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10   **Redox regulation of muscle adaptations to contractile activity and aging**

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29 **Abstract**

30 Superoxide and nitric oxide are generated by skeletal muscle and these species are increased by

31 contractile activity. Mitochondria have long been assumed to play the primary role in generation of

32 superoxide in muscle but recent studies indicate that, during contractile activity, membrane-

33 localized NADPH oxidase(s) rapidly generate(s) superoxide that plays a role in redox signaling. This

34 process is important in upregulation of rapid and specific cytoprotective responses that aid

35 maintenance of cell viability following contractile activity, but the overall extent to which redox

36 signaling contributes to regulation of muscle metabolism and homeostasis following contractile

37 activity is currently unclear, as is identification of key redox-sensitive protein targets involved in

38 these processes. Reactive oxygen and nitrogen species have also been implicated in the loss of

39 muscle mass and function that occurs with aging, although recent work has questioned whether

40 oxidative damage plays a key role in these processes. A failure of redox signaling occurs in muscle

41 during aging and may contribute to the age-related loss muscle fibers. Whether such changes in

42 redox signaling reflect primary age-related changes or are secondary to the fundamental

43 mechanisms is unclear. For instance, denervated muscle fibers within muscles from aged rodents or

44 man appear to generate large amounts of mitochondrial hydrogen peroxide that could influence

45 adjacent innervated fibers. Thus, in this instance a 'secondary' source of reactive oxygen species may

46 be potentially generated as a result of a primary age-related pathology (loss of neurons) but

47 nevertheless may contribute to loss of muscle mass and function during aging.

48

49 **Introduction**

50 The Editor-in-Chief of the *Journal of Applied Physiology* invited this review to accompany the

51 presentation of the 2014 Edward F. Adolph lecture to the Environmental and Exercise section of the

52 American Physiological Society, a lecture entitled *30 years of chasing radicals in muscle: Redox*

53 *regulation of muscle adaptations to contractile activity and aging.* My plan is to present a personal

54 (and hence undoubtedly biased) view of how this exciting field has developed over 30 years, the key

55 achievements that have been made and to discuss some of the difficulties involved in studying this

56 area. Of necessity, this is not a comprehensive description of all that has been discovered and is

57 inevitably incomplete, since the field continues to evolve rapidly and relevant data appear on a

58 regular basis that impact on our understanding of the area. Three key topics will be covered to which

59 our research group have contributed a significant number of publications: (i) Generation of reactive

60 oxygen and nitrogen species in contracting skeletal muscle; (ii) Roles of reactive oxygen species in

61 skeletal muscle; (iii) Reactive oxygen species in muscle aging.

62

63 **Generation of reactive oxygen and nitrogen species in contracting skeletal muscle**

64 It is well established that skeletal muscle fibers generate superoxide and nitric oxide (NO) and these

65 parent molecules can be converted to several secondary reactive oxygen species (ROS) and reactive

66 nitrogen species (RNS). Superoxide and NO are generated from various sources within muscle fibers,

67 and superoxide (53, 76), hydrogen peroxide (90), and NO (3, 46) are released into the interstitial

68 space of muscle fibers (or generated on the extracellular side of the muscle plasma membrane).

69 Contractile activity has been shown to increase the intracellular content or activities of superoxide,

70 hydrogen peroxide, and NO (66, 75, 76, 84), while superoxide, hydrogen peroxide, hydroxyl radical

71 and NO have been detected in the muscle interstitial space (53, 67, 90).

72

73 A number of different approaches have been used to demonstrate the increase in ROS that occurs

74 during contractile activity. Although most data to date have been generated using non-specific

75 approaches, techniques have become increasingly sophisticated such that (for instance) new

76 specific, genetically encoded fluorescent probes, such as *HyPer,* can report changes in single species

77 in defined sub-cellular compartments (see Figure 1 for examples of approaches that have been

78 used). Much of the initial work in this area was based on the assumption that mitochondria were the

79 main source of the ROS generated during contractile activity in muscle, but several recent

80 publications disagree with this possibility (73). There is some debate about the precise location of

81 NAD(P)H oxidase(s) that have been claimed as alternative sources, but the presence of this enzyme

82 in the skeletal muscle plasma membrane (41), sarcoplasmic reticulum (100) and the T-tubules (19)

83 has been reported. The T-tubule localized enzyme appears to be particularly relevant since it has

84 been claimed to be specifically activated by contractions (19). In recent studies we have examined

85 the potential contribution of mitochondrial and non-mitochondrial sources to the acute increase in

86 superoxide seen during muscle contractions (69, 79) and concluded that NADPH oxidase effects

87 predominated over mitochondria during the short contraction periods (10-15 minutes) that were

88 studied. Thus current data appear to indicate that a non-mitochondrial NADPH oxidase (likely to be

89 the Nox2 isoform) is the major source of generation of superoxide during short term contractile

90 activity. The Nox4 isoform of NADPH oxidase has also been reported to be expressed in

91 mitochondria and sarcoplasmic reticulum of skeletal muscle (79, 85), but any role in contraction-

92 induced superoxide generation is unclear.

93

94 A number of specific ROS and RNS are detected in the extracellular space of skeletal muscle

95 myotubes or isolated fibers in culture or in microdialysates from muscle interstitial fluid *in vivo.* It

96 appears that muscle fibers may have generating systems for superoxide that release this species into

97 the extracellular space (53, 76). Substantial diffusion of superoxide (or its protonated form) through

98 the plasma membrane seems extremely unlikely (27), but other species that are detected in the

99 muscle extracellular space (e.g. hydrogen peroxide and NO) can potentially diffuse across

100 membranes and hence may originate from intracellular sites. Javesghani et al (41) reported that a

101 plasma membrane-localized NAD(P)H oxidase could release superoxide to the external face of the

102 membrane and Ward and colleagues (96) have decribed a stretch-activated NADPH oxidase (Nox2

103 isoform) that plays a major role in contraction-induced ROS generation in cardiac myocytes. This

104 enzyme is also reported to be present in the skeletal muscle plasma membrane and appears to

105 release superoxide to the outside of the cell. Other NAD(P)H-dependent systems have also been

106 suggested to play a role in release of superoxide from muscle fibers (35). In muscle *in vivo* or intact

107 muscle preparations *ex vivo*, xanthine oxidase enzymes in the endothelium may also play an

108 important role in contraction-induced release of superoxide (24) and this enzyme has been claimed

109 to be important in adaptations of muscle to contractile activity (22). Figure 2 summarises our current

110 understanding of the sites that have been identified for generation of ROS and NO in skeletal muscle

111 fibers.

112

113 **Roles of ROS in skeletal muscle: Oxidative damage or redox signaling ?**

114 Although excess ROS can be deleterious to cells causing oxidative damage to lipids, DNA and

115 proteins (27), these species also appear to act as mediators of some adaptive processes following

116 cellular stresses under normal physiological conditions. ROS mediate regulatory functions that lead

117 to changes in cell and tissue homeostasis through modification of gene expression (17, 28, 36).

118 Modification of specific thiol residues in proteins appears to be the major mechanism by which ROS

119 exert such regulatory roles (40). Contractile activity increases the intracellular generation of

120 superoxide and NO and these species plus a number of secondary ROS and RNS (66, 73, 75) can

121 mediate activation of a number of redox-regulated signaling pathways. The nature of these

122 pathways has been the subject of extensive research and redox-regulated processes (such as

123 activation of NFκB) have been shown to stimulate the expression of genes associated with

124 myogenesis (2), catabolism and mitochondrial biogenesis (4, 71, 87). Our group have been

125 particularly interested in the role of ROS in activation of short-term cytoprotective changes in

126 expression of regulatory enzymes and cytoprotective proteins in response to contractile activity (30,

127   53, 54). This appears to occur through redox-dependent activation of a number of transcriptional

128   pathways including the transcription factors, NFκB, AP-1, HSF-1 and Nrf2 (36, 42, 77, 91), see Figure

129   3A .

130

131   **Potential modulating effects of antioxidant supplements on ROS-stimulated adaptations to**

132   **contractile activity.**

133   Researchers have been attempting to suppress the presumed deleterious effects of reactive oxygen

134   and nitrogen species generated during exercise since the first descriptions of their generation in this

135   situation (e.g. 15). There has been little evidence of beneficial effects on muscle from such

136   interventions, but the realization that these species play important roles in redox signaling has

137   prompted a rethink of what antioxidants might achieve in this situation. Our group initially

138   demonstrated that high doses of vitamin C could inhibit rapid stress responses to acute exercise (45)

139   and this line was pursued by others who reported that high doses of antioxidants could reduce the

140   training effects of exercise on muscle mitochondrial biogenesis, VO_{2max} and improvements in insulin

141   sensitivity (23, 77). The implication of such studies is that reactive oxygen or nitrogen species play a

142   key role in regulating multiple training-induced adaptations to muscle in humans and animals.

143   Unfortunately such findings could not be repeated by other scientists who reported normal

144   adaptations to exercise training despite administration of high dose antioxidants (e.g. 29). This

145   difference resulted in an intense head-to-head debate in the scientific literature from the groups

146   reporting these differing results (e.g. 31). There are a number of differences in experimental design

147   that are likely to underlie the differences in reported outcomes including the study of animals or

148   humans; trained or untrained subjects; the durations and protocols for the training; the choice of

149   markers of oxidative stress; the time points studied; the use of muscle versus blood markers; and

150   many more potential factors. A recent article by Paulsen et al (68) has shed some light on this

151   controversy although this also illustrates the complexity of relating signaling processes to true

152   physiological function. The study appears to confirm that these supplements do not universally

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153 inhibit major physiological adaptations to exercise training, although they did inhibit potentially

154 relevant changes in mitochondrial proteins. A full explanation for the apparent discrepancies in the

155 literature in this area is unlikely to appear until more is known about the scope and importance of

156 redox signaling in muscle, but the current debate highlights the potential unintended consequences

157 of un-targeted use of high dose antioxidant supplements.

158

159 **ROS and muscle aging**

160 Aging leads to a reduction in muscle mass and function that contributes to physical instability and

161 increased risk of falls (98) such that by the age of 70, skeletal muscle cross-sectional area has

162 declined by 25-30% and muscle strength by 30-40% (72). In both humans and rodents there is

163 evidence that the age-related reduction in muscle mass and function is primarily due to decreased

164 numbers of muscle fibers, and atrophy and weakening of the remaining fibers (6, 49, 50), although a

165 recent study suggests atrophy of type II fibers without fiber loss is the major contributor to the

166 decreased muscle mass seen in healthy elderly human subjects (61). Most of the intrinsic and

167 extrinsic changes regulating muscle aging in humans have been observed in rodents, indicating that

168 mice and rats can provide relevant models of human sarcopenia (14). Denervation also contributes

169 to loss of muscle mass in humans and rodents (13, 39). The comparable changes in morphology seen

170 in myofibers of aged rodents and humans suggest the mechanisms leading to muscle loss and

171 atrophy at the cellular level are comparable (57). Muscle from old rodents also shows an increased

172 proportion of more oxidative fibers (13) and an attenuation of various responses to contractile

173 activity including acute stress responses (91), mitochondrial biogenesis (51) and the contraction-

174 induced increase in muscle protein synthesis (12). These are potentially important aspects of the

175 multiple age-related deficits in muscle including contributing to slowed reactions and an inability to

176 fine tune movements, while transgenic studies indicate that correction of specific attenuated

177 responses to contractions can preserve muscle force generation in aged mice (7, 44, 52).

178

Oxidative damage and defective redox signaling in muscle from old mice and humans

An increase in oxidative damage has been reported in tissues (including skeletal muscle) of all aged organisms compared with levels found in young organisms (16, 81, 90). The possibility that increased oxidative damage plays a key role in age-related tissue dysfunction has received considerable attention. In non-mammalian models, interventions designed to reduce the activities of ROS, such as overexpression of CuZn, superoxide dismutase (SOD1), catalase or both in Drosophila (63-65) or treatment with a MnSOD and catalase mimetic in C. Elegans (56) extended lifespan and thus support the hypothesis, but these effects have not been confirmed in other studies (20). In mammals, only a small number of manipulations designed to reduce ROS activities and/or oxidative damage have increased lifespan (82, 97). It therefore appears that increased ROS generation is not the fundamental cause of aging (or more precisely, the fundamental determinant of lifespan). Many studies have reported that mitochondrial ROS generation is increased in skeletal muscle during aging (55, 88 for reviews) in association with impaired function and oxidative damage to mitochondrial components (38, 81). Furthermore other studies indicate that interventions to reduce mitochondrial hydrogen peroxide content (82) or increase cytoprotective proteins that reduce oxidative damage (7) can preserve muscle function during aging. Increased mitochondrial ROS generation has also been proposed to play a key mediating role in pathological changes in muscle in conditions such as disuse atrophy (74).

Modification of muscle ROS during aging: Knockout of key regulatory proteins

A number of studies have examined the effects of deletion of regulatory enzymes for ROS, but despite frequent observation of increased oxidative damage in these models, no clear relationship with skeletal muscle aging was seen (38). The exception to this pattern was in mice with a whole body deletion of SOD1 which show neuromuscular changes with aging that appear to reflect an accelerated skeletal muscle aging process (58). Adult *SOD1KO* mice show a decline in skeletal muscle mass, loss of muscle fibers and a decline in the number of motor units, loss of motor function and

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contractility, partial denervation and mitochondrial dysfunction by 8 months old (37, 47, 92). The

fiber loss in *SOD1KO* mice is accompanied by degeneration of neuromuscular junctions (NMJs; 37).

These changes are also seen in old WT mice, but not until after 22 months of age. Hence we have

proposed that *SOD1KO* mice are a useful model to examine the potential role of ROS in skeletal

muscle aging (32).

It is relevant to consider why only the *SOD1KO* mice shows an accelerated muscle aging phenotype

although other models with knockout of regulatory enzymes for ROS or RNS also show an increase in

oxidative damage to muscle. SOD1 is expressed in both the cytosol of cells and within the

mitochondrial inter-membrane space (IMS) where it is likely to be present at high concentration

compared with cytosolic SOD1 (43). One implication of this is that lack of SOD1 may influence redox

homeostasis in the mitochondria in addition to the cytosol and hence that disturbances in either

cytosolic or mitochondrial redox may underlie the accelerated skeletal muscle aging phenotype seen

in *SOD1KO* mice. In our studies, we examined the nature of the reactive species that are generated

in mice lacking SOD1. Some studies of aging models have suggested that the decline in tissue

function that occurs with aging and the accelerated loss of skeletal muscle fibers in *SOD1KO* mice

may be caused by superoxide toxicity (38, 56). An alternative possibility is that superoxide and NO

may react chemically to form peroxynitrite, a reaction that competes with the dismutation of

superoxide to hydrogen peroxide by SOD (5). In adult SOD1 null mice, the phenotype may therefore

be associated with excess superoxide, but may also be due to increased peroxynitrite or a reduction

in NO bioavailability. We demonstrated that, similar to muscle fibers from old WT mice, those from

adult SOD1 knockout mice showed an increase in oxidation of the non-specific intracellular ROS

probe, 2', 7'-dichlorodihydrofluorescin-diacetate (DCFH) at rest compared with fibers from adult WT

mice (92). Surprisingly the fibers from *SOD1KO* mice showed no increase in DCFH oxidation following

contractile activity, although an increase in DCFH oxidation was seen in muscle fibers from adult WT

mice following contractile activity. The explanation for this is currently unclear, although DCFH is

231 relatively insensitive to oxidation by superoxide, but is oxidized by other ROS, including hydrogen

232 peroxide, hydroxyl radicals, peroxynitrite and nitric oxide(60). Single muscle fibers from *flexor*

233 *digitorum brevis* of WT and *SOD1KO* mice were therefore also loaded with NO-sensitive (4-amino-5-

234 methylamino-2',7'-difluorofluorescein diacetate, DAF-FM) and superoxide-sensitive

235 (dihydroethidium, DHE) probes (78). These studies illustrated that a lack of SOD1 in the fibers from

236 *SOD1KO* mice did not increase superoxide availability at rest since no increase in ethidium or 2-

237 hydroxyethidium (2-HE) formation from DHE was seen in fibers from *SOD1KO* mice compared with

238 those from WT mice. Fibers from *SOD1KO* mice were found to have decreased NO availability

239 (decreased DAF-FM fluorescence), increased 3-nitrotyrosines (3-NT) in muscle proteins indicating

240 increased peroxynitrite formation and increased content of peroxiredoxin V (a peroxynitrite

241 reductase), compared with WT mice. Following contractile activity muscle fibers from *SOD1KO* mice

242 also showed substantially reduced generation of superoxide compared with fibers from WT mice.

243 Inhibition of NOS to reduce NO availability and hence the potential for formation of peroxynitrite did

244 not affect DHE oxidation in fibers from WT or *SOD1KO* at rest or during contractions. In contrast

245 fibers isolated from nNOS transgenic mice showed increased DAF-FM fluorescence and reduced DHE

246 oxidation in resting muscle fibers. These data appear to indicate that peroxynitrite is formed in

247 muscle fibers as a consequence of lack of SOD1 in *SOD1KO* mice and may therefore contribute to

248 fiber loss in this model. More generally these data also support the hypothesis that NO regulates

249 superoxide availability and peroxynitrite formation in muscle fibers (78).

250

251 **Relative role of a lack of SOD1 in muscle or in motor neurons in the accelerated aging phenotype**

252 **seen in *SOD1KO* mice.**

253 In order to specifically examine how changes in muscle SOD1 might influence age-related changes in

254 muscle, mice with muscle specific deletion of SOD1 (*mSOD1KO* mice) were examined (99), but these

255 mice show no evidence of premature NMJ degeneration or loss of muscle fibers and surprisingly

256 showed some muscle hypertrophy (99). We examined whether the changes in ROS generation

257   observed in the global knockout model (*SOD1KO* mice) were also seen in *mSOD1KO* mice. In brief,

258   the multiple changes in markers of oxidative damage and adaptation seen in SOD1KO mice and

259   described above were not observed in the *mSOD1KO* mice including no evidence for the increases in

260   3-NT and peroxiredoxin V previously reported in muscles of *SOD1KO mice* (79, 99).

261

262   To determine the role of motor neurons in the loss of muscle mass and function seen in *SOD1KO*

263   mice, a transgenic *SOD1KO* mouse in which human SOD1 is expressed in neurons under control of a

264   synapsin 1 promoter (*nSOD1Tg-SOD1KO* mice) was established (80). These "nerve rescue" mice

265   expressed SOD1 in central and peripheral neurons but not other tissues. Sciatic nerve CuZnSOD

266   content in *nSOD1Tg-SOD1KO* mice was ~20% of WT control mice, but they showed no loss of muscle

267   mass or maximum isometric specific force production at 8-12 months of age, when significant

268   reductions were seen in *SOD1KO* mice (80). Thus these data appeared to demonstrate that at least

269   20% of WT CuZnSOD levels in neurons is essential in preserving skeletal muscle and NMJ structure

270   and function in SOD1KO mice and implicated a lack of SOD1 specifically in motor neurons in the

271   pathogenesis of the accelerated muscle aging phenotype seen in the whole body SOD1 null mice.

272

273   Adult mice lacking SOD1 therefore replicate many of the features seen in old WT mice and it appears

274   that further examination of this model and variants of the model with tissue specific modification of

275   SOD1 content could identify key mechanisms leading to loss of muscle fibers and function that are

276   relevant to aging of WT mice. The initiating role for the motor neuron in this model provides a

277   means of determining mechanisms by which disruption of redox homeostasis in the motor neuron

278   can cause loss of muscle fibers and we speculate that this may also be important for aging in WT

279   mice. Although *SOD1KO* mice are a model in which fundamental questions about mechanisms that

280   are highly relevant to understanding muscle aging can be addressed, it is reiterated that there is no

281   evidence that a simple lack of SOD1 contributes to aging-related loss of muscle in WT mice or

282   humans.

283

Potential primary and secondary sources of ROS during aging

Increased ROS generation by mitochondria has been implicated in aging of muscle and other tissues

for a considerable period of time. This process was originally claimed to have a primary role in the

aging process (38, 81), but the recent work of Richardson and colleagues (70) and Gemms (20)

argues strongly against a primary role for oxidative damage in skeletal muscle in aging. Other recent

data also indicate that not all mitochondria isolated from aging muscle show increased ROS

generation (21, 25). Despite these contrasting these data, some interventions that specifically

reduce mitochondrial ROS (mice overexpressing catalase in mitochondria - mCAT mice; 82) or

protect against oxidative damage (mice overexpressing heat shock protein 10 – HSP10^{Tg} mice; 44)

appear to preserve muscle mass and function. We have previously proposed that excess generation

of hydrogen peroxide by mitochondrial from aged mice could act to attenuate the ability of muscle

fibers from aged mice to adapt to contractile activity (34) as shown schematically in Figure 3b. It is

therefore relevant to consider whether increased mitochondrial ROS might play a secondary role in

ageing processes and be a consequence of more direct effects of aging. Potential examples of this

may be the increase in muscle mitochondrial ROS that appears to occur secondarily to other age-

related changes in the *SOD1KO* mouse studies described above, and also by the observation that

experimental denervation leads to a very large sustained increase in muscle mitochondrial ROS

generation (59). Data from both of these situations support the possibility that functional

denervation of individual muscle fibers may lead to a fiber specific increase in mitochondrial ROS

generation.

There is extensive evidence that some denervation of muscle fibers occurs with aging. In man a

~25% reduction in the number of motor neurons occurs with aging and although the causes of this

loss are unknown, small motor neurons (which tend to innervate type I fibers) are preserved relative

to large motor neurons. Over time, the loss of large motor neurons appears to be partially

309   compensated by a sprouting phenomenon through which small motor neurons innervate those type

310   II fibers that have become temporarily denervated and hence these fibers acquire a slower

311   phenotype. This process is thought to be incomplete and eventually the new "giant" motor units are

312   lost (13). Studies to determine whether the age-related loss of muscle fibers is associated with loss

313   of motor units in man and rodents indicate that substantial net loss of whole motor units occurs

314   with increasing age in both species (8, 18, 48). Atrophy and loss of axons has been reported in older

315   individuals (93), together with additional abnormalities in peripheral nerves, including segmental

316   demyelination (1, 83), swollen demyelinated and remyelinated axons and denervated Schwann cell

317   columns (26). A variety of changes have been reported in neuromusclular junctions (NMJs) of aged

318   mice including axonal swelling and sprouting, withdrawal of axons from postsynaptic sites, and

319   fragmentation of the postsynaptic structures (10, 86) and there is evidence from older post-mortem

320   studies that such changes are seen in elderly humans (62). Recent data from rodents also indicate

321   that despite the loss of peripheral axons that occurs with aging, the number of motor neuron cell

322   bodies in the lumbar spinal cord are unchanged suggesting that changes may predominantly occur in

323   peripheral regions of motor units (10). Thus it appears that motor axon and NMJ loss with aging

324   occurs in parallel with loss of muscle fibers and diminished muscle function (9, 49, 50) in both man

325   and animals, but it is currently unclear whether either of these is the primary event (48, 95).

326

327   Thus we speculate that a feasible integrating mechanism based on the current data relating to the

328   age-related changes in ROS activities and redox signaling in muscle is that denervation of individual

329   muscle fibers leads to a large increase in mitochondrial ROS generation in the affected fibers. Since

330   the key ROS generated in mitochondria of the denervated fibers appears to be hydrogen peroxide or

331   other peroxides, such species are membrane permeable and could diffuse to adjacent innervated

332   fibers leading to redox-related changes in oxidative damage and redox signaling.

333

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Conclusions

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336   In conclusion, recent data indicate that membrane-localized NADPH oxidase(s) are the source of the

337   superoxide generated in skeletal muscle during contractile activity that play an important role in

338   redox signaling and that these pathways upregulate cytoprotective responses that aid maintenance

339   of cell viability following contractile activity. A failure of this redox signaling pathway appears to

340   occur in muscle during aging and may contribute to the loss muscle fibers, but whether these

341   changes are primary or secondary events in aging is unclear. One possible explanation that provides

342   an explanation for the current data is that a small number of denervated muscle fibers within the

343   muscle may generate large amounts of hydrogen peroxide from mitochondria and that this can

344   influence redox signaling in adjacent innervated fibers and thus provides a secondary source of

345   reactive oxygen species that may contribute to loss of muscle mass and function during aging.

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628   **Legends to Figures**

629   **Figure 1.** Examples of data derived from different approaches to study ROS generation in muscle or
630   muscle fibers. **A**. Reduction in glutathione content of muscles from WT mice *in vivo* following a 15
631   minute period of isometric contractile activity. Redrawn from Vasilaki et al (90). **B**. Increase in
632   interstitial superoxide monitored by microdialysis in the gastrocnemius muscle of mice during a 15
633   minute period of isometric contractile activity. Redrawn from Close et al (11). **C.** Increase in
634   intracellular DCF fluorescence from fibers isolated from the flexor digitorum brevis (FDB) muscle of
635   mice and subjected to 15 minutes of isometric contractile activity *in vitro.* Redrawn from Palomero
636   et al (66). **D.** Increase in hydrogen peroxide content (indicated by increased *HyPer* fluorescence) in
637   fibers isolated from the FDB muscle of mice and subjected to 10 minutes of isometric contractile
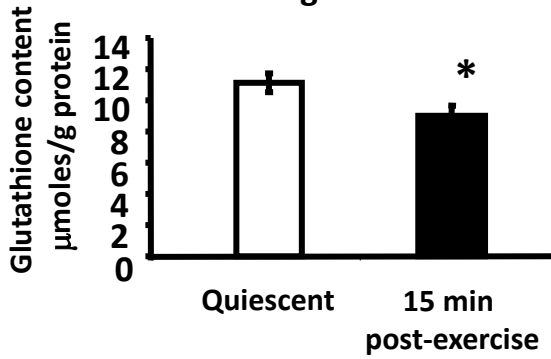638   activity *in vitro*. Redrawn from Pearson et al (69).
639
640   **Figure 2.** Updated working scheme for sites of ROS/RNS generation by skeletal muscle
641   demonstrating the potential role of Nox2 and Nox4 isoforms of NADPH oxidase in generating
642   superoxide in mitochondria and cytosol and acknowledging the lack of evidence for any release of
643   superoxide from mitochondrial during contractile activity. Modified from Jackson (33).
644
645   **Figure 3. A.** Schematic representation of the redox signaling pathways that are postulated to lead to
646   adaptive activation of transcription factors and upregulation of the expression of cytoprotective
647   proteins following contractile activity in skeletal muscle. TF = transcription factor. Redrawn and
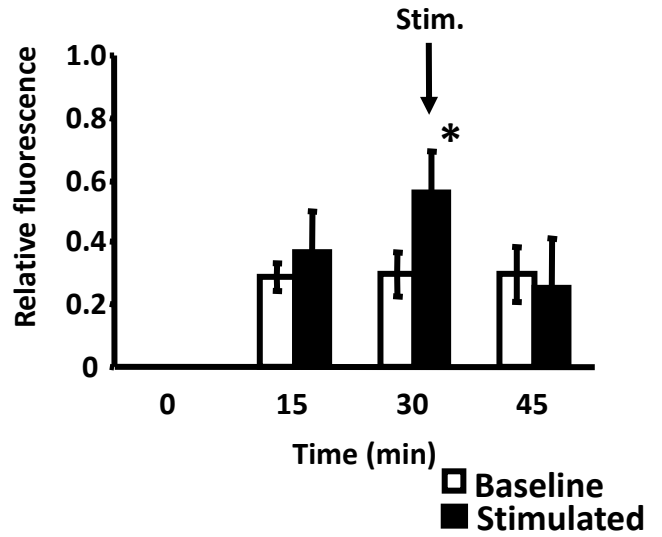648   updated from Jackson (33).
649   **B**. Putative sites at which the redox signaling pathway may be modified in aging leading to a failure
650   of adaptive responses to contractile activity. Excess hydrogen peroxide generated by mitochondria
651   in the muscle during aging may influence the pathway shown in Figure **3A** at multiple points:
652   prevention of activation of NADPH oxidase; a chronic increase in cytosolic hydrogen peroxide;
653   aberrant chronic oxidation of glutathione and other redox sensitive signaling proteins; oxidation of
654   the nuclear environment leading to a failure of TF to activate transcription.
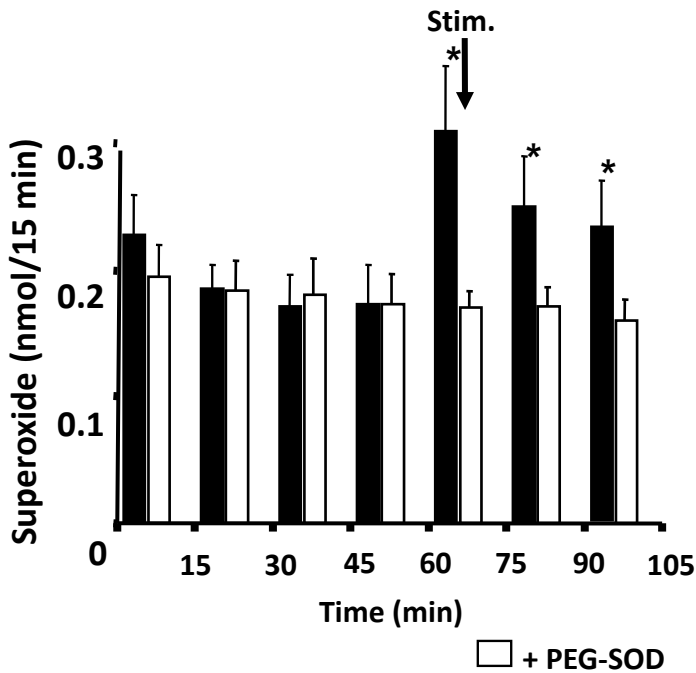655

A. **Effect of contractile activity in vivo on muscle glutathione**

Glutathione content µmoles/g protein

14
12
10
8
6
4
2
0

Quiescent   15 min post-exercise

*

C. **DCF fluorescence from isolated fibers**

Relative fluorescence

Stim.

1.0
0.8
0.6
0.4
0.2
0

0   15   30   45

*

Time (min)

□ Baseline
■ Stimulated

B. **In vivo release of superoxide from muscle**

Stim.
*

Superoxide (nmol/15 min)

0.3

0.2

0.1

0

0   15   30   45   60   75   90   105

*   *

Time (min)

□ + PEG-SOD

D. **HyPer fluorescence from isolated fibers**

Net change in HyPer fluorescence (ratio/10 min)

1.25

1.00

0.75

0.50

0.25

0.00

Stim

0   10   20   30

*

Time (min)

■ Baseline
□ Stimulated

Figure 1

Figure 2

A.

Contractions

↓

Activation of NADPH oxidase (Nox2)

↓

↑ H_2O_2 → Oxidation of GSH and adjacent redox-sensitive proteins → Secondary oxidation by disulphide exchange of key signaling molecules

↓

Activation of TFs (NFκB, AP1, HSF1, Nrf2 etc)

↓

Nucleus

Reduced environment

TF

B.

Contractions

↓

No activation of NADPH oxidase (Nox2)

↑ H_2O_2 → Oxidation of GSH and adjacent redox-sensitive proteins → Secondary oxidation by disulphide exchange of key signaling molecules

↓

Activation of TFs (NFκB, AP1, HSF1, Nrf2 etc)

↓

Nucleus

Oxidised environment

TF

H_2O_2

$O_2^{\cdot-}$

Mitochondria

Figure 3