people ([Young & Skelton, 1994](#_ENREF_83)). Analysis of post-mortem human vastus lateralis muscles have shown a 40% reduction in total muscle area accompanied by ~50% loss of muscle fibers between 50 and 80 years of age (Lexell et al., 1988). Old rodents also show reductions in muscle fiber number with aging (Larkin et al., 2011). Despite the importance of this area limited progress has been made in understanding the mechanisms responsible for age-associated muscle atrophy and weakness.

**3.1 *Oxidative damage in aging muscle***

Markers of oxidative damage have been examined in tissues of many species and it is apparent that all tissues, including skeletal muscle, of old organisms contain greater oxidative damage to lipids, DNA and proteins in comparison with those found in younger organisms (e.g. Vasilaki et al, 2006a). There have been numerous attempts to modify the amount of oxidative damage and in non-mammalian models, initial interventions to reduce the ROS activities throughout life were reported to extend lifespan (Orr & Sohal, 2003), but more recent work in mammalian (Perez et al, 2009) and non-mammalian (Gems and Doonan, 2009) models did not shown any true correlation between the level of oxidative damage and lifespan in different models and argues strongly against a primary role for oxidative damage in aging (Gems & Doonan, 2009). In mammals, few genetic manipulations to reduce ROS activities resulted in increased lifespan (e.g. Schriner et al, 2005). It therefore appears clear that levels of ROS generation and oxidative damage are not the fundamental determinants of lifespan. It is now recognised that the free radical theory and its various derivatives cannot exclusively explain the aging process (Romano et al, 2010; Pulliam et al, 2013).

Despite these studies indicating that oxidative damage does not play a primary role in the aging process, data indicate that mitochondrial ROS generation is increased in tissues, including skeletal muscle, during aging and that this is associated with impaired mitochondrial function and oxidative damage (e.g. Vasilaki et al, 2006a). Furthermore authors have argued that this increased ROS generation with age may not determine lifespan directly, but is important in contributing to age-related diseases (Muller et al, 2007) and more generally to individual health span (Salmon et al, 2010).

**3.2 *Dysregulation of muscle redox signalling during aging***

The increased oxidation seen in tissues during aging clearly suggests that redox homeostasis is altered at this stage of the lifecycle. There is therefore increased interest in whether this is associated with defective redox signalling and how important this may be for age-related decline in tissue function. Thus for instance in normal physiology, ROS mediate increased expression of HSPs and other cytoprotective proteins in muscle following contractions in adult mice (Vasilaki et al, 2006b; Jackson & McArdle, 2011) and this response is attenuated in old mice (Vasilaki et al, 2006b). Furthermore this attenuated response appears to contribute to age-related loss of muscle mass and function (Jackson & McArdle, 2011). Thus transgenic mouse studies have demonstrated that aberrant activation of adaptive responses plays a key role in age-related muscle dysfunction since lifelong overexpression of cytosolic HSP70 or mitochondrial HSP10 normalised NF-κB activation at rest and reduced functional deficits in muscle of old mice (Kayani et al, 2010).

We have previously proposed that the aberrant H2O2 generation from mitochondria that occurs during aging could explain this attenuation of adaptive responses leading to a failure to induce important cytoprotective and other responses (Jackson and McArdle 2011). Increased H2O2 generation by mitochondria has been described in some (e.g. Vasilaki et al, 2006), but not all studies (Ghosh et al, 2011). An overview of the roles of reactive oxygen species in physiological signalling of adaptations to contracting skeletal muscle and the potential effects of a chronic increase in mitochondrial ROS production during aging is provided in Figure 1.

1. **Effect of a chronic increase in ROS activities on neuromuscular aging, oxidative damage and redox signalling**

In order to attempt to elucidate the role of ROS in neuromuscular aging, oxidative damage and redox signalling, we have collaborated extensively with researchers at the University of Michigan (Drs Susan Brooks, John Faulkner and Lisa Larkin) and University of Oklahoma (Drs Arlan Richardson and Holly Van Remmen) to undertake studies of the effects of deletion of regulatory enzymes for ROS on neuromuscular aging in mice. These models invariably lead to an increase in oxidative damage in muscle and other tissues, but no clear relationship with neuromuscular aging was generally seen. The exception to this pattern was in mice with a whole body deletion of Cu,Zn superoxide dismutase (Sod1) which show neuromuscular changes with aging that have been claimed to reflect an accelerated skeletal muscle aging process (Muller et al., 2006). Adult Sod1KO mice showed a decline in skeletal muscle mass, loss of muscle fibers and a decline in the number of motor units, loss of motor function and contractility, partial denervation and mitochondrial dysfunction by 8 months old (Larkin et al., 2011). These are all changes that are also seen in old WT mice, but not until after 22-24 months of age.

Sod1 is present in both the cytosol of cells and within the mitochondrial inter-membrane space (IMS), (Kawamata and Manfredi, 2010) and hence lack of Sod1 may influence redox homeostasis in the mitochondria and cytosol. Jang et al, (2010) showed that this model was associated with a large increase in mitochondrial H2O2 production and we also showed that, in common with old WT mice, muscles of Sod1KO mice demonstrated a constitutive activation of NF-κB with increased production of pro-inflammatory cytokines and a constitutive increase in the content of a number of HSPs in muscle at rest. Muscles from these mice also failed to further activate cytoprotective adaptive responses to contractile activity and this results in diminished acute additional expression of heat shock proteins (HSPs) and other cytoprotective proteins following contractile activity. This failed activation in response to contractile activity could potentially occur through a lack of induction of additional superoxide and/or hydrogen peroxide during contractile activity due to a failure of activation of muscle NADPH oxidase activity (Sakellariou et al., 2013). Thus, a lack of Sod1 mimics the changes in redox signalling seen in old WT mice that lead to a failure of redox-mediated signalling of adaptive responses to contractile activity.

Our group of investigators has also examined whether the muscle atrophy in this whole body Sod1 knockout model is initiated by changes within muscle fibers or motor neurons. Mice with skeletal muscle specific deletion of Sod1 (mSod1KO mice) showed no evidence of NMJ degeneration or loss of muscle fibers, but showed some muscle hypertrophy (Zhang et al., 2013). The group also studied a transgenic Sod1KO mouse in which human SOD1 under control of a synapsin 1 promoter (nSOD1-Tg-Sod1KO mice) expressed SOD1 in central and peripheral neurons but not other tissues. Sciatic nerve CuZnSOD content in nSOD1-Tg-Sod1KO mice was ~20% of WT control mice, but they showed no loss of muscle mass or maximum isometric specific force production at 8-12 months of age, when significant reductions were seen in Sod1KO mice (Sakellariou et al., 2014). Thus these data implicate a lack of Sod1 specifically in motor neurons in the pathogenesis of the accelerated muscle aging phenotype seen in the Sod1KO mice. We have also recently examined the effect of neuron-specific Sod1 knockout in nSod1KO mice, but this model also does not recapitulate the full sarcopenia phenotype seen in Sod1KO mice and shows only minor changes in muscle mass and function (Sataranatarajan et al, 2015). The implication of this work appears to be that both neurons and muscle contribute to maintenance of neuromuscular function in this model and that deletion of Sod1 in both tissues is necessary to generate the full sarcopenic phenotype.

Thus studies of the Sod1KO model have demonstrated the importance of nerve-muscle interactions in the maintenance of neuromuscular function where ROS homeostasis is compromised during aging. Since adult mice lacking Sod1 replicate many of the features seen in old WT mice they may indicate key mechanisms that lead to loss of muscle fibers and function that are relevant to aging of WT mice.

1. **Changes in motor neurons and NMJ during aging**

Muscle fiber loss in aging is associated with a loss of motor units (Campbell et al., 1973; Larson and Ansved, 1995) and the numbers of motor axons innervating skeletal muscles has been found to be decreased in old rodents (Larson and Ansved, 1995) and old humans (Krantic et al, 2005). The surviving motor neurons show axonal sprouting that has been proposed to rescue muscle fibers that have become temporarily denervated, resulting in an increase in average motor unit size (Brown et al., 1981). It has been proposed that the ability of motor units to increase their size is limited, and muscle fibers and motor units are eventually lost ([Delbono, 2003](#_ENREF_12)). Aging is also associated with numerous pre- and post-synaptic structural abnormalities in peripheral nerve endings, including segmental demyelination([Adinolfi et al., 1991](#_ENREF_1)) demyelinated and remyelinated axonsand denervated Schwann cell columns ([Grover-Johnson and Spencer, 1981](#_ENREF_21)), synaptic detachment, partial or complete withdrawal of axons from postsynaptic sites, and fragmentation of postsynaptic motor endplates ([Chai et al., 2011](#_ENREF_10), Jang et al., 2010). Some data from rodents also indicate that changes in the peripheral regions of motor units occur prior to any loss in number of motor neuron cell bodies in the lumbar spinal cord (Chai et al., 2011), suggesting that degenerative processes in the peripheral regions of motor nerves may be particularly important.

***5.1 Changes in the redox status of motor nerves during aging***

We have also recently examined whether peripheral nerves show evidence for increased oxidative damage or defective redox signalling during aging (McDonagh et al, 2016). We examined the sciatic nerve of old mice at an age when loss of tibialis anterior muscle mass and function is apparent. Sciatic nerve from old mice did not show an equivalent increase in oxidative damage to that seen in skeletal muscles, but electron paramagnetic resonance (EPR) studies indicated an increase in the activity of superoxide and/or peroxynitrite in the nerves of old mice at rest that was further exacerbated by electrical stimulation of the nerve to activate muscle contractions. Proteomic analyses also indicated that specific redox-sensitive proteins are increased in content in the nerves of old mice that may reflect an adaptation to regulate the increased superoxide/peroxynitrite and maintain redox homeostasis, while analysis of redox active cysteines showed some increase in reversible oxidation in specific proteins in nerves of old mice. Detailed analysis of the redox-active cysteine in one protein in the nerve of old mice that is key to redox signalling (Peroxiredoxin 6, Cys 47) showed a minor increase in reversible oxidation that would be compatible with a change in its redox signalling function. Thus these data indicate that sciatic nerve from old mice does not show a gross increase in oxidative damage similar to that seen in the TA and other muscles that are innervated by this nerve, but support the possibility that an adaptation to increased oxidation has occurred and minor changes in oxidation of key cysteines are present that may contribute to defective redox signalling in the nerve.

1. **Does hydrogen peroxide play a role in redox cross-talk between neurons and muscle?**

The situation cited above for the nerve rescue Sod1KO mice provides an example of how restoration of neuronal ROS homeostasis can restore defective function in muscle mitochondria that is associated with increased ROS generation. An analogous situation appears to occur in experimental denervation or nerve crush which has been found to lead to activation of a number of degenerative pathways in the denervated muscle, including an increased mitochondrial generation of reactive oxygen species (Muller *et al*, 2006) and increased generation of pro-inflammatory cytokines (Cea *et al*, 2013). The reason for this rapid activation of hydrogen peroxide generation and release of cytokines is unclear, but it is feasible that initially this may reflect an attempt to restore innervation, since products such as cytokines are released from the muscle fiber and some cytokines have been claimed to stimulate axonal sprouting. Motor neurons have the capacity to upregulate endogenous regulatory proteins for ROS and other cytoprotective proteins in response to exogenous reactive oxygen and nitrogen species (Bishop et al., 1999) and neurotrophic factors (e.g. GDNF and BDNF) have been reported to promote neuronal survival by increasing defences against oxidative damage (Gabaizadeh et al., 1997). In addition the peripheral axons of motor nerves are exposed to increased extracellular activities of ROS derived from the muscle fibers during contractile activity (Vasilaki et al., 2006a). Previous studies of ROS derived from contracting skeletal muscle indicate that they cause transient oxidation in other non-contracting tissues (Close et al., 2007) and we hypothesise that this level of oxidation is unlikely to produce substantial oxidative damage, but acts as a stimulus for up-regulation of cytoprotective systems.

We therefore postulate that redox-cross talk between muscle and neurons occurs through release of H2O2 (and potentially other ROS). Recent data from model systems supports this indicating a new paradigm for the regulation of tissue homeostasis whereby H2O2 attracts nerves and nerves control H2O2 levels in a positive feedback loop (Meda et al, 2015). This work focusses on sensory neurons in a zebrafish fin amputation model, but we speculate that this may also apply in mammalian systems. Thus in both pathological models of muscle denervation (e.g. ALS models) and experimental denervation of muscle in young mice very large increases in the mitochondrial generation of H2O2 and other peroxides have been reported in the denervated muscle (Muller et al, 2006). This increased H2O2 generation precedes atrophy and loss of fibers in the denervated muscle. Although the increase may stimulate degenerative pathways including apoptosis in the muscle fiber, it is also possible that the H2O2 release may reflect an end-organ response to stimulate re-innervation through promotion of axonal sprouting and nerve regrowth. Other studies in zebrafish indicated that H2O2 promoted axonal regeneration in skin (Rieger & Sagasti, 2011) and a single report suggested that physiological levels of H2O2 could stimulate cell proliferation in mammalian neurons (Min et al, 2006). Thus our current hypothesis is that loss of neuronal input to muscle in neuromuscular disorders and aging leads to the generation of large amounts of H2O2 and potentially other ROS by the muscle mitochondria. This release may (at least) initially stimulate sprouting and regrowth of local motor axons, but also leads to a failure of adaptation of muscle fibers to contractile activity and if sustained will increase oxidative damage to muscle fibers (a schematic representation of this hypothesis is shown in Figure 2).

1. **Conclusions**

Finding effective ways of ameliorating the negative physical, mental and social effects of aging is a major global challenge. Physical frailty is driven by loss of muscle mass and function and hence preventing this is key to reduction in frailty. Our current understanding of the key areas in which ROS contribute to age-related deficits in muscle is through increased damage cell constituents and through induction of defective redox signalling. While much published data has focussed on changes in oxidative damage, there is increasing interest in more subtle changes in redox signalling pathways that may have important effect to reduce the integrity of the neuromuscular system. Understanding these pathways may provide opportunities to identify targeted interventions that have the potential to help prevent age-related neuromuscular decline with a consequent improvement in quality of life for older people.

1. **Acknowledgments**

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**Legends to Figures**

**Figure 1.** (1)Schematic illustration of a key redox signalling pathway leading to some acute responses to contractile activity in young/adult mice. Details of the pathways involved are described in the text. The bracketed area: “*local thiol oxidation*” indicates the poorly understood mechanisms whereby ROS generated by the signalling NADH oxidase interacts with established signalling pathways such as NF-κB, AP-1, HSF-1 and nrf2 to induce changes in gene expression.

(2) Schematic illustration of how an age-related chronic increase in ROS generation by mitochondria leads to oxidative damage and disrupts the redox signalling pathway described above.

**Figure 2.** Hypothetical representation of the way in which a failure of innervation of a single muscle fiber leads to local concentrations of H2O2 and other ROS which are sufficiently high to induce local oxidative damage, and able to diffuse to other fibers at a sufficiently high concentration to chronically activate redox-sensitive pathways in those normally innervated fibers and attenuate responses to contractions. We also hypothesise that the high local concentrations of H2O2 and other ROS may feedback on the peripheral axons to stimulate regeneration and sprouting in an attempt to promote re-innervation of the denervated fiber.

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