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TOPICAL REVIEW

Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training?

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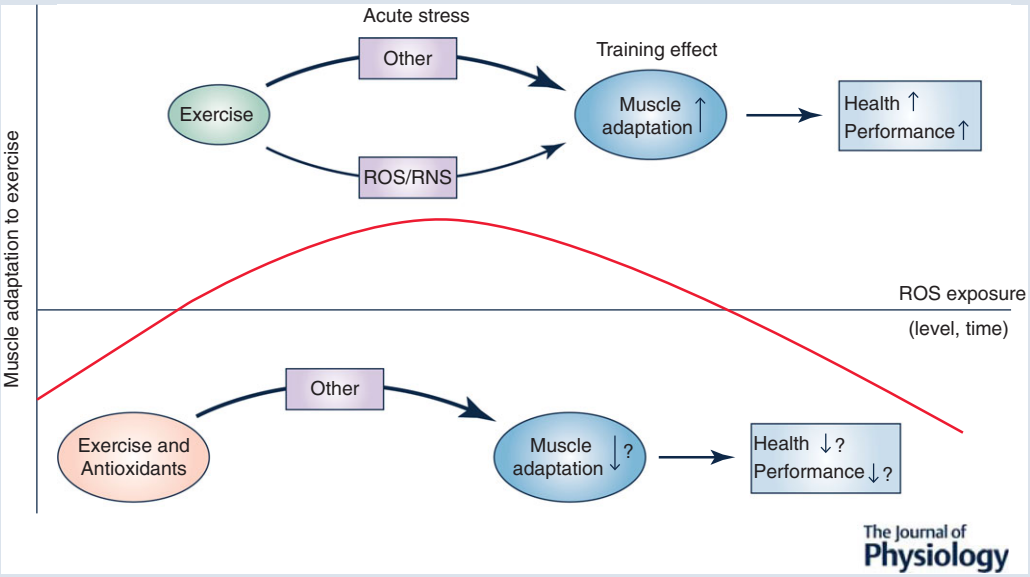

Abstract A popular belief is that reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced during exercise by the mitochondria and other subcellular compartments ubiquitously cause skeletal muscle damage, fatigue and impair recovery. However, the importance of ROS and RNS as signals in the cellular adaptation process to stress is now evident. In an effort to combat the perceived deleterious effects of ROS and RNS it has become common practice for active individuals to ingest supplements with antioxidant properties, but interfering with ROS/RNS signalling in skeletal muscle during acute exercise may blunt favourable adaptation. There is building evidence that antioxidant supplementation can attenuate endurance training-induced

Troy Merry obtained his PhD from the University of Melbourne investigating the role or reactive oxygen species (ROS) and reactive nitrogen species (RNS) in regulating skeletal muscle glucose uptake during exercise. He is now a post-doctoral research fellow at the ETH Zurich and his research focuses on understand the molecular mechanisms underlying metabolic disease and metabolic adaptation to exercise, and the involvement of ROS in this regard. **Michael Ristow's** research is focused on the biochemical and molecular basis of longevity – in particular the role played by mitochondria in lifespan regulation and prevention of metabolic diseases. Contrary to the widely re-iterated Free Radical Theory of Ageing, Ristow was the first to show that the health-promoting effects associated with low caloric intake, physical exercise and other lifespan-extending interventions like sirtuin signalling are caused by increased formation of reactive oxygen species within the mitochondria, causing a vaccination-like adaptive response that culminates in increased stress resistance and extended longevity, a process called mitohormesis.

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and ROS/RNS-mediated enhancements in antioxidant capacity, mitochondrial biogenesis, cellular defence mechanisms and insulin sensitivity. However, this is not a universal finding, potentially indicating that there is redundancy in the mechanisms controlling skeletal muscle adaptation to exercise, meaning that in some circumstances the negative impact of antioxidants on acute exercise response can be overcome by training. Antioxidant supplementation has been more consistently reported to have deleterious effects on the response to overload stress and high-intensity training, suggesting that remodelling of skeletal muscle following resistance and high-intensity exercise is more dependent on ROS/RNS signalling. Importantly there is no convincing evidence to suggest that antioxidant supplementation enhances exercise-training adaptions. Overall, ROS/RNS are likely to exhibit a non-linear (hormetic) pattern on exercise adaptations, where physiological doses are beneficial and high exposure (which would seldom be achieved during normal exercise training) may be detrimental.

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Abstract figure legend Acute exercise increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which contribute to the signalling of skeletal muscle adaptations that occur with training. Preventing ROS/RNS stress during exercise through antioxidant supplementation could potentially impair the adaptation process. ROS/RNS are likely to exhibit a hormetic effect on skeletal muscle adaptations during exercise, with physiological increases promoting, and very low or very high exposure potentially hampering adaptation.

Abbreviations AMPK, 5′-adenosine monophosphate-activated protein kinase; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CK, creatine kinase; Gpx, glutathione peroxidases; IL-6, interleukin 6; HSP, heat shock protein; L-NAME, N^G-nitro-L-arginine; MAPK, mitogen-activated protein kinases; MHC, myosin heavy chain; mTOR, mechanistic target of rapamycin; mtTFA, mitochondrial transcription factor A; NADPH, nicotinamide adenine dinucleotide phosphate; NFAT, nuclear factor of activated T-cells; NFE2l2, nuclear factor erythroid 2-related factor 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NOS, nitric oxide synthase; NRF1, nuclear respiratory factors; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; $\dot{V}_{O_2max}$, maximal oxygen uptake.

Reactive oxygen species and exercise training

Regular exercise improves the function of multiple organs in the human body, and reduces the risk of developing diseases such as type 2 diabetes, cardiovascular disease, cancer and dementia, and by that increases life expectancy (Warburton *et al.* 2006). One of the most plastic and responsive organs to exercise training is skeletal muscle (Bassel-Duby & Olson, 2006). Physiological stress applied through repeated bouts of acute exercise (training) induces skeletal muscle adaptations that improve the muscles ability to deal with future exercise stress, and the potential to cope with other similar stressors which may be associated with disease development. Adaptations that occur in skeletal muscle in response to exercise training are dependent on the parameters of the exercise stress applied, with resistance training promoting hypertrophy and endurance training having greater effects on muscle oxidative capacity (Bassel-Duby & Olson, 2006). One of the many stressors that are imposed through exercise is an increased exposure to reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Powers & Jackson, 2008).

During exercise skeletal muscle ROS are produced from both mitochondrial and non-mitochondrial sources, which include xanthine oxidases, NADPH oxidases and phospholipase A2, while the parent RNS nitric oxide (NO) comes from NO synthase (NOS) (Powers & Jackson, 2008). Under most exercise conditions oxidative balance is maintained within physiological limits, minimizing the potential for oxidative damage (Powers & Jackson, 2008). This is achieved by a multifaceted antioxidant defence system that consists of antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidases (Gpx), peroxiredoxins and thioredoxins that reduce ROS, and endogenous antioxidant substrates like glutathione that scavenge ROS and RNS (Powers & Jackson, 2008). Traditionally, however, exercise-induced ROS has been seen as an unavoidable by-product of increased oxidative metabolism that impairs muscle function and causes oxidative damage. This notion coupled with the association between oxidative stress (where ROS production is continuously greater than anti-oxidant capacity) (Sies, 1985), tissue damage and disease has led to the popular dogma that ROS and RNS produced

during exercise are detrimental to health and muscle function. As such, supplements that have the potential to reduce ROS levels are marketed for their 'health promoting antioxidant properties' (Fig. 1). It has become common practice for athletes and health conscious physically active individuals to chronically supplement their normal diet with high doses of antioxidants including vitamins C (ascorbic acid) and E, α-lipoic acid, selenium, amino acids and coenzyme Q10 (Gomez-Cabrera *et al.* 2008*b*), but this practice may actually be hampering normal skeletal muscle adaptation process (Fig. 1).

ROS and RNS are important molecular messengers that act through direct revisable interactions with redox-sensitive proteins to specifically regulate many physiological processes including insulin sensitivity, vasodilatation, mitochondrial biogenesis, immune response and growth factor signalling (Grodstein *et al.* 2013; Chandel & Tuveson, 2014; Ristow, 2014; Yun & Finkel, 2014). The laboratory of Michael Reid was the first to show a non-liner relationship between ROS production and muscle function (Reid, 2001), with basal and physiological increases of ROS being required for optimal skeletal muscle force production, and higher levels being associated with a decline in function and oxidative damage. This is consistent with hormesis, whereby favourable biological adaptations occur in response to low continuous or higher intermitted exposure to a stressor that would be harmful at large or chronic doses (Ristow, 2014; Yun & Finkel, 2014). Indeed, antioxidants can ameliorate adaptions in cellular defences induced by ROS (Ristow, 2014), making the cell less tolerant of subsequent stress. It is, therefore possible that antioxidant supplementation during exercise training may negate favourable adaptations brought about through exercise-induced increases in ROS and RNS (Fig. 1).

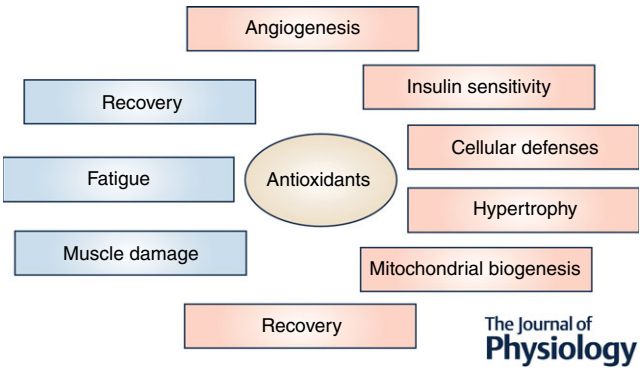

Figure 1. Effects of antioxidants on skeletal muscle during exercise training
Effectors of antioxidants that may be negatively affected are marked in red, effectors of antioxidants that may be beneficially affected are marked in blue.

Antioxidant supplementation and skeletal muscle adaptations to exercise training

Antioxidant treatment modifies skeletal muscle signalling during and immediately following a single bout of exercise and this can result in alterations in glucose uptake, force production, sodium–potassium pump function, mitochondrial biogenesis markers and insulin sensitivity (Powers & Jackson, 2008). Transient induction of acute signalling in response to an exercise stress is required for exercise training adaptations; however, antioxidants effects on a single bout of exercise may be overcome by training. This review focuses primarily on whether chronic antioxidant supplementation affects exercise training-induced skeletal muscle adaptations. We have also limited ourselves to studies that have utilised supplements whose primary function is ROS or RNS prevention. As such, polyphenols and flavonoids with antioxidant properties like resveratrol and epigallocatechin gallate, which rather act as indirect antioxidants or may have additional antioxidant-independent effects, are not considered.

Antioxidants defences

Exercise acts as an antioxidant by up regulating endogenous antioxidant defence (Gomez-Cabrera *et al.* 2008*b*). Since oxidative stress is associated with the pathogenesis of numerous diseases, including diabetes, cancer, cardiovascular disease and neurodegenerative diseases, the natural antioxidant effect of exercise is likely to be one of the mechanisms underlying the health-promoting benefits of regular exercise. However, if this is the case then why does antioxidant supplementation appear not to affect, or may even increase, the incidence of disease in humans (Ristow, 2014)? The answer may relate to the types of antioxidants supplemented, which are normally generalized, non-target scavengers of all ROS such as vitamin C and E. In contrast to these universal ROS-scavenging antioxidants, our endogenous regulation of cellular redox status relies on a complex and compartmentalized network of ROS producing and eliminating systems (Powers & Jackson, 2008). These systems allow for discrete, controlled localized production of specific types of ROS. Disruption of this system by over-supplementation with exogenous generalised antioxidants will not only affect potentially harmful ROS/RNS but also ROS/RNS-related signalling that is required for cellular adaptation. In contrast, exercise and other stressors are likely to regulate antioxidant responses in a more specific manner, negating possible unwanted effects of non-discriminately scavenging ROS/RNS.

One of the early focuses of antioxidant supplementation on skeletal muscle adaptions was the antioxidant response to exercise. Work from several independent laboratories

has provided data that antioxidant supplementation during an acute bout of exercise in humans or rodents, or the disruption of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α; a central mediatory of exercise adaptation; Gomez-Cabrera *et al.* 2008*b*), can attenuate exercise-induced increases in skeletal muscle antioxidant gene expression. Since then a number of groups have assessed whether antioxidant supplementation during training reduces the normal antioxidant effect of exercise.

In rodents, supplementation with various general antioxidants during endurance training has been shown to attenuate increases in Gpx, SOD and peroxiredoxin mRNA levels (Gomez-Cabrera *et al.* 2008*a*; Meier *et al.* 2013), but effects on antioxidant protein or activity levels are less commonly observed (Strobel *et al.* 2011). This perhaps suggests that assessing gene expression at a single time point is insufficient to determine changes at the protein level during exercise training protocols. Supplementation with the xanthine oxidase inhibitor allopurinol alone does not appear to be sufficient to alter training-induced increases in SOD1 or SOD2 mRNA levels (Wadley *et al.* 2013). During very strenuous exercise training (6 h day^{-1}) vitamin C/E supplementation does not appear to affect short-term training (3 days) increases in SOD1 or SOD2 protein expression (Higashida *et al.* 2011), but does attenuate increases in catalase and Gpx activities and suppresses reductions in SOD activity associated with longer duration strenuous training (6 days week^{-1}, 8 weeks) (Chang *et al.* 2007).

Surprisingly few studies have thoroughly assessed whether antioxidant supplementation in humans influences exercise training-induced alterations in antioxidant capacity. Ristow *et al.* (2009) reported that vitamin C/E supplementation during 4 weeks of endurance training attenuated increases in skeletal muscle SOD1, SOD2 and Gpx1 mRNA levels. However, Yfanti *et al.* (2010) did not observe any effect of vitamin C/E supplementation on increases in muscle SOD2 protein expression following 12 weeks of training, while Cumminings *et al.* (2014*b*) saw little effect of either exercise training or antioxidant supplementation on Gpx1 or SOD2 expression. It appears, therefore, that antioxidant supplementation is detrimental to acute exercise antioxidant response and can impair endurance training-induced increases in antioxidant enzyme mRNA levels, but the effects on endogenous antioxidant enzyme activities and protein levels following training are not clear.

Exercise performance

Successful exercise training enhances the ability of the body to deal with the physiological stress of a given exercise task, and thus allows the task to be completed more efficiently. Improvements in exercise performance, especially by competitive athletes, are often used to determine the success of a training programme. Studies showing that antioxidant treatment can delay fatigue of skeletal muscles contracted *in vitro* and *in situ* and during electrically stimulated contractions in humans has prompted the investigation of antioxidants as ergogenic aids (Reid, 2001; Lamb & Westerblad, 2011). Through studying isolated muscle and muscle fibres, ROS and RNS have been shown to alter the contractile apparatus Ca^{2+} sensitivity (Lamb & Westerblad, 2011). The conditions of muscle contraction (muscle type, ROS levels, contraction protocol and temperature) influences the effect ROS has on Ca^{2+} sensitivity and determines if antioxidants promote or impair skeletal muscle contraction force (Powers & Jackson, 2008; Lamb & Westerblad, 2011).

The effect antioxidant supplementation has on acute *in vivo* exercise performance is difficult to interpret due to the diverse exercise protocols employed, and different types of antioxidants and supplementation procedures utilised. An in-depth analysis on the topic is beyond the scope of this review and readers are referred to Powers & Jackson (2008) and Braakhuis & Hopkins (2015). Suffice it to say that there are a handful of studies that have reported acute antioxidant supplementation can enhance performance of a single bout of exercise, but most have yielded negative results showing no effect or even impairment of performance. In general, those that have reported antioxidants to improve performance employed fatiguing-type exercise and supplementation either immediately before or during exercise, while the adverse effect of antioxidants on performance are more commonly associated with longer duration (several days or longer) supplementation protocols, the exception being that longer supplementation period may be beneficial during tournament-type situations, where multiple high-intensity exercise sessions are completed in close succession (Cobley *et al.* 2011).

Although there may be some evidence to support acute antioxidant supplementation immediately prior to certain types of acute exercise where performance is important, it is far more common practice for athletes to continuously take antioxidants during training. The premises for this is that antioxidants will reduce muscle damage and fatigue, thus boosting recovery (although this may not be the case; see Recovery section) and allowing improved training in subsequent sessions. Several studies have assessed the effect of antioxidant supplementation on short-term training-induced increases in performance. Overall, the results have been disappointing for those that market antioxidants as a training supplement, with no studies convincingly showing that antioxidant supplementation during exercise training further enhances performance, and several reporting attenuated improvements (Braakhuis & Hopkins, 2015).

Endurance performance. In humans the improvement in maximal oxygen uptake ($\dot{V}_{O_2max}$) achieved with 4–12 weeks of training appears not to be affected by supplementation with vitamin C (Gomez-Cabrera *et al.* 2008*a*; Roberts *et al.* 2011), or a combination of vitamins E and C (Yfanti *et al.* 2010; Paulsen *et al.* 2014*a*). While $\dot{V}_{O_2max}$ measures maximal aerobic capacity, it does not always reflect endurance performance, which is commonly assessed with work or time trials, or time to exhaustion during fixed intensity exercise. Although Comez-Cabrera *et al.* (2008*a*) showed that vitamin C supplementation blocked improvements in endurance capacity in rats following 6 weeks of training, improvements in human (Roberts *et al.* 2011; Braakhuis *et al.* 2014) and mouse (Abadi *et al.* 2013; Meier *et al.* 2013) endurance performance appears not to be greatly affected by antioxidant supplementation during training. These contrasting results may reflect species-specific exercise adaptation processes, or be a result of different antioxidant supplementation protocols utlised. Gomer-Cabrera *et al.* (2008) used 500 mg kg^{-1} day^{-1} vitamin C, while the human studies used a much lower dose of 13–16 mg kg^{-1} day^{-1}, and mouse studies employed antioxidant cocktails containing such antioxidants as *N*-acetylcysteine, vitamin E and coenzyme Q10. It is also worth noting that some studies have reported what appeared to be reduced performance during the training itself (Braakhuis *et al.* 2014) and blunted reductions in physiological stress (Sharman *et al.* 1971; Paulsen *et al.* 2014*a*) when exercise training is combined with vitamin C/E supplementation. In contrast, Asha Devi *et al.* (2003) reported that vitamin E supplementation during 12 weeks of swimming training improves endurance capacity of rats. Unfortunately, these results are difficult to interpret since endurance capacity was not assessed prior to the exercise training intervention, and only three rats were used per group. Interestingly, Aguilo *et al.* (2007) observed that 3 months of daily supplementation with vitamin E and β-carotene lead to greater training-induced improvements in anaerobic threshold; however, this did not translate to improved performance as determined by $\dot{V}_{O_2max}$ or maximal work output trial.

High intensity and resistance exercise performance. While there is little evidence to suggest that antioxidant supplementation affects training-induced improvements in endurance performance, several studies have reported a detrimental effect of antioxidant supplementation on high-intensity exercise performance. This may suggest that ROS are more pivotal in regulating adaptations to high-intensity exercise. Indeed, this would fit with the observation that higher levels of ROS are produced during more intense muscle contractions (Pattwell *et al.* 2004), and that improvements in exercise performance that have been seen following acute antioxidant supplementation are generally associated with highly fatiguing exercise (Medved *et al.* 2004; Matuszczak *et al.* 2005; McKenna *et al.* 2006). In two separate studies Malm *et al.* (1996, 1997) report that supplementation with the antioxidant coenzyme Q10 during short-term training attenuated increases in peak and mean power output for repeated bouts of 10 s all-out cycling. This is consistent with the observation that greyhound racing dogs ran slower during 500 m races over 4 weeks when supplemented with vitamin C (Marshall *et al.* 2002). However, vitamin C supplementation was not seen to affect training improvements in intermitted high-intensity shuttle runs (Roberts *et al.* 2011), suggesting less of a role of ROS in intermittent endurance exercise training adaptations.

Recently, Paulsen *et al.* (2014*b*) assessed the effects of daily supplementation with a combination of vitamins C and E on strength following 10 weeks of resistance training. They showed that antioxidant supplementation attenuated increases in upper body strength, and a similar trend was noted for maximal voluntary contractions in the lower body. However, this does not appear to be a universal finding and may apply more to concentric-type exercise since vitamin C/E supplementation does not affect isometric knee extension performance following eccentric exercise training (Theodorou *et al.* 2011) or strength gains during resistance training in the elderly (Bobeuf *et al.* 2011), while improvements in muscle function were reported in old rats supplemented with vitamin E during exposure to 4.5 weeks of repetitive muscle loading (Ryan *et al.* 2010).

Mitochondrial biogenesis

Aerobic exercise training increases skeletal muscle mitochondrial content (by number and volume) and function, and this is one of the central adaptations through which training enhances oxidative capacity. The increase in energy demand and calcium turnover that occurs during exercise is sensed by a number of proteins including 5′-adenosine monophosphate-activated protein kinase (AMPK), mechanistic target of rapamycin (mTOR), Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), sirtuins and mitogen-activated protein kinases (MAPKs) (Bassel-Duby & Olson, 2006), which activate several key transcription factors, namely PGC-1α, nuclear respiratory factor (NRF) 1 and 2, and mitochondrial transcription factor A (mtTFA), which regulate mitochondrial biogenesis. There is now strong evidence that ROS and RNS can mediate mitochondrial biogenesis, and that antioxidants interfere with acute exercise-induced increases in mitochondrial biogenesis signalling intermediates p38 MAPK, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), AMPK, and several transcription factors (Gomez-Cabrera *et al.* 2005; Wadley *et al.* 2013) (Fig. 2).

However, whether this suppression of acutely regulated mitochondrial biogenesis markers translates to impaired exercise training-induced increases in mitochondrial biogenesis is less clear.

In 2008 Gomez-Cobrera *et al.* (2008*a*) published the first study linking antioxidant supplementation to impaired exercise training-induced mitochondrial biogenesis. They, and subsequently others (Strobel *et al.* 2011), showed that daily antioxidant supplementation in rats during treadmill training prevented increases in hind-limb muscle mitochondrial biogenesis markers (including PGC1α, mtTFA, NRF1) as well as mitochondrial protein and activity levels. Since the initial observations that antioxidants can blunt training-induced increases in mitochondrial biogenesis, several other rodent studies have reported antioxidants not to affect exercise training-induced increase in mitochondrial biogenesis.

Antioxidant treatment of mice was shown to suppress acute exercise and some exercise training-induced increases in mitochondrial biogenesis markers (Abadi *et al.* 2013; Meier *et al.* 2013); however, this did not affect exercise training-induced increases in mitochondrial mass (Abadi *et al.* 2013; Meier *et al.* 2013). In agreement, Wadley *et al.* (2013) recently reported that allopurinol (an xanthine oxidase inhibitor) attenuates acute exercise, but not exercise training-induced increases in mitochondrial biogenesis. While initial studies in rats combined anti-oxidant supplementation only during exercise training (Gomez-Cabrera *et al.* 2008*a*; Strobel *et al.* 2011), Higashida *et al.* (2011) examined the effect of vitamin C/E supplementation for 5 weeks prior to, and during 3 weeks of very high volume (6 h day^{-1}, 6 days week^{-1}) swimming training. They report that supplementation did not affect increases in triceps mitochondrial proteins. Possibly this suggests that both the type of antioxidant employed (general *vs.* targeted) and duration of treatment (pre-treatment *vs.* treatment only during training) and/or volume of training are factors determining the effect antioxidant supplementation has on exercise training adaptations.

Soon after antioxidant supplementation was reported to impair mitochondrial biogenesis in rodents (Gomez-Cabrera *et al.* 2008*a*), Ristow *et al.* (2009) reported that in humans vitamin C/E supplementation during exercise training attenuated increases in PGC1α mRNA. This work has recently been supported by Paulsen *et al.* (2014*a*) who showed that endurance training-induced increases in PGC1α protein and the mitochondrial protein cytochrome oxidase 4 expression were prevented by vitamin C/E supplementation. In contrast, Yfanti *et al.* (2010) report that vitamin C/E supplementation during 12 weeks of cycling training does not affect increases in citrate synthase activity. Although no mitochondrial biogenesis markers or protein levels were reported, this suggests that mitochondrial volume was not affected by antioxidants.

Nitric oxide can promote mitochondrial biogenesis; however, its role in exercise-induced mitochondrial biogenesis is not clear. In muscle cells the NOS inhibitor N^G-nitro-L-arginine (L-NAME) can attenuate increases in mitochondrial biogenesis markers stimulated by compounds that mimic exercise stress such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; an AMPK activator) and caffeine (which stimulates calcium release) (McConell *et al.* 2010). Treatment of rats with L-NAME or the separate disruption of neuronal or endothelial NOS in mice does not impair, and may enhance, acute exercise-induced increases in PGC1α (Wadley *et al.* 2007; Wadley & McConell, 2007). L-NAME treatment did, however,

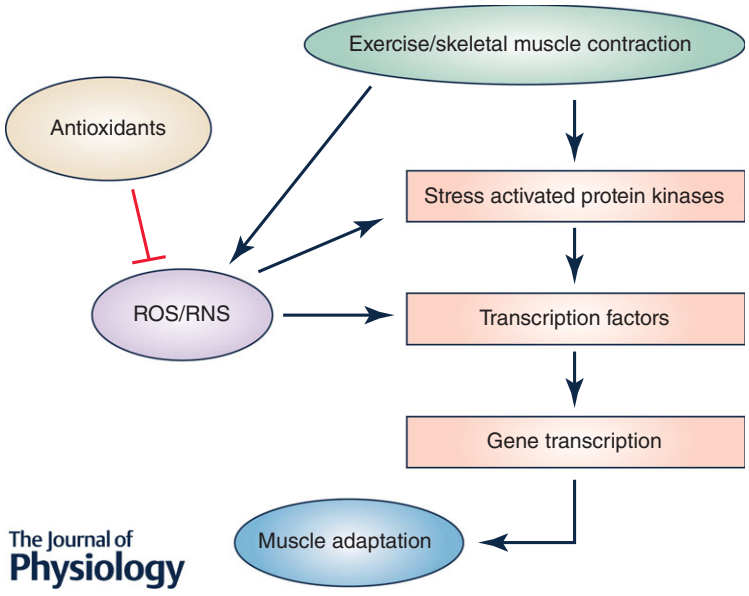

Figure 2. Acute skeletal muscle signalling during exercise, and interference of antioxidants to hamper training adaptations

attenuate exercise-induced increases in the downstream regulator of mitochondrial biogenesis mtTFA in some muscles (Wadley & McConell, 2007). It must be noted that potentially NO may only be required from one of the NOS isoforms to effectively modulate exercise-induced mitochondrial biogenesis. Unfortunately, the effect neuronal or endothelial NOS disruption has on training adaptations has only been assessed following short-term (9 day) training that had little effect on mitochondrial biogenesis (Wadley *et al.* 2007).

The discrepancy in the effect of antioxidants on exercise training-induced mitochondrial biogenesis could suggest that there is redundancy in the mitochondrial biogenesis signalling allowing, in certain conditions, for defects in ROS signalling brought about through antioxidant supplementation to be overcome. As such the extent to which mitochondrial biogenesis is affected by antioxidant supplementation could depend on numerous factors including duration and timing of supplementation, type of antioxidant and parameters of the training protocol.

Insulin sensitivity

Regular physical exercise improves glucose regulation by transiently enhancing insulin sensitivity for several hours following a single bout of exercise, and by inducing adaptations that result in longer-term improvements in glucose homeostasis. There has been limited research into whether antioxidants affect acute exercise- or exercise training-induced increases in insulin sensitivity. Ristow *et al.* (2009) provided evidence that vitamin C/E supplementation in humans during short-term (4 weeks) exercise training can impair improvements in insulin sensitivity, as assessed by glucose infusion rates during a hyperinsulinaemic, euglycaemic clamp. However, this effect appears to be overcome by longer-term training, with Yfanti *et al.* (2011) reporting that vitamin C/E supplementation does not affect enhancement of insulin sensitivity or expression of insulin signalling-related proteins following 12 weeks of endurance training. In rats, Higashida *et al.* (2011) observed that three consecutive days of 6 h exercise training enhanced glucose transporter 4 expression and *ex vivo* insulin-stimulated glucose uptake, and this was not affected by vitamin C/E supplementation. However, the short-term very high volume nature of the training protocol questions whether the effects were the result of normal exercise training adaptations.

In two separate studies, Saengsirisuwan *et al.* (2001; 2004) has shown that the antioxidant α-lipoic acid in combination with exercise training has an additive effect on enhancing insulin-mediated *ex vivo* glucose uptake of muscle from genetically obese Zucker rats. This suggests that exercise training and α-lipoic acid have a synergistic effect on improving insulin sensitivity in obese rats, and that under conditions of obesity α-lipoic does not interfere with exercise training-induced adaptations that enhance insulin sensitivity. In partial agreement, vitamin C supplementation in rats fed a high-fat diet does not affect training-induced enhancement in insulin sensitivity (Picklo & Thyfault, 2015).

Interesting, ROS have been implicated in the enhancement of insulin sensitivity following an acute bout of exercise. Loh *et al.* (2009) showed that the enhancement in insulin sensitivity seen acutely following exercise is augmented in mice that do not express the antioxidant enzyme Gpx1, an effect that was abolished by treatment with the antioxidant *N*-acetylcysteine. This may suggest that enhancing ROS promotes, while decreasing ROS via antioxidant supplementation may attenuate, post-exercise enhancement of insulin sensitivity. Indeed, it has recently been reported that infusion of *N*-acetylcysteine to humans during exercise attenuates post-exercise insulin sensitivity (Trewin *et al.* 2015)

Hypertrophy

Much of the research regarding antioxidants and exercise training has focused on endurance adaptions. However, recent studies have sought to determine the effect anti-oxidant supplementation may have on overload-induced muscle hypertrophy in rodents and humans. Makanae *et al.* (2013) found that hypertrophy induced in the plantaris muscle through the surgical remove of synergistic muscles (mechanical overload model) was attenuated by daily vitamin C supplementation. In humans vitamin C/E supplementation during resistance training does not appear to affect hypertrophy in young participants (Paulsen *et al.* 2014*b*), while in older participants vitamin C has been report to attenuate gains in lean mass seen during 12 weeks (Bjornsen *et al.* 2015), but not 6 months, of resistance training (Bobeuf *et al.* 2011). Furthermore, antioxidant supplementation reduces hypertrophy signalling (namely phosphorylation of ERK1/2, p38 MAPK and p70S6 kinase) and strength gains in muscle following resistance exercise (Makanae *et al.* 2013; Paulsen *et al.* 2014*b*).

Through the use of the plantaris mechanical overload model and neuronal nitric oxide synthase (nNOS) null mice, Ito *et al.* (2013) has provided a mechanism through which antioxidants can impair muscle hypertrophy. They show that following muscle overload, NO interacts with superoxide (produced by NADPH oxidase 4) to generate peroxynitrite. The latter then acts via transient receptor potential cation channel, subfamily V, member 1 and Ca^{2+} signalling to induce hypertrophy via the activation of mTOR and downstream target p70S6 kinase. As such, NOS inhibition and several different antioxidants were shown to severally attenuate overload-induced hind-limb muscle hypertrophy. Therefore, antioxidant supplementation during

resistance training could potentially blunt signalling of adaptive hypertrophy.

Muscle recovery

Antioxidant supplements are often taken during exercise training in an effort to reduce exercise-related oxidative stress damage and hasten recovery. Experiments conducted *in situ* and *ex vivo* have shown that the parental ROS, superoxide, plays a role in the impairment of muscle force production at submaximal intensities following a series of maximal contractions. The over-expression of the mitochondrial antioxidant enzyme SOD2, or treatment with reducing agents has been shown to improve short-term recovery in muscle function (Powers & Jackson, 2008; Lamb & Westerblad, 2011). In support, supplementation with the antioxidant *N*-acetylcysteine attenuates deterioration in performance of repeated bouts of high-intensity intermittent exercise (Cobley *et al.* 2011). Therefore, there is some evidence to support supplementing with antioxidants when optimal muscle performance is required following short recovery intervals, where muscle adaptation is inconsequential.

Antioxidants can prevent protein oxidation during exercise (Gomez-Cabrera *et al.* 2005); however, there is little *in vivo* evidence to suggest that antioxidant supplementation is sufficient to attenuate exercise-induced muscle damage or improve recovery during longer rest periods. Several studies have monitored plasma creatine kinase (CK; an index of muscle damage) following a single bout of exercise (Malm *et al.* 1996; Teixeira *et al.* 2009) or exercise during training (Avery *et al.* 2003). Surprisingly, the majority report that antioxidant supplementation augmented exercise-induced increases in plasma CK levels (Childs *et al.* 2001; Close *et al.* 2006; Bailey *et al.* 2011; Cobley *et al.* 2011). Some (Close *et al.* 2006) but not all (Bailey *et al.* 2011) found that this resulted in delayed recovery in muscle function, but did not appear to affect ratings of muscle soreness (Close *et al.* 2006; Bailey *et al.* 2011), while treatment with the NOS inhibitor L-NAME may delay muscle regeneration following acute eccentric exercise (Sakurai *et al.* 2013).

In contrast to the effects on CK levels, there is evidence that antioxidant supplementation can attenuate exercise-induced increases in inflammatory markers and cytokines (Peake *et al.* 2007) including interleukin 6 (IL-6) and tumour necrosis factor (TNF) (Vassilakopoulos *et al.* 2003; Fischer *et al.* 2004), although this is not seen by all (Aguilo *et al.* 2007; Teixeira *et al.* 2009; Yfanti *et al.* 2010; Bailey *et al.* 2011). Muscle injury-associated inflammation can cause pain but the up-regulation of inflammatory mediators is essential for muscle regeneration (Arnold *et al.* 2007). It is questionable whether blunting of the inflammatory response to exercise by antioxidants is beneficial to long-term muscle adaptation. In agreement, Yfanti *et al.* (2010) report that exercise training induced decreases in circulating IL-6, and muscle mRNA levels were attenuated by vitamin E/C supplementation suggesting that normal exercise training modulation of cytokine levels is modified by antioxidant supplementation.

Other exercise-mediated adaptations

Fibre type transition. An important adaptation to training is the transition between fibre types, with endurance training promoting the transition of fast-type anaerobic fibres to slower contracting more oxidative fibres. One of the mechanisms controlling this fibre type transition is the nuclear translocation of nuclear factor of activated T-cells (NFAT), which can regulate the isoform-specific regulation of the contractile protein myosin heavy chain (MHC) (Bassel-Duby & Olson, 2006). There is evidence to suggest that NO can regulate NFATc1 nuclear accumulation and this increases the expression of the slow oxidative MHC-I. Importantly, the ingestion of the NOS inhibitor L-NAME during 10 days of low-intensity muscle stimulation prevents faster to slower muscle fibre transition in rodents (Martins *et al.* 2012). It must be noted, however, that chronic low-intensity stimulations applied to the peroneal nerve for 12 h day^{-1} result in substantial fibre type transitions, and shifts of this magnitude are not seen during normal *in vivo* exercise training. Therefore, whether antioxidants prevent fibre type transitions during exercise training in humans, and whether this accounts for antioxidant-induced impairments in endurance training adaptations need to be investigated in detail.

Heat-shock proteins. Acute non-damaging concentric exercise increases skeletal muscle heat-shock protein (HSP) levels, and increased HSP expression may confer some of the cytoprotective effect of exercise training. Several groups have shown that antioxidant supplementation attenuates increase in HSPs in response to acute exercise (Khassaf *et al.* 2003; Fischer *et al.* 2006). There appears to be little effect of exercise training or the combination of exercise training and antioxidant supplementations on basal HSP expression. One study (Cumming *et al.* 2014*a*) has examined whether antioxidant supplementation affects training-induced alteration in HSP response to acute exercise. Cytosolic HSP27 levels following acute exercise were seen to decrease in the antioxidant but not the placebo group. Unfortunately, the effect that the acute exercise protocol had on HSP27 levels prior to training was not assessed making it difficult to interpret the interaction between training and antioxidant supplementation.

Transcriptions factors. Exercise activates transcription factors that control how skeletal muscle adapts (Fig. 2). ROS can activate transcription factors by direct oxidation or through interactions with upstream protein targets. Several transcription factors have been identified as being sensitive to alterations in cell redox status that occur during exercise. PGC1α is viewed as a key regulator of skeletal muscle adaptation to exercise, and antioxidant supplementation can impair acute exercise and exercise training-induced increases in PGC1α and other mitochondrial biogenesis transcription factors (see Mitochondrial biogenesis section). The transcription factors NF-κB and NFE2L2 also appear to be both redox and exercise sensitive (Gomez-Cabrera *et al.* 2008*b*; Narasimhan *et al.* 2014). NF-κB and NFE2L2 have been suggested to regulate exercise-induced changes in endogenous antioxidant expression and cellular defences. Indeed, treatment with allopurinol can attenuate exercise-induced increases in NF-κB in both rodents and humans (Gomez-Cabrera *et al.* 2005, 2008*b*). Disruption of NFE2L2 interferes with skeletal muscle antioxidant responses to exercise (Narasimhan *et al.* 2014), whereas antioxidant supplementation does not affect increases in NFE2L2 skeletal muscle protein concentration that occur with training (Wadley *et al.* 2013). Overall there is building evidence that antioxidant supplementation can affect exercise activation of transcription factors; whether supplementing with antioxidants during training alters the post-training response to acute exercise has received little attention.

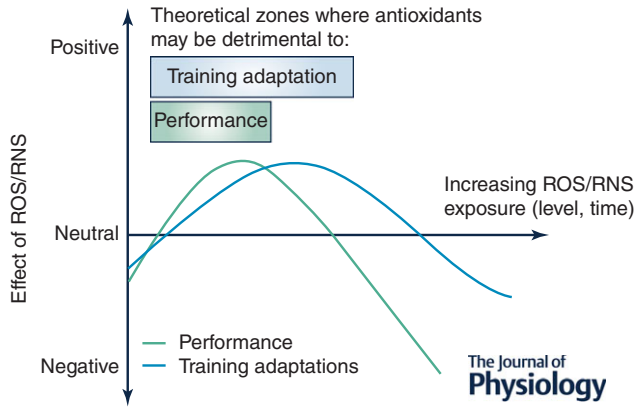

Figure 3. Non-linear/hormetic effects of ROS/RNS on exercise training adaptations and performance
Lower doses of ROS/RNS exert beneficial effects, whereas theoretical high ROS/RNS exposure (which would rarely be achieved during training) would impair training adaptations and performance. Antioxidant supplementation during an exercise task that results in an increase in ROS/RNS production which promotes training adaption and improves performance would be detrimental (blue and green bars, respectively). However, if the exercise task resulted in ROS/RNS exposure that exceeds this level then antioxidant supplementation may potentially be beneficial. We suggest that the level of ROS/RNS stress required to reduce performance would be lower than what would be necessary to hamper training adaptation.

Angiogenesis. Exercise training induces angiogenesis, increasing capillary density of skeletal muscle, and allows for enhanced blood flow and therefore oxygen (O_2) delivery. Although antioxidant supplementation can impair exercise-mediated vasodilatation and O_2 delivery (Copp *et al.* 2009), exercise-induced increases in mRNA for vascular endothelial growth factor, a central regulator of angiogenesis, are not affected by supplementation with antioxidants (Hellsten *et al.* 2007). Interestingly, Wary *et al.* (2009) report that exercise training-induced improvements in exercising blood pressure and flow-mediated dilatation were prevented by acute antioxidant supplementation prior to exercise. This may suggest that training enhances ROS regulation of local blood flow during exercise and this is prevented by antioxidant supplementation.

Limitations and future directions

To date most studies that have assessed the effect of antioxidant supplementation on muscle signalling and training adaptations have focused on aerobic endurance exercise. More recent studies, however, have begun to provide evidence of a potential negative role of antioxidant supplementation on resistance training adaptations, while few have investigated muscle adaptation in response to anaerobic training.

Global antioxidants, which act by non-specifically scavenging all free radicals, and include vitamin C/E as well as coenzyme Q and lipoic acid, have most often been used in this field of research. This is partly due to these antioxidants being the most commonly utilised by the public, but also because they are easily obtainable for use in research. Given the discrete compartmentalized nature of ROS signalling, to really understand the effect ROS and antioxidants are having on particular training adaptions more targeted approaches are required. Allopurinol has been utilized to examine the role of xanthine oxidase-produced ROS in the exercise response, and impairments in acute signalling (Gomez-Cabrera *et al.* 2005; Wadley *et al.* 2013) but not training-induced adaptation (Wadley *et al.* 2013) have been reported. Preventing ROS derived from the NADPH oxidase enzyme impairs overload-induced muscle adaptation (Ito *et al.* 2013), and mitochondrial targeted antioxidants can protect against muscle atrophy (Min *et al.* 2011). However, the effects of antioxidants that target the NADPH oxidases, mitochondria and other ROS production sites have on exercise-induced adaptions has received little attention.

An alternative approach to organelle-specific antioxidants is to utilize genetic rodent models that allow the tissue-specific overexpression or disruption of

endogenous antioxidant, respectively, to assess the potential role of ROS in mediating exercise-training adaptation. In addition, antioxidants that are targeted to the mitochondria are available, at least for non-human studies (Murphy & Smith, 2007). These approaches would help to address questions such as what type, location of production and target sites of ROS are important for training adaptations, and what are the best types of antioxidants to use to enhance performance and recovery without blunting training responses.

Summary

Taken together, the effect antioxidant supplementation has on skeletal muscle adaptation to exercise training is still equivocal. While there is no convincing evidence to support antioxidant supplementation in regards to training adaptations, there is a growing body of literature suggesting it may hamper or prevent the signalling of important adaptations such as muscle mitochondrial biogenesis, insulin sensitivity and hypertrophy. This is consistent with hormesis where stressors induce ROS that act as intracellular signalling molecules to promote adaptations that equip the cell to better tolerate future stress (Fig. 3). It is theoretically possible that antioxidants may aid exercise training if the exercise stress was sufficient to chronically elevate ROS to levels which impair function and cause damage; however it is unlikely that such levels would be achieved solely through exercise training (Fig. 3). This does not preclude the potential for acute antioxidant supplementation to enhance performance of certain types of exercise where following adaptive responses are irrelevant. The level of ROS required to impair muscle performance is likely to be lower than that which would hamper muscle adaption (Fig. 3). Given the potential for antioxidants to suppress some training adaptations with little evidence to suggest any positive effects, the authors tend to reject the use of such supplements.

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Additional information

Competing interests

The authors declare no competing interests.

Author contributions

Both authors contributed the writing of this manuscript and design of the figures. Both 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.

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