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Symposium Two: Ageing and Multimorbidity

Reactive oxygen species in age-related musculoskeletal decline: implications for nutritional intervention

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Musculoskeletal disorders and age-related musculoskeletal decline are major contributors to the burden of ill health seen in older subjects. Despite this increased burden, these chronic disorders of old age receive a relatively small proportion of national research funds. Much has been learned about fundamental processes involved in ageing from basic science research and this is leading to identification of key pathways that mediate ageing which may help the search for interventions to reduce age-related musculoskeletal decline. This short review will focus on the role of reactive oxygen species in age-related skeletal muscle decline and on the implications of this work for potential nutritional interventions in sarcopenia. The key physiological role of reactive oxygen species is now known to be in mediating redox signalling in muscle and other tissues and ageing leads to disruption of such pathways. In muscle, this is reflected in an age-related attenuation of specific adaptations and responses to contractile activity that impacts the ability of skeletal muscle from ageing individuals to respond to exercise. These pathways provides potential targets for identification of logical interventions that may help maintain muscle mass and function during ageing.

Keywords: Ageing: Skeletal muscle: Redox

Chronic conditions associated with musculoskeletal ageing are a major burden experienced by rapidly ageing populations in all countries. The two most common chronic musculoskeletal disorders, osteoarthritis (OA) and osteoporosis contribute significantly to the high prevalence of disability in older adults and together with age-related loss of skeletal muscle mass and function (commonly described as sarcopenia), affect 30–60% of people over 65 years of age in the UK[1,2]. The greatest risk factor for OA, osteoporosis and sarcopenia is age and the number of older people in countries such as the UK continues to increase dramatically[1]. There is therefore a clear need to identify and test new strategies to reduce the incidence and consequences, of common age-related chronic disease. This is particularly true for debilitating age-related disorders of the musculoskeletal system since these have major adverse effects on independence and quality of life of older individuals and limit physical activity, amplifying age-related risks of multiple cardio-metabolic diseases, major cancers and neurodegenerative diseases[3,4].

Ageing of skeletal muscle is characterised by loss of mass and contractile force and has a profound impact on the quality of life of older people. Loss of skeletal muscle begins in middle age and continues until the end of life[5]. In older people, declining muscle mass and function

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causes instability and increased risk of falls with a loss of independence[6]. By age 70, the cross-sectional area of skeletal muscle is reduced by 25–30% and muscle strength by 30–40%[7]. Both a decrease in the number of muscle fibres and atrophy and weakening of those fibres remaining[8–10] appear to contribute to the reduction in muscle mass and function with age in humans and rodents. This is termed sarcopenia and the intrinsic and extrinsic changes regulating muscle ageing in humans occur in rodents, indicating that ageing mice and rats are relevant models of human sarcopenia[11,12]. While there is undoubtedly a major effect of the ageing process on the loss of muscle mass and weakness seen in elderly populations, multiple other factors play a role in individuals, including lack of exercise, increased sedentary behaviour, sub-optimal nutrition, social isolation and sub-optimal health care[13].

There have been dramatic advances in understanding the fundamental mechanisms underlying the ageing process in non-mammalian and mammalian models[14,15] and this information is also informing investigations of the mechanisms underlying age-related degeneration of single tissues, including the musculoskeletal tissues, such as skeletal muscle, bone and cartilage and of interventions to ameliorate such degeneration[16]. The focus of this review will be on one of these fundamental mechanisms, redox regulation and the role of redox changes in age-related loss of skeletal muscle mass and function (sarcopenia).

Reactive oxygen species (ROS) in ageing

A factor clearly associated with loss of function during ageing in numerous tissues is oxidative damage and experimental evidence from humans and rodents indicates that skeletal muscles and other musculoskeletal tissues show age-dependent increases in the products of oxidative damage to biomolecules including proteins, lipids and nucleic acids[17–20]. Various reports have attributed the positive correlation between age and oxidative damage to age-related changes in reactive oxygen species (ROS) production, with skeletal muscles from old mice exhibiting a higher intracellular ROS generation in comparison to muscles from young mice[21,22]. The loss of muscle that occurs with ageing occurs in parallel with loss of motor units in both humans and rodents[23,24]. A 25–50% reduction in the number of motor neurons occurs in both man and rodents with ageing[25,26]. Loss of innervation of individual fibres occurs in muscles of aged humans and animals and our study which indicated that ~15% of muscle fibres from old mice are completely denervated and ~80% of neuromuscular junctions (NMJs) showed some disruption[27]. Recent data indicate that this loss of innervation may play a fundamental role in in the changes in ROS generation that occur in ageing skeletal muscle[28,29]providing evidence for important inter-tissue interactions affecting muscle viability during ageing[30].

Roles of ROS in physiology of the musculoskeletal system

The term oxidative stress as it related to oxidative damage to cells and tissues was coined by Helmut Sies and colleagues in 1985[31] and is defined as "a disturbance of the pro-oxidant-antioxidant environment in favour of the former". The implications of this definition were originally that oxidative stress was potentially deleterious to tissues and cells and that inhibition or reversal of the stress on cells and tissues would generally be beneficial. This could be potentially achieved by a reduction in the promotors of oxidation (usually free radicals or ROS), or an increase in substances or pathways that decrease oxidation (antioxidant substances or regulatory proteins). These assumptions underlined many of the original studies to investigate the roles of ROS and antioxidants in skeletal muscle and in exercise[32–35]. Particularly prominent in these studies was the assumption that nutritional antioxidants would be beneficial and many of the early studies included a component to examine the possibility that antioxidant supplementation would suppress effects to demonstrate the possible negative role of free radicals or ROS[32,34]. As further studies were undertaken, it rapidly became clear that skeletal muscle could not only generate ROS, but also that it could respond to that generation by upregulation of regulatory pathways[36,37] which prevented the potential for subsequent oxidative damage to the tissue. Thus, ROS in this situation were not necessarily damaging but inducing adaptive changes in tissues. These apparent contrasting roles of ROS have subsequently been described as redox signalling effects compared with oxidative stress and damage and described more specifically by Helmut Sies and colleagues as oxidative eustress and oxidative distress[38]).

Redox signalling in skeletal muscle

Signalling by ROS is mainly achieved by targeted modifications of specific residues in proteins[39,40]. Muscle fibres respond to contractions by an increase in the intracellular generation of superoxide and nitric oxide (NO) with the formation of secondary ROS and reactive nitrogen species[41–43]. This leads to activation of a number of transcription factors, including NF-κB, AP-1 and HSF-1[36,44–46] and an increased expression of regulatory enzymes and cytoprotective proteins[37,47,48]. Redox-regulation is also apparent for genes associated with catabolism[49–51] and mitochondrial biogenesis[52,53]. Identification of the specific redox mediated steps in adaptive pathways to exercise has proven difficult to define in skeletal muscle. Studies in humans and animals using very high levels of antioxidants have provided evidence that these interventions inhibited cytoprotective responses (e.g., exercise-induced increase in heat shock and other stress proteins)[54], reduced mitochondrial biogenesis[55–57], prevented an increase in muscle insulin sensitivity[55] and inhibit the release of cytokines and inflammatory mediators[58]. These antioxidant supplementation studies have been controversial[59,60], but additional adaptations potentially activated by ROS have been identified in genetic knockout mouse models, designed to delete

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ROS-generating enzymes. For instance, NADPH oxidase 2 (Nox2) knockout mice show reductions in post-exercise glucose uptake via impaired GLUT4 translocation[61,62], Nox4 knockout in mice was found to lead to development of insulin resistance[63] and specific endothelial Nox4 knockout leads to impaired metabolic adaptations to chronic exercise[64]. Thus, together these studies indicate that the range of adaptive pathways activated during exercise and regulated by redox pathways is likely to be extensive. Key processes involved in muscle adaptations to exercise have been intensively studied for a number of years and among these, multiple pathways have been identified where redox regulation appears important including the key pathways leading to the adaptations described above[65].

Is redox signalling disrupted during ageing in the musculoskeletal system?

It now seems clear that the level of ROS generation and oxidative damage is not a fundamental determinant of lifespan although some authors have argued that the age-related changes in ROS activities and oxidative damage are important mediators of age-related disorders[66]. Several of the ROS-stimulated responses to exercise are attenuated in old mice including increased stress responses[46] and mitochondrial biogenesis[67,68]. Mitochondrial peroxide generation has also been repeatedly reported to be increased in skeletal muscle during ageing[69,70]. In order to decipher the effects of ROS in musculoskeletal ageing, a number of studies have examined the effects of deletion of regulatory enzymes for ROS in mammalian models. Despite frequent observations of increased oxidative damage in these models of dysregulated ROS homeostasis, no clear relationship with skeletal muscle ageing was seen[70]. Studies of muscle ageing in mice predominantly use the C57Bl strain of laboratory mice which reach maturity at 4–6 months of age and in many laboratory animal facilities they show age-related loss of muscle force production and loss of muscle mass from approximately 22 months of age. In the relevant studies described here, mice were examined at 6–8 months of age (adult mice) and 22–26 months of age (old mice).

Mice with a whole body deletion of SOD1 (Cu, Zn superoxide dismutase) differed from all of the other models studied and showed an increase in tissue oxidative damage associated with neuromuscular changes with ageing. This was described by the discoverer as "accelerated age-related loss of muscle mass"[71,72]. Adult *Sod1KO* mice show a decline in skeletal muscle mass, loss of muscle fibres and a decline in the number of motor units, loss of motor function and contractility, partial denervation and mitochondrial dysfunction by 8 months old[73,74][75]. The fibre loss in *Sod1KO* mice is accompanied by degeneration of NMJs[73]. These changes are also seen in old control wild type (WT) mice, but not until after 22 months of age. These mice also show the attenuation of redox-mediated responses to contractile activity that is seen in ageing mice[46]. Hence, *Sod1KO* mice have been proposed as a useful model to examine the potential role of ROS in skeletal muscle ageing[76]. The only known function of Sod1 is to catalyse the dismutation of superoxide to hydrogen peroxide, a reaction that also occurs chemically in the absence of Sod1 but at a much slower rate[77]. Superoxide also reacts rapidly with nitric oxide (NO) to generate peroxynitrite, a reaction that is approximately 3 times faster than the chemical dismutation of superoxide to hydrogen peroxide (Fig. 1). Furthermore, the muscle cytosolic concentration of NO is many fold higher than superoxide. We have demonstrated increased generation of peroxynitrite in muscles of *Sod1KO* mice providing a potential mechanism by which the lack of this protein specifically leads to accelerated muscle loss[77].

The *Sod1KO* mouse shows many of the muscle phenotypes of old WT mice at a much earlier age and the major effect of the lack of Sod1 appears to be through effects at the level of the motor neuron. This model has also been proposed as a useful experimental model of frailty since *Sod1KO* mice exhibit four characteristics that have been used to define human frailty: weight loss, weakness, low physical activity and exhaustion. In addition, *Sod1KO* mice show increased inflammation and sarcopenia, which are strongly associated with human frailty[78]. A series of tissue-specific *Sod1KO* mice have been generated to establish the key tissue and cellular locations at which the lack of Sod1 exerts an effect to lead to skeletal muscle loss. In recent studies, we have examined in detail the changes in motor neurons and the NMJ, which occur in inducible neuron-specific *Sod1KO* mice (*i-mnSod1KO mice*) which present with an early onset of muscle loss[79]. Surprisingly no specific effect of a lack of neuronal Sod1 was seen, but rather all of the changes seen in ageing were accelerated. We concluded that neuronal deletion of Sod1 induced exaggerated loss of muscle in old mice and this deletion leads to a reduced axonal area, increased proportion of denervated NMJ and reduced acetylcholine receptor complexity and other changes in nerve and NMJ structure that are also seen in WT mice at a more advanced age[79]. Thus, the *Sod1KO* mouse model and its tissue specific derivatives have provided a great deal of valuable information on the tissue interactions and mechanisms that lead to muscle loss in ageing. It is clear that a simple lack of Sod1 does not occur during ageing in WT animals or humans but the detailed analogies in phenotype and mechanisms seen in ageing WT mice and *Sod1KO* mice provide confidence in the relevance and utility of this model.

The current data therefore suggest that aberrant ROS generation and subsequent defective redox signalling and is a feature of ageing in skeletal muscle and contributes to attenuated responses to contractile activity and diminished efficacy of adaptations to contractile activity. This appears to be an important component in maintenance of muscle mass and function during ageing since studies in mice have shown that restoration of some stress responses helps maintain muscle mass and function in aged cohorts[80,81] although comparable human studies have not been undertaken.

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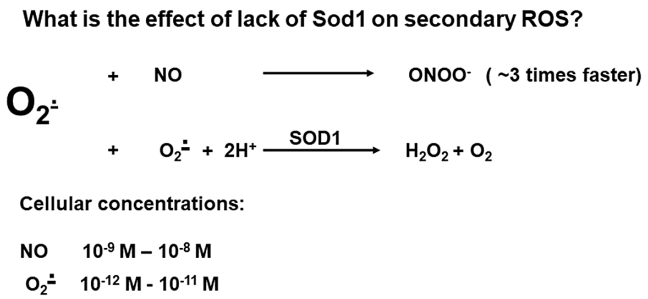


Fig. 1. Schematic illustrating the reactions of superoxide to generate hydrogen peroxide via Sod1-catalysed dismutation and the chemical reaction with NO to generation peroxynitrite. Data presented in Sakellariou *et al.*, (2011) indicate increased peroxynitrite generation occurs in muscle fibres in the *Sod1KO* mice[77].

Potential role of skeletal muscle mitochondria in aberrant redox signalling in ageing

Studies in ageing models suggest that early in the ageing process mitochondria show a change to a phenotype reflecting modified fusion, together with a change in orientation more perpendicular to the fibre axis[82] in association with other changes in mitochondrial dynamics[83]. Mitochondria play a central role in regulation of muscle protein synthesis and degradation through multiple signalling pathways, including energy production, generation of ROS, modified calcium handling and cytochrome C release initiating apoptotic pathways[84,85]. Mitochondrial signalling to activate these various pathways has been linked to failure of protein homeostasis in muscle atrophy through, for example, altered mitochondrial ATP generation leading to energy dependent dephosphorylation of AMPK[86], increased ubiquitination due to increased ROS generation[87,88] and activation of apoptotic pathways due to increased cytochrome c release[89,90]. The role of mitochondria as a potential master regulator of muscle mass and function in a variety of different models seems clear, but due to the varying aetiologies of the onset of different conditions leading to muscle loss, common initiating factors that lead to mitochondrial disruption have not been recognised. The loss of muscle with ageing occurs with loss of motor units in both humans and rodents[23,24], and loss of innervation of individual fibres has been reported in aged muscles. We found that ~15 % of muscle fibres in old mice were completely denervated and ~80 % of NMJs showed disruption[27]. Studies of mice lacking Sod1 (a model of accelerated skeletal muscle ageing)[91–95] have highlighted the role of disruption of neuromuscular integrity in regulation of muscle mitochondrial ROS generation. These data, combined with studies of transection of the innervating nerve, which also caused a large increase in muscle mitochondrial peroxide generation[91], identified a key role for motor neuron and NMJ integrity in regulation of muscle mitochondrial ROS generation in old mice. We examined the effect of partial denervation of the mouse tibialis anterior (TA) muscle and found a substantial increase in mitochondrial peroxide generation in the denervated fibres and also in neighbouring innervated fibres (Fig. 2)[28]. These data suggest that loss of innervation in fibres contributes to increased mitochondrial ROS generation[28] and associated mitochondrial degeneration[96] in ageing.

We have linked the attenuation of responses to contractile activity seen in both aging and the *Sod1KO* mice to an increase in muscle mitochondrial hydrogen peroxide production in both situations. We speculated that the increase in mitochondrial hydrogen peroxide would lead to an increased expression of regulatory enzymes for reactive oxygen species (Prx, GPx, TrX etc) which would suppress the likelihood of oxidation of critical cysteines in signalling proteins during contractions[65]. Furthermore since cycles of localised denervation and re-innervation appear to occur throughout life and may contribute to disrupted mitochondrial peroxide generation[65] and mitochondrial structure and function[96], we speculated that the focal denervation seen in both adult *Sod1KO* and old WT mice leads to the increased mitochondrial peroxide production in both the denervated and neighbouring innervated muscle fibres which would drive the attenuation of redox-regulated adaptive mechanisms[65] (Fig. 3). This provides a testable mechanism by which focal and intermittent denervation during aging have a deleterious effect in suppressing key responses of muscle to exercise.

Implications for nutritional interventions

Most of the current interest in nutritional interventions to ameliorate or prevent age-related loss of muscle mass and function is concerned with the protein content or composition of the diet and potential protein supplements[97]. Such approaches offer considerable promise in helping maintain muscle bulk in the elderly[98]. Interventions that affect the ageing process *per se* also appear a potential route to preservation of muscle, but currently these are focussed on experimental models and primarily involve pharmacological approaches[99]. There has been considerable speculation and preliminary studies examining whether "antioxidant" nutrients may be beneficial in prevention or treatment of sarcopenia, but intervention studies have been disappointing (e.g. see recent reviews on vitamins E and C[100–102]). Epidemiological data also support a potential beneficial effect of a Mediterranean diet high in antioxidants as protective against sarcopenia.

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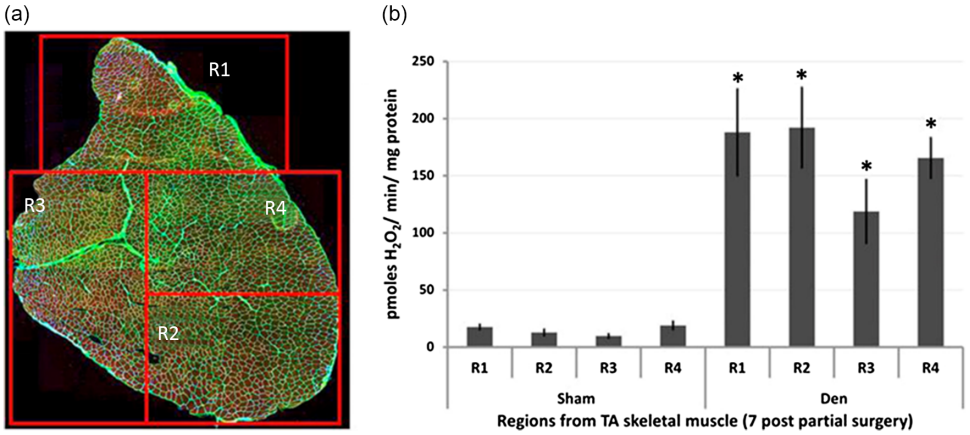


Fig. 2. **(a)** Effect of partial denervation of the Tibialis Anterior (TA) muscle on peroxide generation by mitochondria from different regions of the muscle. The four regions identified contained fibres that were either fully innervated (Region R1), fully denervated (Region R3) or partially denervated (Regions R2 and R4) and small bundles of fibres were obtained from each region. **(b)** These fibres were permeabilised and state 1 mitochondrial peroxide generation examined at 7 d post-surgery in comparison with sham-operated control muscles; $^*P < 0.05$ compared with fibres from the same region of sham-operated muscles. Modified from reference[28].

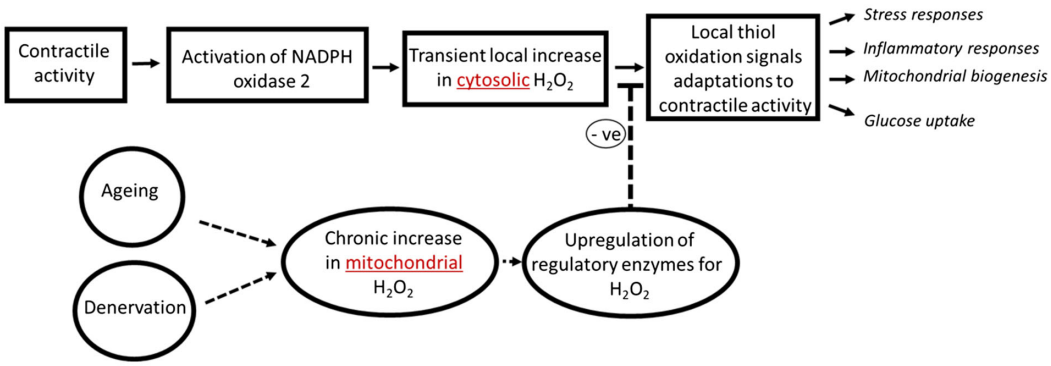


Fig. 3. Schematic illustrating the outline mechanism by with contractile activity in skeletal muscle leads to increased cytosolic hydrogen peroxide, thiol oxidation and activation of adaptive signalling pathways. In ageing or denervation, a chronic increase in mitochondrial hydrogen peroxide generation leads to attenuation of these responses by inducing a chronic upregulation of expression of various regulatory proteins for hydrogen peroxide. See text for further details.

A recent systematic review concluded that Mediterranean diet adherence had a positive effect in maintaining muscle mass and muscle function in older subjects, although the results were less clear with regard to muscle strength[103].

The mechanistic data described above also indicate that aberrant mitochondrial ROS generation and defective redox signalling are features of ageing in skeletal muscle and contribute to attenuated responses of skeletal muscle to contractile activity and diminished adaptations to exercise[104]. The scheme shown in Fig. 3 suggests multiple sites at which pharmacological or nutritional interventions may interact to prevent or reverse the changes in redox signalling mechanisms that occur with ageing in skeletal muscle.

Restoration of redox homeostasis

It is tempting to speculate that antioxidant supplements may be beneficial is restoring redox homeostasis in skeletal muscle, but intervention studies have been disappointing and evidence from supplementation studies in exercising humans indicate that high dose supplementation with vitamins E and C suppress adaptive responses of muscle to exercise[54–57]. These studies were controversial in that the same suppression of training effects was not seen by all investigators[59,60], but no clear beneficial effects of the supplements were seen in any studies. Since similar effects of low dose supplements or changes in diet have not been observed, it is interesting to speculate whether this reflects a "U" shaped response curve with high dose antioxidant supplements having a deleterious effect at high concentrations. In spite of this, these data do provide an important insight into range of the physiological roles of redox signalling in muscle[105].

In contrast our data indicate that targeted antioxidant administration aimed at suppression of a chronic increase specifically in mitochondrial ROS may offer an alternative approach. While this topic is still relatively underexplored, a number of compounds have been described that may specifically reduce mitochondrial

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ROS. In many cases these compounds are within the classification of pharmaceutics, but might also include some dietary components that have affinity for mitochondrial membranes[106]. Examples of these latter compounds include Astaxanthin and vitamin E which are reported to preferentially localise to plasma and mitochondrial membranes in skeletal muscle[107–109].

Synthetic mitochondrial antioxidants

Several synthetic antioxidant compounds have been developed that specifically target mitochondria. These include SS peptides, such as SS-31 which concentrates up to 1000 fold in mitochondria and is thought to interact with cardiolipin in the inner mitochondrial membrane (IMM)[110]. Whilst this compound does not appear to directly scavenge ROS, an indirect effect to reduce mitochondrial ROS levels has been shown[111] and beneficial effects on models of muscle atrophy have been reported[111,112].

An alternative approach has been to link a lipophilic cation, such as tetraphenylphosphonium, to a small molecular weight antioxidant to generate compounds that accumulate in mitochondria up to 100–1000 fold[113]. This has resulted in a number of agents such as MitoQ[113,114], SkQ1/SkQR1[115] and XJB-5-131[116]. None of these compounds have any tissue specificity, but positive effects on muscle function[117] and no effect on muscle ageing[118] have been reported.

In conclusion our research has demonstrated that ageing is associated with a disruption of redox signalling of beneficial adaptations to contractile activity in skeletal muscle. Studies with basic models of muscle loss in ageing indicate that maintenance of these pathways is an important factor in maintain muscle in ageing. Previous studies utilising antioxidant supplements have been disappointing in terms of prevention or treatment of sarcopenia, but mechanistic studies suggest that interventions targetted at restoring muscle mitochondrial redox status may hold promise to help maintain muscle mass and function during ageing.

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