TOPICAL REVIEW

Reactive oxygen/nitrogen species and contractile function in skeletal muscle during fatigue and recovery

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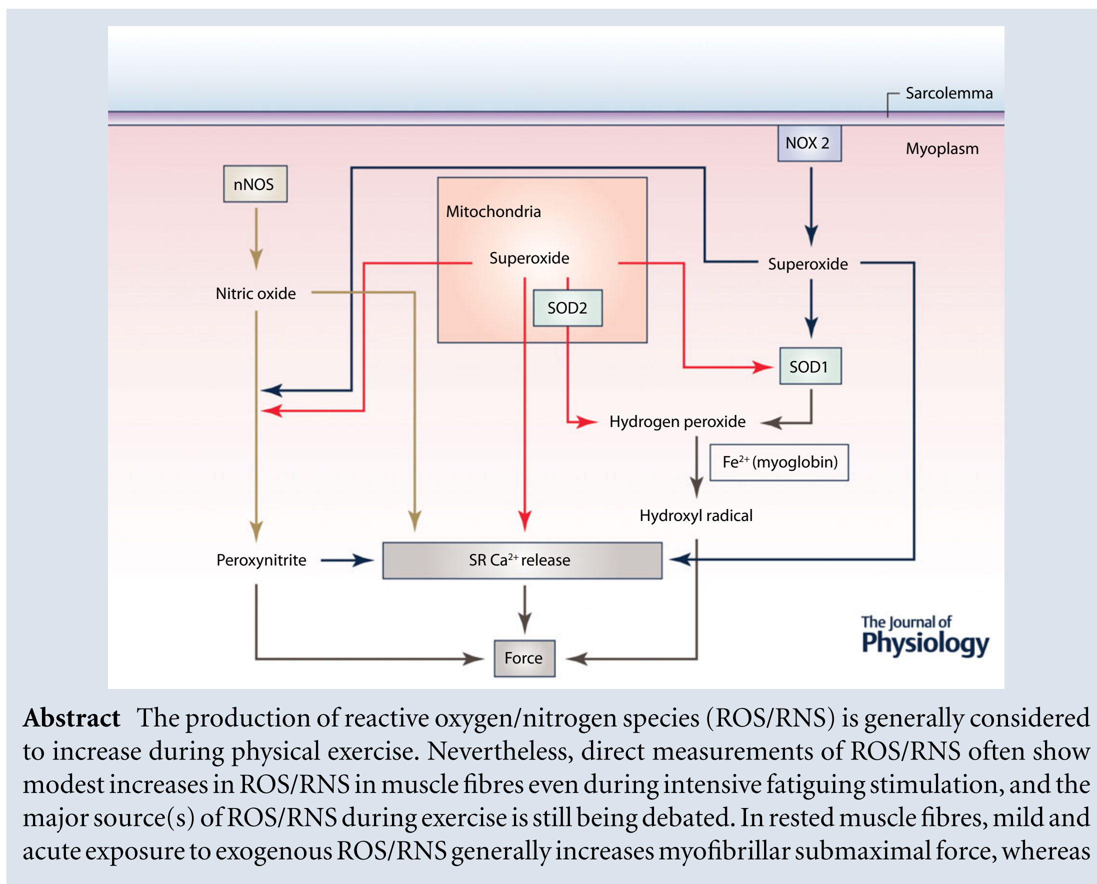

Abstract The production of reactive oxygen/nitrogen species (ROS/RNS) is generally considered to increase during physical exercise. Nevertheless, direct measurements of ROS/RNS often show modest increases in ROS/RNS in muscle fibres even during intensive fatiguing stimulation, and the major source(s) of ROS/RNS during exercise is still being debated. In rested muscle fibres, mild and acute exposure to exogenous ROS/RNS generally increases myofibrillar submaximal force, whereas

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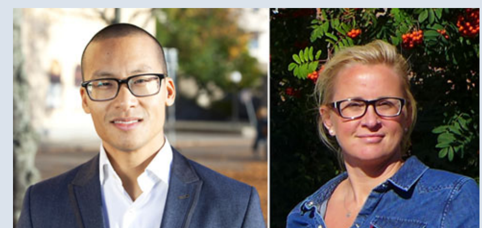

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DOI: 10.1113/JP270650

The Journal of Physiology

stronger or prolonged exposure has the opposite effect. Endogenous production of ROS/RNS seems to preferentially decrease submaximal force and positive effects of antioxidants are mainly observed during fatigue induced by submaximal contractions. Fatigued muscle fibres frequently enter a prolonged state of reduced submaximal force, which is caused by a ROS/RNS-dependent decrease in sarcoplasmic reticulum Ca^{2+} release and/or myofibrillar Ca^{2+} sensitivity. Increased ROS/RNS production during exercise can also be beneficial and recent human and animal studies show that antioxidant supplementation can hamper the beneficial effects of endurance training. In conclusion, increased ROS/RNS production have both beneficial and detrimental effects on skeletal muscle function and the outcome depends on a combination of factors: the type of ROS/RNS; the magnitude, duration and location of ROS/RNS production; and the defence systems, including both endogenous and exogenous antioxidants.

(Received 29 September 2015; accepted after revision 23 December 2015; first published online 9 February 2016)
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Abstract figure legend Schematic diagram showing major sources and interactions of ROS/RNS that affect sarcoplasmic reticulum Ca^{2+} release and force production during skeletal muscle fatigue and recovery.

Abbreviations $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; DTT, dithiothreitol; eNOS, endothelial $NO^{\bullet}$ synthase; FDB, flexor digitorum brevis; GSH, reduced glutathione; iNOS, inducible $NO^{\bullet}$ synthase; NAC, *N*-acetylcysteine; NOS, $NO^{\bullet}$ synthase; nNOS, neuronal $NO^{\bullet}$ synthase; NOX, NADPH oxidase; PLFFD, prolonged low-frequency force depression; RNS, reactive nitrogen species; roGFP, redox-sensitive green fluorescent protein; ROS, reactive oxygen species; RyR, ryanodine receptor; SOD, superoxide dismutase; SR, sarcoplasmic reticulum; *t*-BOOH, *tert*-butyl hydroperoxide; Tn, troponin.

Introduction

In a classical study, Reid *et al.* showed that the general antioxidant *N*-acetylcysteine (NAC) mitigated the force decline when human subjects performed repeated submaximal contractions (Reid *et al.* 1994). These results imply that the production of reactive oxygen/nitrogen species (ROS/RNS) increases in skeletal muscle during physical exercise and ameliorating the resulting 'oxidative stress' with antioxidants lessens the force decrease. Moreover, several studies show increases in ROS/RNS in conditions with skeletal muscle dysfunction and muscle weakness, such as rheumatoid arthritis (Yamada *et al.* 2015*a*,*b*), Duchenne muscle dystrophy (Khairallah *et al.* 2012), malignant hyperthermia (Lanner *et al.* 2012), and in normal ageing (Andersson *et al.* 2011). On the other hand, submaximal force in intact and skinned fast-twitch skeletal muscle fibres has been shown to increase with acute exposure to the ROS hydrogen peroxide (H_2O_2) (Andrade *et al.* 1998*a*, 2001; Murphy *et al.* 2008; Mollica *et al.* 2012). Furthermore, recent human and animal studies show that treatment with antioxidants hampers the beneficial effects of endurance training (Gomez-Cabrera *et al.* 2008; Ristow *et al.* 2009; Paulsen *et al.* 2014). Thus, increases in ROS/RNS can have both beneficial and detrimental effects on skeletal muscle contractile function and fitness, and the outcome probably depends on a combination of factors: the type of ROS/RNS, the size of ROS/RNS increase, the duration of ROS/RNS elevation (e.g. milliseconds *vs.* hours), and the localization of ROS/RNS production and accumulation (Droge, 2002; Westerblad & Allen, 2011; Ristow, 2014). In the first part of this review, we will discuss the metabolism and sources of ROS/RNS that are likely to increase during exercise and ways to measure them. In the latter part, we will discuss ROS/RNS effects on muscle fibre contractility during exercise (i.e. during induction of fatigue) and in the subsequent recovery phase.

ROS/RNS in contracting muscle: metabolism, sources and methods to measure

A free radical is an atom, molecule or ion with unpaired valency electron(s), generally making them highly unstable and reactive (Halliwell & Gutteridge, 1984). The dominant ROS in cells are superoxide ($O_2^{\bullet -}$) and its downstream derivatives, such as H_2O_2. Similarly, the central RNS in cells are nitric oxide ($NO^{\bullet}$) along with its downstream derivatives, such as peroxynitrite ($ONOO^{\bullet -}$). ROS/RNS production in skeletal muscle is generally considered to increase during physical exercise (Powers & Jackson, 2008). However, the direct mechanisms and sources of the increased ROS/RNS production during exercise remain uncertain and they are likely to differ depending on the type of activity, e.g. endurance *vs.* resistance training or short-term high-intensity *vs.* prolonged low-intensity exercise. Moreover, direct measurements of ROS/RNS during exercise are relatively rare and the increases detected

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with conventional fluorescent indicators are often rather modest (e.g. Reid *et al.* 1992; Pye *et al.* 2007; Sakellariou *et al.* 2013; Cheng *et al.* 2015), which implies that such measurements are methodologically problematic.

Superoxide. $O_2^{\bullet -}$ is generated through either incomplete reduction of oxygen in the mitochondrial electron transport chain or as a specific product of enzymatic reactions. $O_2^{\bullet -}$ is negatively charged and hence relatively impermeable to cell membranes. It has a relatively long half-life (~1 μs), which permits diffusion within the muscle cell and allows interaction with several cellular targets (Winkler *et al.* 1999). Calculations in endothelial cells indicate that the steady-state cellular concentration of $O_2^{\bullet -}$ is in the pico- to nanomolar range (Carballal *et al.* 2014).

Complexes I and III are the two major sites of $O_2^{\bullet -}$ production in the mitochondria (Cadenas *et al.* 1977; Turrens & Boveris, 1980; Murphy, 2009; Tahara *et al.* 2009; Quinlan *et al.* 2012). Early reports suggested that 2–5% of the total oxygen consumed by mitochondria was reduced to $O_2^{\bullet -}$ (Boveris & Chance, 1973; Loschen *et al.* 1974). This measure of $O_2^{\bullet -}$ production seems far too high in contracting skeletal muscle fibres, because oxygen consumption increases massively during intense exercise and prolonged exercise would then result in dangerously high ROS levels, severe oxidative stress, and muscle damage (Westerblad & Allen, 2011). Accordingly, more recent data indicate that only ~0.1–0.2% of the oxygen consumed by the mitochondria forms $O_2^{\bullet -}$ (St-Pierre *et al.* 2002; Murphy, 2009; Tahara *et al.* 2009; Brand, 2010). The extent of mitochondrial $O_2^{\bullet -}$ production in muscle also critically depends on other factors, such as sub- *vs.* supramaximal exercise intensity or sufficient *vs.* limited O_2 delivery. For instance, mitochondrial ROS production appears to be higher during state 4 (basal) than during state 3 (maximal ADP-stimulated) respiration, and the latter dominates during aerobic exercise (Powers & Jackson, 2008). Thus, only a very small fraction of the oxygen consumed by the mitochondria during exercise is reduced to $O_2^{\bullet -}$ and there is no fixed relation between the rates of mitochondrial oxygen consumption and $O_2^{\bullet -}$ production.

Enzymes that produce $O_2^{\bullet -}$ in skeletal muscle include NADPH oxidase (NOX) (Pal *et al.* 2013), phospholipase A_2 (Nethery *et al.* 1999), xanthine oxidase (Gomez-Cabrera *et al.* 2010) and uncoupled $NO^{\bullet}$ synthase (NOS) (Stuehr *et al.* 2001). Of these enzymes, NOX has received most recent attention and is proposed to be a major contributor to $O_2^{\bullet -}$ production in skeletal muscle both at rest and during contractile activities (Michaelson *et al.* 2010; Pal *et al.* 2013; Sakellariou *et al.* 2013). A skeletal muscle fibre expresses two NOX isoforms, NOX2 and NOX4, and of these NOX2 has received most attention (Sakellariou *et al.* 2014). NOX2 has several subunits and is localized in the sarcolemma, either at the surface or in the t-tubular system (Javeshghani *et al.* 2002; Hidalgo *et al.* 2006; Sakellariou *et al.* 2014). NOX4 is less studied in skeletal muscle. It has been proposed to be localized in the sarcoplasmic reticulum (SR) where it may affect the Ca^{2+} release channel (ryanodine receptor 1; RyR1) (Sun *et al.* 2011; Sakellariou *et al.* 2014).

For decades, mitochondria have been considered as the major site for $O_2^{\bullet -}$ production in contracting skeletal muscle. Accordingly, a recent study using mouse flexor digitorum brevis (FDB) fibres transfected with a novel mitochondrial-targeted superoxide biosensor (mt-cpYFP) shows strictly localized mitochondrial $O_2^{\bullet -}$ production during repetitive contractions (Wei *et al.* 2011). Moreover, pretreatment with a mitochondrial-targeted antioxidant (SS-31) decreased ROS production, as measured with the fluorescent ROS indicator MitoSOX Red, in isolated mouse muscle fibres during repeated tetanic contractions (Cheng *et al.* 2015). Conversely, recent studies propose NOX2 as the main producer of ROS in contracting skeletal muscles, because the contraction-mediated increase in cytosolic ROS was prevented by pharmacological inhibition or genetic knockdown of NOX2 (Michaelson *et al.* 2010; Pal *et al.* 2013; Sakellariou *et al.* 2013). Thus, there are conflicting results regarding the importance of different sources of $O_2^{\bullet -}$ in contracting muscle and further studies are required to resolve this issue.

Hydrogen peroxide. Dismutation of $O_2^{\bullet -}$, both spontaneous and catalysed by superoxide dismutase (SOD), constitutes the major source of H_2O_2 in muscle cells ($2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$). Two out of three SOD isoforms are highly abundant (~10–20 μM) within the skeletal muscle fibres: SOD1 requires copper–zinc as a cofactor and is located in the cytosol and in the mitochondrial intermembrane space; SOD2 uses manganese as a cofactor and is located in the mitochondrial matrix (Powers & Jackson, 2008). Of the total SOD activity in skeletal muscle fibres, ~15–35% is in the mitochondria and the remaining 65–85% in the cytosol. The highest SOD activities are present in oxidative slow-twitch muscle fibres and endurance exercise increases SOD activity in both slow- and fast-twitch fibres (Higuchi *et al.* 1985; Powers *et al.* 1994; Oh-ishi *et al.* 1997; Bruton *et al.* 2008). H_2O_2 is cell permeable and relatively stable with a half-life of seconds to minutes. The concentration of H_2O_2 in skeletal muscle has been calculated to be ~10–100 nM at rest and to increase to ~100–200 nM during contractions (Vasilaki *et al.* 2006). In comparison to other ROS, H_2O_2 is a relatively weak oxidizing agent, but in the presence of Fe^{2+} it can be converted into the highly reactive and cytotoxic hydroxyl radical ($OH^{\bullet}$) (Halliwell & Gutteridge, 1984; Powers & Jackson, 2008). In skeletal muscle, H_2O_2 is metabolized to H_2O via three major antioxidant enzymatic systems: glutathione peroxidases ($H_2O_2 + 2GSH \rightarrow 2H_2O +$

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GSSG), where GSH is reduced glutathione and GSSG is oxidized glutathione; catalase ($2H_2O_2 \rightarrow 2H_2O + O_2$); and peroxiredoxins (PRX(reduced) + $H_2O_2 \rightarrow$ PRX(oxidized) + $2H_2O$) (Sakellariou *et al.* 2014).

Methods to measure ROS. Quantitative measurements of ROS in skeletal muscle are rare and this is to a large extent due to the fact that methods available to measure $O_2^{\bullet-}$ and H_2O_2 production have severe shortcomings. Many studies of mitochondrial ROS production are performed with *in vitro* measurements on isolated mitochondria. This approach is problematic because the techniques used to isolate mitochondria from muscle alter mitochondrial structure and function by, for instance, causing fragmentation, loss of soluble matrix enzymes and altered respiratory rates and $O_2^{\bullet-}/H_2O_2$ production (Schwerzmann *et al.* 1989; Picard *et al.* 2011*a*,*b*). Fluorescent ROS indicators (e.g. dichlorofluorescein, dihydroethidium, MitoSOX Red) commonly used to assess changes in ROS in intact muscle fibres have limitations in that they, for instance, are not specific to a certain ROS (Kalyanaraman *et al.* 2012). Moreover, increases in the overall fluorescence of these indicators in response to repeated contractions are relatively small (e.g. Reid *et al.* 1992; Pye *et al.* 2007; Sakellariou *et al.* 2013; Cheng *et al.* 2015) and therefore it is difficult to assess contraction-induced ROS changes in a quantitative and spatially/temporally confined manner. To deal with the limited specificity of these fluorescent ROS indicators, it has been suggested that they should be combined with HPLC-based methods (Kalyanaraman *et al.* 2012), but such methods are cumbersome when following real-time changes in ROS, e.g. during repeated contractions and in the following recovery period.

Promising new tools for dynamic and site-specific assessment of ROS have recently been developed and these will hopefully lead to a better understanding of the role(s) of ROS in muscle biology. For instance, genetically engineered fusion of redox-sensitive green fluorescent protein (roGFP) and the peroxide Orp1 has been used to measure the concentration of H_2O_2 (Gutscher *et al.* 2009). Furthermore, Rodney and co-workers recently used roGFP fused to the regulatory subunit $p47^{phox}$ of NOX2 for real-time ROS measurements in contracting mouse skeletal muscle fibres (Pal *et al.* 2013). Furthermore, roGFP-based ROS indicators are relatively sensitive, reversible, ratiometric (i.e. independent of changes in indicator concentration) and pH insensitive (Hanson *et al.* 2004; Gutscher *et al.* 2009; Meyer & Dick, 2010; Pal *et al.* 2013). Thus, genetically engineered fusion proteins constitute a novel group of ROS indicators that allow more specific detection of one ROS and can be targeted to organelles or proteins (e.g. mitochondria or NOX2).

Nitric oxide. $NO^{\bullet}$ is a versatile biological signalling molecule that is generated via enzymatic reactions of nitric oxide synthase (NOS) and the production increases in muscle fibres during repeated contractions (Pye *et al.* 2007; Cheng *et al.* 2015). $NO^{\bullet}$ can also be formed from the inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) (Weitzberg *et al.* 2010). Skeletal muscle constitutively expresses neuronal and endothelial NOS (nNOS and eNOS, respectively), whereas inducible NOS (iNOS) is upregulated in response to acute inflammatory insults. Despite its name, nNOS is expressed at higher levels in human skeletal muscle than in human brain (Nakane *et al.* 1993). $NO^{\bullet}$ is synthesized by NOS from L-arginine, NADPH and O_2. nNOS and eNOS are activated by increases in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Forstermann *et al.* 1994). The K_m of $[Ca^{2+}]_i$ for the activation of nNOS is ~200 nM and $[Ca^{2+}]_i$ reaches higher levels during contractions (>1 μM), hence nNOS will become highly active during contractile activities (Bredt & Snyder, 1990). Under normal conditions, nNOS is mostly compartmentalized to submembrane scaffolds that are part of the dystrophin glycoprotein complex (Brenman *et al.* 1995). In addition, minor fractions of nNOS are detectable in association with the SR and with mitochondria (Buchwalow *et al.* 2005). In line with this, data from our group and others show that some nNOS co-localize with the RyR1 in skeletal muscle from mouse and human subjects (Salanova *et al.* 2008; Yamada *et al.* 2015*b*). Interestingly, the amount of nNOS co-localized with RyR1 was markedly increased in muscles from mice with collagen-induced arthritis (Yamada *et al.* 2015*b*).

$NO^{\bullet}$ on its own is relatively stable but it reacts rapidly with numerous cellular targets, which results in a biological half-life time of ~0.3 s in skeletal muscle under physiological conditions (Thomas *et al.* 2001). Nevertheless, $NO^{\bullet}$ is small, uncharged and freely diffusible through membranes and is therefore considered to exert effects over distances even exceeding 100 μm (Thomas *et al.* 2001). The concentration of $NO^{\bullet}$ has been calculated to be ~20 nM in resting rat diaphragm muscle fibres (Boczkowski *et al.* 1999).

Peroxynitrite. $ONOO^{\bullet-}$, formed when $NO^{\bullet}$ reacts with $O_2^{\bullet-}$, is a potent oxidizing and nitrating agent able to react with a wide range of biomolecules. The biological half-life under physiological conditions is ~10 ms and it is estimated to influence cellular targets within ~5–20 μm (Radi, 1998; Romero *et al.* 1999; Szabó *et al.* 2007; Carballal *et al.* 2014). Calculations in endothelial cells indicate that the steady-state level of $ONOO^{\bullet-}$ is ~1 nM (Carballal *et al.* 2014).

The rate constant of $ONOO^{\bullet-}$ formation has been estimated to be within the range of (4–16) × 10^9 M^{-1} s^{-1} (Goldstein & Czapski, 1995; Botti *et al.* 2010), which is higher than the rate of SOD conversion of $O_2^{\bullet-}$ to H_2O_2 ((1–2) × 10^9 M^{-1} s^{-1}) (Klug-Roth *et al.* 1973; Hsu *et al.*

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1996). Thus, when $NO^{\bullet}$ is produced at a high rate, it will rapidly react with $O_2^{\bullet-}$ to produce significant amounts of $ONOO^{\bullet-}$ even in the presence of the high physiological concentrations of SOD (∼10–20 μM). In fact, the formation of $ONOO^{\bullet-}$ from $NO^{\bullet}$ and $O_2^{\bullet-}$ can occur about six times faster than the rate at which SOD can convert $O_2^{\bullet-}$ to H_2O_2 (Beckman & Koppenol, 1996). Peroxiredoxins represent an efficient detoxification system for $ONOO^{\bullet-}$ and CO_2 accounts for another fraction of the $ONOO^{\bullet-}$ consumption by forming carbonate radicals and nitrogen dioxide (Carballal *et al.* 2014). On the other hand, GSH (present at mM concentration in muscles) does not react sufficiently fast *in vivo* to directly scavenge $ONOO^{\bullet-}$; instead, glutathione may counteract $ONOO^{\bullet-}$-dependent processes via reactions with secondary radicals (Ferrer-Sueta & Radi, 2009).

Acute effects of ROS/RNS on myofibrillar function in skeletal muscle

The intracellular events leading to contraction of skeletal muscle fibres start with RyR1-mediated Ca^{2+} release from SR. Ca^{2+} then binds to the troponin (Tn) complex consisting of TnC, TnI and TnT. Ca^{2+} binding to the Tn–Ca^{2+} complex moves the position of the tropomyosin filaments, hence turning on cross-bridge cycling and contraction by uncovering active sites of actin for myosin binding (Gordon *et al.* 2000). In addition, the degree of myofibrillar activation depends on the kinetics of cross-bridge attachment and detachment to actin, because an actin-bound cross-bridge exerts mechanical impact on tropomyosin that facilitates the binding of neighbouring cross-bridges (Brenner, 1988; Gordon *et al.* 2000). The result of these interacting processes is the steep, sigmoidal force–$[Ca^{2+}]_i$ relationship. Changes in the Tn–Ca^{2+} interaction and/or cross-bridge kinetics can affect the steepness of the force–$[Ca^{2+}]_i$ relationship as well as the position on the $[Ca^{2+}]_i$ axis; for simplicity, we will refer to alterations in the latter parameter as changes in myofibrillar Ca^{2+} sensitivity. Generally, acute ROS/RNS-mediated changes in force generation are more marked with activation at submaximal than at maximal frequencies (Lamb & Westerblad, 2011). Submaximal contractions occur on the steep part of the force–$[Ca^{2+}]_i$ relationship, which means that even small changes in myofibrillar Ca^{2+} sensitivity, or SR Ca^{2+} release, have large effects on the generated force (Fig. 1). In this context it is worth noting that everyday activities generally require low to moderate forces and the firing frequencies of motor units are therefore set to produce submaximal contractions (Marsden *et al.* 1971; Grimby & Hannerz, 1977)

Intact fast-twitch mouse muscle fibres acutely exposed to the reducing agent dithiothreitol (DTT) showed decreased submaximal (30–60 Hz) force. Acute exposure to H_2O_2, or its non-metabolizable analogue *tert*-butyl hydroperoxide (*t*-BOOH), has the opposite effect, that is, it increased submaximal force (Andrade *et al.* 1998*a*, 2001; Cheng *et al.* 2015). The effect on $[Ca^{2+}]_i$ during contractions was small both with DTT and H_2O_2/*t*-BOOH exposure, hence the changes in force production were explained by altered myofibrillar Ca^{2+} sensitivity. The force-potentiating effect of H_2O_2/*t*-BOOH exposure was transient and prolonged (more than 5 min) exposures resulted in a marked decrease in submaximal force, again accompanied by only minor changes in tetanic $[Ca^{2+}]_i$ (Andrade *et al.* 1998*a*, 2001; Cheng *et al.* 2015) (Fig. 2*A*). Intriguingly, the depression of submaximal force induced by prolonged exposure to H_2O_2 was reversed by exposure to DTT and, vice versa, the depression induced by initial exposure to DTT was reversed by H_2O_2 (Andrade *et al.* 1998*a*) (Fig. 2*B*). To sum up, myofibrillar Ca^{2+} sensitivity is highly susceptible to acute exogenous exposure to oxidizing and reducing agents, the effect is readily reversed by the opposite redox challenge, and rested muscle fibres appear to be in a suboptimally reduced state (Andrade *et al.* 1998*a*; Lamb & Westerblad, 2011; Powers *et al.* 2011).

The effect of H_2O_2 application on the myofibrillar Ca^{2+} sensitivity of skinned fast-twitch fibres differs markedly depending on the presence or absence of myoglobin and glutathione, which are normally present in intact skeletal muscle fibres (Murphy *et al.* 2008; Lamb & Westerblad, 2011). Thus, application of H_2O_2 on its own had little effect in skinned rat fast-twitch fibres, whereas myofibrillar Ca^{2+} sensitivity was severely decreased in the presence of myoglobin. The proposed mechanism was that H_2O_2, through the Fenton reaction, interacts with Fe^{2+} on myoglobin to produce the highly reactive $OH^{\bullet}$

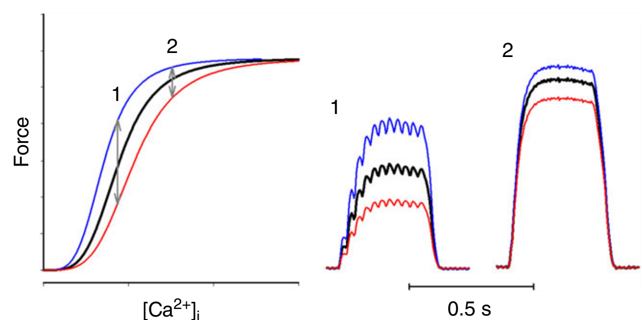

Figure 1. Changes in myofibrillar Ca^{2+} sensitivity have a much larger effect on submaximal than on maximal force
Schematic representation of the effect of increased (blue lines) and decreased (red lines) myofibrillar Ca^{2+} sensitivity. (1) Stimulation at frequencies giving unfused tetani results in forces on the steep part of the force–$[Ca^{2+}]_i$ relationship and changes in sensitivity have a large effect (∼40% in the example) on force output. (2) Conversely, the same changes in sensitivity have little effect (here ∼10%) at higher stimulation frequencies and fused tetani. Similar changes occur with changes in tetanic $[Ca^{2+}]_i$, i.e. larger effects in unfused contractions.

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(Murphy *et al.* 2008). On the other hand, application of H_2O_2 to skinned fast-twitch fibres in the presence of myoglobin *and* glutathione resulted in an initial increase in myofibrillar Ca^{2+} sensitivity followed by a decrease (Murphy *et al.* 2008), i.e. a pattern very similar to that observed when intact fast-twitch fibres are exposed to H_2O_2 or *t*-BOOH (see Fig. 2). Subsequent experiments on skinned fast-twitch rat and human muscle fibres revealed a likely mechanism for the initial increase in myofibrillar Ca^{2+} sensitivity: GSH reacts with oxidized cysteine residues on the fast isoform of TnI (TnI_f; probably Cys133) and the resulting *S*-glutathionylation increases myofibrillar Ca^{2+} sensitivity (Mollica *et al.* 2012). Accordingly, human muscle showed a marked increase in TnI_f *S*-glutathionylation following 40 min of low-intensity cycling at ∼60% peak oxygen consumption (Mollica *et al.* 2012), and a recent study shows that repeated stimulation can increase myofibrillar Ca^{2+} sensitivity by *S*-glutathionylation of TnI_f in *in situ* experiments performed on rat gastrocnemius muscles (Watanabe *et al.* 2015). Notably, no corresponding increase in myofibrillar Ca^{2+} sensitivity was observed in mammalian slow-twitch fibres, or in chicken and toad fibres, which have TnI isoforms that lack the equivalent of Cys133 (Mollica *et al.* 2012).

Intact fast-twitch fibres showed several changes (albeit relatively minor, ∼5%) in cross-bridge function in response to acute exposure to either H_2O_2 or *t*-BOOH: decreased maximum shortening velocity, increased maximum force production (i.e. force at saturating $[Ca^{2+}]_i$), and increased rate of force redevelopment after shortening (Andrade *et al.* 2001). The same treatment resulted in increased myofibrillar Ca^{2+} sensitivity and the faster force redevelopment might contribute to this increase (Brenner, 1988). The concentration of peroxide used by Andrade *et al.* (1 μM) would be within or close to the physiological range and hence endogenous increases in H_2O_2 occurring during, for instance, high-intensity exercise are likely to affect cross-bridge function (Andrade *et al.* 2001).

What about the effects of acute exposure to ROS/RNS other than H_2O_2 on myofibrillar function? Myofibrillar Ca^{2+} sensitivity appears not to be affected by $O_2^{\bullet-}$ (Bruton *et al.* 2008; Murphy *et al.* 2008). Acute exposure to $NO^{\bullet}$ donors leads to decreased myofibrillar Ca^{2+} sensitivity (Andrade *et al.* 1998*b*; Dutka *et al.* 2011). Studies on intact muscle fibres exposed to $NO^{\bullet}$ donors indicate that the $NO^{\bullet}$-induced decrease in Ca^{2+} sensitivity is not caused by altered cross-bridge kinetics (Morrison *et al.* 1996; Andrade *et al.* 1998*b*) and therefore it would be due to impaired Ca^{2+}–Tn interaction. $ONOO^{\bullet-}$ is known to decrease both myofibrillar Ca^{2+} sensitivity and maximum force in slow- and fast-twitch muscles (Dutka *et al.* 2011).

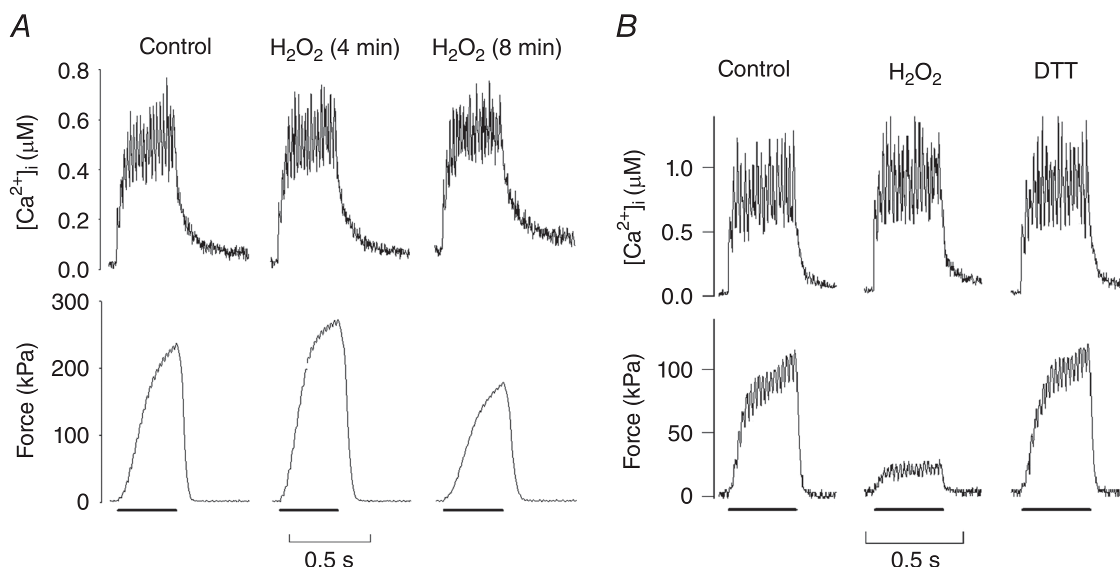

Figure 2. Transient and reversible effects of H_2O_2 on myofibrillar contractile function
Original $[Ca^{2+}]_i$ and force records from submaximal (50 Hz) contractions of single FDB muscle fibres exposed to H_2O_2 (300 μM) for up to 8 min (*A*) and H_2O_2 for 6 min followed by exposure to the reducing agent DTT (1 mM) for 10 min (*B*). Note that H_2O_2 causes major force changes whereas $[Ca^{2+}]_i$ is little affected, which means that H_2O_2 mainly acts at the myofibrillar level. The effects on force are time dependent with brief H_2O_2 exposure resulting in increased submaximal force, which is followed by a progressive force decline (*A*). Furthermore, the force depression caused by prolonged H_2O_2 exposure is reversed by reduction with DTT (*B*). Conversely, prolonged exposure to DTT results in depressed submaximal force that can be reversed by application of H_2O_2 (not shown). Figure adapted from Andrade *et al.* (1998).

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Effects of ROS/RNS on contractile function during fatigue and recovery

Studies of human exercise show a clear positive effect of decreased ROS/RNS on endurance when fatigue is induced by submaximal contractions, whereas the effect is small or absent with maximal contractions (Reid *et al.* 1994; Powers *et al.* 2011). 'Classical' fatigue-causing factors may dominate during the latter type of exercise and potential effects of ROS/RNS would therefore be difficult to discern (Allen *et al.* 2008). Such 'classical' factors that contribute to the force decrease in acute fatigue include accumulation of inorganic phosphate ions due breakdown of creatine phosphate (Dahlstedt *et al.* 2003), depletion of inter- and intramyofibrillar glycogen (Ørtenblad *et al.* 2013; Nielsen *et al.* 2014), and impaired action potential propagation (Pedersen *et al.* 2004; de Paoli *et al.* 2013). In a general sense, the positive effects of reducing ROS/RNS during fatigue with submaximal contractions fit with the fact that acute ROS/RNS effects are most marked on the steep part of the force–Ca^{2+} relationship (see above). However, acute exogenous application of H_2O_2 results in a transient increase in myofibrillar Ca^{2+} sensitivity (see Fig. 2) and some skinned fibre experiments show increased rather than decreased myofibrillar Ca^{2+} sensitivity after fatiguing contractions (Gejl *et al.* 2016; Watanabe *et al.* 2015). Thus, these results suggest that reducing ROS/RNS during fatigue would impair rather than improve performance. One tentative explanation for this apparent conflict is that exogenously applied and endogenously produced H_2O_2 have different effects. Another tentative explanation is that the effect of increases in ROS/RNS other than H_2O_2 dominates; for instance, both $NO^•$ and $ONOO^{•-}$ have been shown to decrease myofibrillar Ca^{2+} sensitivity (Andrade *et al.* 1998*b*; Dutka *et al.* 2011). Furthermore, deleterious effects of oxidants may overpower any potentiating effects when the physical activity is prolonged and/or highly intense (Lamb & Westerblad, 2011).

In contrast to the situation during the actual induction of acute fatigue, obvious effects of increased ROS/RNS are seen during the subsequent recovery period (Westerblad & Allen, 2011). For instance, isolated mouse soleus fibres did not fatigue prematurely when exposed to severe oxidative stress (fatigued at 43°C in the presence of 10 μM H_2O_2 or *t*-BOOH), but contractures developed and the fibres died ∼10 min after the end of stimulation (Place *et al.* 2009). Under less extreme conditions, fatigued muscle fibres frequently enter a prolonged state of severely depressed submaximal force, i.e. prolonged low-frequency force depression (PLFFD) (Allen *et al.* 2008). At the muscle fibre level, depressed submaximal force can be due to decreased SR Ca^{2+} release

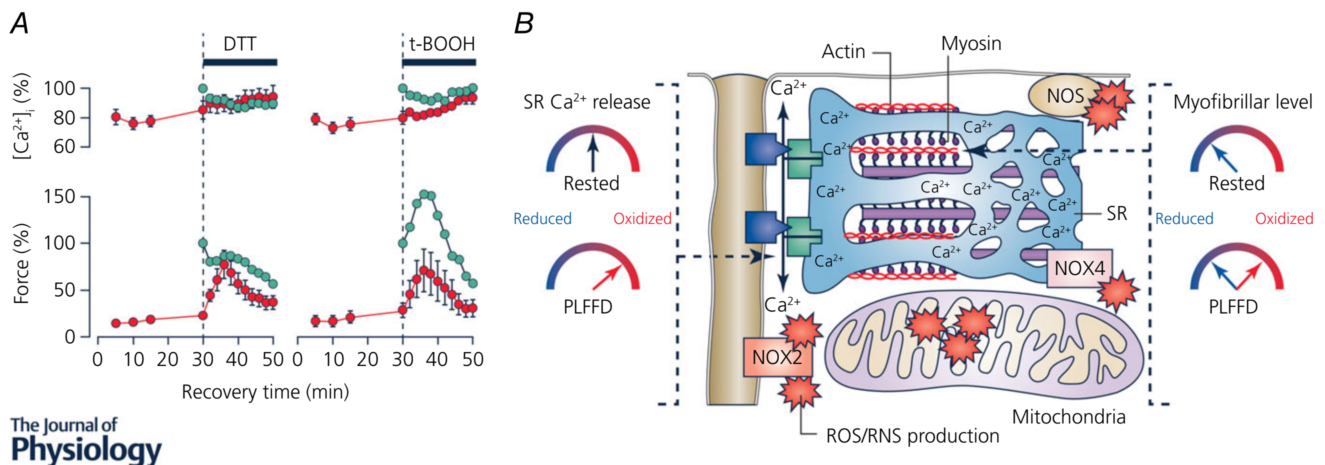

Figure 3. The prolonged low-frequency force depression (PLFFD) after fatiguing stimulation is the result of complex ROS/RNS effects on SR Ca^{2+} release and myofibrillar Ca^{2+} sensitivity
A, $[Ca^{2+}]_i$ (upper panel) and force (lower panel; mean data ± SEM) in 30 Hz contractions produced during PLFFD (red circles) initially in standard Tyrode solution, followed by addition of DTT (1 mM) or the non-metabolizable analogue of H_2O_2, *t*-BOOH (10 μM). The effect of the same exposures on unfatigued fibres are also shown (green circles). *B*, simplified model of ROS/RNS effects on SR Ca^{2+} release and myofibrillar Ca^{2+} sensitivity. Key proteins for SR Ca^{2+} release are the t-tubular voltage sensors, the dihydropyridine receptors (blue boxes), and the SR Ca^{2+} release channels, RyR1 (green boxes). These proteins appear to be in an optimal redox state at rest and become overly oxidized during fatiguing stimulation resulting in decreased $[Ca^{2+}]_i$, which is not affected by application of either *t*-BOOH or DTT (see *A*). In the rested state, myofibrillar proteins are in a suboptimal reduced state. Some myofibrillar proteins become overly oxidized during induction of fatigue and the resulting force decrease is transiently counteracted by application of DTT. Intriguingly, other myofibrillar proteins apparently remain reduced during fatigue since application of the oxidizing agent *t*-BOOH temporarily improves force generation, i.e. similar to the effect in the rested state (see *A*). Figure adapted from Cheng *et al.* (2015).

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and/or reduced myofibrillar Ca^{2+} sensitivity. Acutely fatigued fast-twitch mouse FDB fibres displayed a marked PLFFD that is mainly caused by decreased SR Ca^{2+} release (Westerblad *et al.* 1993). Intriguingly, the cause of PLFFD changes towards reduced myofibrillar Ca^{2+} sensitivity in genetically modified mouse FDB fibres overexpressing the mitochondrial matrix redox enzyme SOD2 (Bruton *et al.* 2008), in rat FDB fibres that endogenously express more SOD2 (Bruton *et al.* 2008), and in mouse FDB fibres treated with the mitochondria-targeted antioxidant SS-31 or the NOS inhibitor L-NAME (Cheng *et al.* 2015). In a recent study we show that PLFFD is accompanied by RyR1 fragmentation in vastus lateralis muscles of recreationally active human subjects after one session of high-intensity interval training (6 × 30 s all-out cycling) (Place *et al.* 2015). Conversely, when elite endurance athletes performed the same exercise, a similar PLFFD was observed but the RyR1 remained intact. The elite endurance athletes had higher levels of the antioxidant enzymes SOD2 and catalase in their muscles than recreationally active subjects. Moreover, a similar RyR1 fragmentation could be induced by high-intensity stimulation of isolated mouse FDB muscles and this fragmentation was blocked by the antioxidant NAC (Place *et al.* 2015). Collectively, these results indicate that accumulation of mitochondrially generated $O_2^{\bullet-}$, or $ONOO^{\bullet-}$, preferentially affects SR Ca^{2+} release, probably via redox modifications of RyR1 (Bellinger *et al.* 2008*a*,*b*; Andersson *et al.* 2011; Lanner *et al.* 2012). Conversely, when $O_2^{\bullet-}$ is more efficiently metabolized, the resulting increase in H_2O_2, or downstream products, preferentially leads to changes in myofibrillar Ca^{2+} sensitivity. In other words, antioxidants do not prevent PLFFD, but they can change the underlying mechanism from impaired SR Ca^{2+} release to reduced myofibrillar Ca^{2+} sensitivity. The question is then: Does this matter? Our answer is: Yes, it probably does.

Impaired SR Ca^{2+} release caused by redox modifications of RyR1 is associated with increased SR Ca^{2+} leak at rest, and the resulting increase in resting $[Ca^{2+}]_i$ may stimulate mitochondrial biogenesis and thereby improve muscle endurance (Wright *et al.* 2007; Bruton *et al.* 2010). On the other hand, major adaptations are unlikely to be triggered when PLFFD is caused by decreased myofibrillar Ca^{2+} sensitivity. Accordingly, prolonged changes in gene expression were recently shown after one session of high-intensity interval training that induced PLFFD accompanied by ROS/RNS-dependent RyR1 fragmentation in recreationally active subjects. Conversely, prolonged changes in mRNA levels were not observed in elite endurance athletes where PLFFD occurred while RyR1 remained intact (Place *et al.* 2015). Thus, ROS/RNS-induced changes in RyR1 structure and function provide a mechanism as to why treatment with antioxidants hamper the beneficial effects of endurance training (Gomez-Cabrera *et al.* 2008; Ristow *et al.* 2009; Paulsen *et al.* 2014).

A recent study from our laboratory highlights the complexity of fatigue-induced redox effects (Cheng *et al.* 2015). Neither exposure to DTT nor *t*-BOOH had any clear-cut effect on the impaired SR Ca^{2+} release during PLFFD. Unexpectedly, application of DTT as well as *t*-BOOH resulted in a major, but transient, increase in myofibrillar Ca^{2+} sensitivity during PLFFD. The effect of DTT during PLFFD was opposite to that in the rested state, where DTT decreased force production, which implies that redox-sensitive sites were suboptimally reduced under resting conditions, became too oxidized during fatigue, and this was reversed by DTT. The effect of *t*-BOOH was more intriguing since the results showed a transiently improved myofibrillar force production both in the unfatigued state and during PLFFD. Thus, some functionally important sites on myofibrillar proteins appeared to remain in a suboptimally reduced state during fatigue and the function of these was then temporarily improved by exogenous peroxide application (Fig. 3). While these intriguing findings illustrate the complexity of redox effects, further studies are clearly required before they can be understood at a more precise mechanistic level.

Conclusions

In this review we discuss intricate ROS/RNS effects on myofibrillar contractile function. While mechanisms underlying some changes in myofibrillar function are fairly well established, others are more uncertain. For instance, it is fairly well established that *S*-glutathionylation of TnI_f can cause an initial increase in myofibrillar Ca^{2+} sensitivity during exogenous H_2O_2 exposure, whereas the mechanism(s) behind the decreased sensitivity during prolonged exposure remains uncertain. Due to such uncertainties we find it premature to discuss complex changes in contractile function, e.g. mechanisms underlying PLFFD, in terms of one specific ROS/RNS acting on one specific molecular target. This type of knowledge would, for instance, require improved methods to measure changes in a specific ROS/RNS with good temporal and spatial resolution. Moreover, an obvious risk with reductionistic approaches to assess the complex ROS/RNS effects is that simplistic cellular or subcellular experiments are prioritized to get at *the* molecular mechanism. Our opinion is that such experiments often provide the correct answer to the wrong question.

In studies of ROS/RNS-induced changes in myofibrillar function, we find it essential to measure both force and $[Ca^{2+}]_i$, since both are likely to be affected. Experiments on enzymatically isolated muscle fibres are technically much easier to perform than experiments on dissected

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fibres. However, enzymatically dissociated fibres lack tendons and force measurements become cumbersome. Intact whole muscles are also easier to use than dissected single muscle fibres, but $[Ca^{2+}]_i$ is then difficult to measure, especially in deeper parts of the muscle. The mechanism of PLFFD shifts from decreased tetanic $[Ca^{2+}]_i$ in wild-type muscles to reduced myofibrillar Ca^{2+} sensitivity in SOD2-overexpressing muscles (Bruton *et al.* 2008). Experiments with enzymatically dissociated fibres would detect decreased tetanic $[Ca^{2+}]_i$ during recovery of wild-type fibres, whereas $[Ca^{2+}]_i$ returned to the pre-fatigue level with SOD2 overexpression. Thus, the conclusion would be that increased SOD2 activity prevents PLFFD. Conversely, experiments with intact whole muscles would detect a similar force depression during recovery of wild-type and SOD2-overexpressing muscle, and the conclusion would be that SOD2 overexpression has no effect on PLFFD. Thus, the correct conclusion requires experiments with simultaneous measurements of force and $[Ca^{2+}]_i$ in fully intact fibres, or the combination of experiments with enzymatically dissociated fibres *and* whole muscles.

A wide range of antioxidants, including gp91ds-tat and SS-31, could not prevent the loss of force observed after acute fatiguing stimulation of healthy mouse muscle fibres (Cheng *et al.* 2015). Moreover, improved antioxidant capacity in muscles of endurance athletes did not prevent the development of PLFFD after a session of high-intensity interval training (Place *et al.* 2015). On the other hand, prolonged administration of antioxidants (e.g. vitamin C and E) has been shown to hamper the beneficial effects of endurance training in humans and rodents (Gomez-Cabrera *et al.* 2008; Ristow *et al.* 2009; Paulsen *et al.* 2014). Thus, the overall impact of antioxidant supplementation in association with endurance training is negative: antioxidants do not prevent the exercise-induced prolonged ROS/RNS-dependent decline in contractile function, whereas they can hamper the beneficial adaptations that come with endurance exercise.

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Additional information

Competing interests

None declared.

Author contributions

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

The authors acknowledge support from the Swedish Research Council and the Swedish National Centre for Sports Research.

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