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Abstract

The exogenous antioxidants vitamin C (ascorbate) and vitamin E (α-tocopherol) often blunt favourable cell signalling responses to exercise, suggesting that redox signalling contributes to exercise adaptations. Current theories posit that this antioxidant paradigm interferes with redox signalling by attenuating exercise-induced reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation. The well-documented *in vitro* antioxidant actions of ascorbate and α-tocopherol and characterisation of the type and source of the ROS/RNS produced during exercise theoretically enables identification of the redox-dependent mechanism responsible for the blunting of favourable cell signalling responses to exercise. This review aimed to apply this reasoning to determine how the aforementioned antioxidants might attenuate exercise-induced ROS/RNS production. The principal outcomes of this analysis are (1) neither antioxidant is likely to attenuate nitric oxide signalling either directly (reaction with nitric oxide) or indirectly (reaction with derivatives, e.g. peroxynitrite) (2) neither antioxidant reacts appreciably with hydrogen peroxide, a key effector of redox signalling (3) ascorbate but not α-tocopherol has the capacity to attenuate exercise-induced superoxide generation and (4) alternate mechanisms, namely pro-oxidant side reactions and/or reduction of bioactive oxidised macromolecule adducts, are unlikely to interfere with exercise-induced redox signalling. Out of all the possibilities considered, ascorbate mediated suppression of superoxide generation with attendant implications for hydrogen peroxide signalling is arguably the most cogent explanation for blunting of favourable cell signalling responses to exercise. However, this mechanism is dependent on ascorbate accumulating at sites rich in NADPH oxidases, principal contributors to contraction mediated superoxide generation, and outcompeting nitric oxide and superoxide dismutase isoforms. The major conclusions of this review are: (1) direct evidence for interference of ascorbate and α-tocopherol with exercise-induced ROS/RNS production is lacking (2) theoretical analysis reveals that both antioxidants are unlikely to have a major impact on exercise-induced redox signalling and (3) it is worth considering alternate redox-independent mechanisms.

Key words: Vitamin C, Vitamin E, antioxidant, reactive oxygen species, reactive nitrogen species, exercise adaptations, oxidative stress

Abbreviations: 5LOX: 5-lipoxygenase; AP-1: Activating Protein 1; cGMP: Cyclic Guanosine Monophosphate; ERK: Extracellular Signal-Regulated Kinase; GSH: Glutathione (reduced); GSSG: Glutathione (oxidised); H_2O_2; Hydrogen Peroxide; HIF-α: Hypoxia Inducible Factor Alpha; HSF-1: Heat Shock Factor 1; HSP90: Heat Shock Protein 90; JNK:c-Jun N-terminal Kinase; KEAP-1: Kelch-like ECH-Associated Protein 1; NADPH oxidase: Nicotinamide Adenine Dinucleotide Phosphate-Oxidase; NF-κB: Nuclear Factor Kappa Beta; NO: Nitric Oxide; NOS: Nitric Oxide Synthease; Nrf2: Nuclear Factor (erythroid-derived 2)-like 2; p38 MAPK: p38 Mitogen Activated Protein Kinase; PGC-1α: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha; PTEN: Phosphatase and Tensin Homolog; SHP-2: Src Homology Protein-2; SOD: Superoxide Dismutase; Src: STAT3: Signal Transducer and Activator of Transcription 3

Introduction

In the last year, many studies have observed that exogenous antioxidant supplementation, principally ascorbate and α-tocopherol co-supplementation, blunts favourable molecular responses to exercise training [1-3]. These findings confirm some [4-7] but not others [8-14] in this area [reviewed in 15-18]. Irrespective of the outcome, all of the aforementioned studies share a common mechanistic rationale that depends on the antioxidant action of ascorbate and α-tocopherol (see figure 1A). This redox dependent mechanism is often assumed, yet seldom confirmed by any biochemical measurements. That is, evidence to support the postulate that redox-dependent mechanisms are responsible for the observed results is rarely presented. A redox-dependent mechanism of action principally rests on the assumption that ascorbate and α-tocopherol react appreciably with reactive oxygen species (ROS) and reactive nitrogen species (RNS) implicated in redox signalling (see box 1). In line with a recent commentary [19] the terms ROS/RNS are not used hereafter for two reasons (1) they convey limited mechanistic information and (2) the two electron oxidants that principally mediate redox signalling (e.g. peroxynitrite) are known. The well-documented *in vitro* antioxidant actions of ascorbate and α-tocopherol and characterisation of the sources of superoxide and nitric oxide (NO) generation, precursors of hydrogen peroxide (H_2O_2) and peroxynitrite, during exercise in skeletal muscle enables the veracity of this assumption to be explored (see figure 1B). Possible redox-dependent mechanisms for these results are appraised herein.

Redox signalling

Cell signalling enables cells to integrate information provided by internal and external cues into an orchestrated biological response [20-22]. A fundamental aspect of cell signalling is the propagation, via regulated biochemical reactions, of specific and reversible compartmentalised signals [20-22]. There is an increasing realisation and indeed evidence base supporting the notion that redox-dependent mechanisms contribute to cell signalling processes [23-29]. The basic premise of redox signalling is that two electron oxidants, principally H_2O_2, regulate specific and reversible post-translational modifications to thiol (SH^-) moieties on target proteins implicated in cell signalling [27]. Salient modifications include *inter alia*: disulphide formation, sulfenic acid formation, S-nitrosylation and S-glutathionylation [23-31]. Of course, redox signalling is not limited to thiol modification with other processes contributing, notably oxidation of other amino acids (e.g. methionine) and oxidised macromolecule adducts (e.g. 4-hydroxynonenal [25, 32-33]). Whilst the biological importance of redox signalling is clear, the underpinning mechanisms are unresolved [23-25, 34]. This is best evidenced by the chemical constraints that could limit the reaction of H_2O_2 with thiol moieties on target proteins (see below [24-25]). It is, therefore, clear that redox signalling is important but that elucidating the underpinning mechanisms requires further research.

Exogenous antioxidants, exercise and redox signalling

One conceptual model of exercise adaptation posits that 'exercise signals' (e.g. altered Ca^{2+} flux and energy status) during acute exercise bouts activate signalling pathways, that with repeated activation (multiple exercise bouts), yield exercise adaptations [35-37]. From a redox perspective, increased exercise-induced superoxide, NO, peroxynitrite and H_2O_2 generation is an 'exercise signal' implicated in the regulation of beneficial cyto-protective and mitochondrial exercise adaptations [38-41]. Cyto-protective adaptations confer increased resistance to oxidative stress owing to increased glutathione content, antioxidant enzyme activity and content coupled to up-regulation of cyto-protective proteins, notably heat shock proteins [42-45]. Mitochondrial adaptations are principally manifested by increased mitochondrial content and consequent metabolic adaptations post-training [46-49]. At the molecular level, increased contraction-mediated superoxide, NO, peroxynitrite and H_2O_2 generation is implicated in the regulation of several signalling proteins, including kinases (e.g. p38 MAPK [50]), transcriptional co-activators (e.g. PGC-1α [51]) and transcription factors (e.g. NF-κB, HSF-1, AP-1 and Nrf2 [38-41; 52]). Akin to the parent discipline, knowledge of mechanisms underpinning exercise-induced redox signalling is fragmentary. That is, how contraction-mediated superoxide, NO, peroxynitrite and H_2O_2 generation impacts the post-translational state of redox-sensitive signalling proteins remains to be fully resolved and demonstrated in an exercise setting. Exercise-induced redox signalling could involve free radical (e.g. superoxide) and non-radical mediated (e.g. peroxynitrite) mechanisms [26-28]. The aforementioned mechanisms will next be considered in turn but it is emphasised that the impact of ascorbate and α-tocopherol cannot be fully appraised until the mechanistic nature of exercise-induced redox signalling is better understood. The need to advance knowledge of exercise-induced redox signalling constitutes a major theme of this review.

Direct signalling

Skeletal muscle contractions are associated with a transient increase in superoxide and NO generation, secondary to NADPH oxidase and nitric oxide synthease (NOS) isoform activation, respectively [53-56]. It is, therefore, necessary to consider whether (1) direct redox signalling by superoxide and NO is possible (2) ascorbate and α-tocopherol react appreciably with either radical (3) this reaction out-competes other reactions and (4) any reaction interferes with compartmentalised redox signalling.

Superoxide

There are several sources of superoxide in skeletal muscle, including: mitochondrial electron transport chain complex I and III, NADPH oxidases, dual oxidases, xanthine oxidase, uncoupled NOS isoforms, phospholipases and lipoxygenases [57-59]. Recent data suggest that NADPH oxidases are the principal contributors to contraction mediated superoxide generation [60-61]. NADPH oxidases are expressed at several locations in skeletal muscle, including: mitochondria, sarcolemma, transverse tubules and sarcoplasmic reticulum [60-64]. From a signalling perspective, superoxide does not react appreciably with thiols ($k \sim 10^3$ M^{-1}

s^{-1} [65]) and any reaction would have to outcompete the kinetically favourable ($k \sim 10^9$ M^{-1} s^{-1}) reaction of superoxide with superoxide dismutase (SOD) isoforms [66]. Hence, signalling via this mechanism is unlikely *in vivo* [23, 66]. It should be noted that the reaction of superoxide with thiols is complex and involves intermediate thiyl radicals that ultimately result in the regeneration of superoxide [29, 65]. It is also of note that superoxide is not that reactive with most biomolecules [66-67]. Indeed, superoxide is more of a reductant than an oxidant unless protonated [66-67]. Nevertheless, we do not exclude the possibility that elevated superoxide concentrations allied to target co-localisation might overcome this kinetic constraint [28, 68]. Whilst the reaction with thiols might be unlikely, superoxide can react with protein metal centres directly [69]. One example relevant to exercise is the involvement of superoxide in the regulation of HIF-α, a protein that regulates exercise-induced angiogenesis [70-71]. Superoxide can react with the metal centre of propyl hydroxylase, an inhibitor of HIF-α, converting Fe^{2+} to Fe^{3+} and inactivating the enzyme [72]. Direct signalling by superoxide is, therefore, possible but comes with the caveat that this mechanism is not well characterised and thiol oxidation seems unlikely.

Although, under-characterised and indeed unlikely in some contexts (e.g. thiol oxidation) superoxide may contribute to exercise-induced redox signalling. Providing a potential mechanism for ascorbate and α-tocopherol to blunt exercise-induced redox signalling provided either antioxidant reacts appreciably with superoxide. α-tocopherol does not react appreciably with superoxide, partly owing to its poor solubility in aqueous solution and the negative charge of superoxide that restricts diffusion across biological membranes [69, 73]. It follows that α-tocopherol is extremely unlikely to interfere with exercise-induced redox signalling in this fashion. A redox-independent mechanism is possible via inhibition of 5-lipoxygenase (5-LOX) activity [74-75] but this has not been demonstrated in skeletal muscle cell lines.

Ascorbate can directly react with superoxide ($k \sim 10^5$ M^{-1} s^{-1} [76]). From a kinetic perspective, therefore, ascorbate mediated scavenging of superoxide with attendant implications for redox signalling is possible. Human skeletal muscle is highly responsive to ascorbate supplementation [77-78]. Indeed, levels can be increased by ~3.5 fold post-supplementation [77]. Elevated ascorbate concentrations post-supplementation increase the likelihood of the ascorbate-superoxide reaction occurring. This could have signalling implications provided (1) ascorbate out-competes other reactants and (2) reacts in the relevant microdomain. Whether ascorbate out-competes other reactants, namely SOD isoforms and NO for superoxide [78], is not known. It is unlikely however, that ascorbate out-competes the diffusion-limited superoxide-NO reaction [78]. Redox signalling is compartmentalised and subject to intricate spatiotemporal regulation [80-86]. Spatiotemporal regulation of different redox-sensitive networks is controlled, in part, by various subcellular redox couples (e.g. GSH/GSSG) that are not in equilibrium [80-86]. That is, redox couples in different microdomains and organelles exhibit different redox potentials and are not necessarily interlinked [80-86]. For instance, a signalling event might involve oxidation of the cytoplasmic but not nuclear GSH pool [80-82]. It follows that, the reaction of ascorbate with superoxide requires spatial context for proper interpretation. For example, if it is assumed

that exercise-induced redox signalling occurred in the caveolae of the plasma membrane following NADPH oxidase activation and resultant superoxide generation. Then ascorbate would need to be present in this microdomain to effect a reduction in the amount of superoxide available for reaction with a target or dismutation to H_2O_2. In this scenario, the initial signalling event would be unperturbed by reaction of ascorbate with superoxide in other microdomains (e.g. cytoplasm). Signalling requires only a small proportion of the total target protein population to be modified hence it is noted that signalling could still proceed despite some reduction in superoxide and target protein modification levels. Whether ascorbate is present in the relevant microdomains remains an open question. Overall, ascorbate reacts with superoxide but the spatiotemporal nature of this reaction and its relevance to exercise-induced redox signalling requires further investigation.

Nitric oxide

NOS isoforms utilise L-arginine to catalyse NO production [87]. The principal NOSs in skeletal muscle are nNOS (localised to the sarcolemma), eNOS (localised to the mitochondria) and iNOS the inducible isoform [88-89]. Skeletal muscle contractions increase intra and extracellular NO generation [55-56]. NO activates guanylate cyclases, via reversible heme group binding, to generate the signalling biomolecule cGMP [87]. This signalling mechanism is associated with several physiological outcomes, notably vasodilation following NO generation by vascular endothelial cells [90], but is not generally considered to be redox signalling *per se* [25]. Rather, NO based redox signalling is typically indirect in nature, proceeding through reaction of NO with other radicals [28]. Any reaction of exogenous antioxidants with NO directly would, therefore, be of consequence for indirect signalling. In this regard, NO reacts rapidly with other ROS/RNS, notably superoxide, but reacts slowly with other cellular biomolecules [91]. Hence, ascorbate and α-tocopherol have limited ability to suppress NO directly [69]. It is, however, recognised that ascorbate could influence NO bioavailability with possible implications for indirect signalling [92-93]. NOS mediated NO generation is contingent upon several co-factors, notably tetrahydrobiopterin (BH_4 [94]). Low levels of BH_4 and/or ablated BH_4 binding uncouple NOS isoforms resulting in the production of superoxide [93]. NOS uncoupling is implicated in the pathophysiology of cardiovascular disease [95]. Ascorbate is suggested to prevent NOS isoform uncoupling and thus enhance NO bioavailability [92]. The underpinning mechanisms remain to be fully resolved but might involve superoxide suppression [92], reduction in BH_4 oxidation and/or reduction of oxidised intermediaries (e.g. BH_3 [93]). The implication of this is unclear from a signalling perspective and may not be relevant in non-pathological settings. Overall, neither antioxidant can interfere with NO signalling by direct reaction but ascorbate might influence NO bioavailability, the outcome of this being unclear in an exercise setting.

Indirect signalling

Peroxynitrite

Peroxynitrite, a term encompassing peroxynitrite anion and its protonated form peroxynitrous acid, is an extremely labile reactive species generated by the diffusion controlled reaction

between NO and superoxide (k ~ 4-16 ^{x} 10^9 M^{-1} s^{-1} [97-100]). The aforementioned reaction proceeds at a significantly faster rate than the reaction of superoxide with SOD isoforms (k ~ 1-2 ^{x} 10^9 M^{-1} s^{-1} [88, 101]), rendering peroxynitrite generation a likely fate of NO and superoxide produced during muscle contractions [102]. From a signalling perspective, direct signalling by peroxynitrite is unlikely owing to rapid reaction with peroxiredoxins (k ~ 10^6-10^7 M^{-1} s^{-1} [99, 103-106]) and CO_2 (k ~ 5.8 ^{x} 10^4 M^{-1} s^{-1} [99, 104, 107-108]). The rather slow reaction (k ~ 10^2 M^{-1} s^{-1} for ascorbate [69]) of both ascorbate and α-tocopherol with peroxynitrite is unlikely to outcompete the aforementioned rapid reactants. It is improbable that this reaction out-competes the moderate reaction of peroxynitrite with glutathione (k = 1.35 x 10^3 M^{-1} s^{-1} [77]), given the abundance, present at millimolar concentrations in most cells, of glutathione. Further, diffusion of peroxynitrite across biological membranes is limited, rendering reaction with α-tocoperhol unlikely [77]. It is necessary, therefore, to consider whether ascorbate or α-tocopherol can modulate indirect peroxynitrite signalling.

Indirect peroxynitrite signalling could proceed via (1) coupled sensing and metabolism mechanism, wherein peroxiredoxins function as sensor proteins that transmit the signal (2) reaction with glutathione and generation of thiyl radicals and/or (3) radical derivatives of the reaction of peroxynitrite with CO_2 [25, 28]. Ascorbate and α-tocopherol are unlikely to interfere with any peroxiredoxin associated sensing-metabolism signalling. This would necessitate outcompeting two highly abundant and efficient reactants, CO_2 and peroxiredoxins, for peroxynitrite and hence will not be further considered herein. Analogously, neither antioxidant will likely out-compete glutathione to blunt any thiyl radical associated signalling. In any case, the principal biological fate of peroxynitrite is rapid reaction with CO_2 to generate short-lived intermediaries (e.g. nitrosoperoxocarbonate) that can form radical products following homolysis, notably carbonate radical and nitrogen dioxide [99, 104, 107-108]. It is possible that signalling proceeds through carbonate radical and nitrogen dioxide, as both are one electron oxidants [109] that could be implicated in thiol based signalling [28]. The capacity of these radicals to be second messengers in redox signalling might be limited by their non-selective reaction with protein thiols. Both radicals can initiate protein nitration with attendant implications for redox signalling [110]. For instance, nitration of HSP90 at specific residues (Tyr 33 & 56) inducts neuronal apoptosis via the Fas pathway [110]. It can also inactivate antioxidant enzymes (e.g. SOD2 and GPx1 [111-113]), which could facilitate transient transmission of a redox signal [114-115]. As a signalling paradigm, protein nitration could be limited by its random nature and lack of reversibility. Nevertheless, ascorbate or α-tocopherol mediated scavenging of carbonate radical and nitrogen dioxide could blunt subsequent thiol and/or protein nitration based signalling.

Ascorbate reacts with both carbonate radical and nitrogen dioxide [109]. In particular, the reaction of ascorbate with nitrogen dioxide (k ~ 3.5 ^{x} 10^7 M^{-1} s^{-1}) is similar to glutathione (k ~ 2 ^{x} 10^7 M^{-1} s^{-1}) and the reaction of nitrogen dioxide with tyrosine radical (k ~ 3.2 ^{x} 10^5 M^{-1} s^{-1}), an intermediate in the formation of nitrated proteins [77, 116]. Increased ascorbate concentrations post-supplementation could facilitate scavenging to attenuate nitrogen dioxide mediated protein nitration or thiol oxidation. The relevance of this for redox signalling is ill

defined and this represents a considerable caveat. Further, ascorbate would have to attenuate nitrogen dioxide formation proximal to the signalling reaction (nitrogen dioxide-protein tyrosine residue) as blunting signalling depends on interfering with spatially regulated cascades [80-83]. Distal reactions would be likely to just attenuate macromolecule damage without impinging redox signalling [80-83]. Any reaction of α-tocopherol with carbonate radical is likely biologically irrelevant, since the charge state of carbonate radical restricts diffusion through lipid bilayers [109, 117]. In contrast, nitrogen dioxide is uncharged and can react with α-tocopherol ($k \leq 10^6$ M^{-1} s^{-1}[116]). However, α-tocopherol is not considered an efficient nitrogen dioxide scavenger [116] and is likely out-competed by other reactants (e.g. glutathione), despite any increases in α-tocopherol membrane content post-supplementation. Overall, it is clear that (1) neither antioxidant is likely to interfere with indirect signalling associated with peroxiredoxins or glutathione (2) α-tocopherol is unlikely to interfere with any carbonate and nitrogen dioxide signalling but this is theoretically possible for ascorbate and (3) the importance of carbonate radical and nitrogen dioxide for redox signalling is unclear, questioning the biological relevance of any interference.

Hydrogen peroxide

Several aspects of redox signalling have been attributed to H_2O_2, a relatively stable and membrane permeable reactive oxygen species [23-29, 118-121]. The basic mechanism of H_2O_2 mediated signalling involves changes in target protein function following oxidation of cysteine residues to form sulfenic acid and disulphide bonds [26-27]. The reaction of H_2O_2 with highly abundant enzymes, notably glutathione peroxidase (k ~10^8 M^{-1} s^{-1} [122]), catalase (k ~2.0 x 10^7 M^{-1} s^{-1} [123]) and peroxiredoxins (10^5-10^8 M^{-1} s^{-1} [106, 124]), proceeds at a significantly faster rate than its reaction with reactive cysteine residues on low abundant signalling proteins (e.g. KEAP1 estimated k ~140 M^{-1} s^{-1} [125]). It would, at first glance, seem that H_2O_2 signalling would be precluded, owing to the H_2O_2 signal being metabolised before reaction with target proteins [23, 25]. There are several explanations for redox signalling proceeding despite this chemical bottleneck (see 28, 125), however three are particularly cogent. First, the H_2O_2 metabolising enzymes could act as sensors themselves, as has been suggested for peroxiredoxin isoforms [25; 126]. Indeed, peroxiredoxin 2 acts as a signal receptor and transmitter in STAT3 signalling [127]. Second, post-translational modifications (e.g. phosphorylation) could alter the catalytic efficiency of H_2O_2 metabolising enzymes, permitting transient transmission of a redox signal [25, 114-115]. Third, co-localisation of target and source allied to a favourable target protein microenvironment, principally manifested by an exposed thiol with low pK_a [23-29; 128-129]. It is apparent that the mechanistic details of H_2O_2 mediated signalling require further investigation [23].

Despite the aforementioned mechanistic considerations, H_2O_2 mediated signalling is implicated in the regulation of kinases, phosphatases, transcriptional co-activators and transcription factors in various subcellular compartments [23-29; 125]. For instance, kinases and phosphatases modulate cell signalling via catalysing phosphorylation and dephosphorylation of protein residues, respectively [129-130]. Oxidation of cysteine residues in the catalytic domain of these enzymes, results in reversible activation of tyrosine kinases (e.g. Src [130]) and inactivation of phosphatases (e.g. PTEN and SHP-2 [131]). This redox

signalling paradigm is important for the propagation of growth factor signalling (e.g. epidermal growth factor), as demonstrated by genetic over-expression of H_2O_2 metabolising enzymes [132]. Indeed, growth factor activation stimulates localised H_2O_2 generation in several cell types, probably owing to NADPH oxidase mediated superoxide production and subsequent dismutation to H_2O_2 [130]. In an exercise setting, H_2O_2 mediated inactivation of mitogen activated protein kinase phosphatase could promote p38 MAPK, JNK and ERK activation, proteins implicated in exercise-induced cell signalling [36]. Although, the precise events have yet to be defined, H_2O_2 is likely a key effector of exercise-induced redox signalling.

It is noteworthy that neither ascorbate nor α-tocopherol react appreciably with H_2O_2 [133] and hence, *prima facie*, have limited capacity to directly impact this important redox signalling mechanism. Even if they could react with H_2O_2, both ascorbate and α-tocopherol would be unlikely to out-compete endogenous H_2O_2 reactants, such as peroxiredoxins [24]. There are, however, two indirect mechanisms that warrant consideration. First, SOD isoforms catalyse the dismutation of superoxide to H_2O_2 [134]. Ascorbate could indirectly attenuate the H_2O_2 signal via reaction with superoxide, provided spatiotemporal concerns are satisfied, localised reaction with superoxide in the relevant microdomain (see superoxide section), and other reactants are outcompeted (e.g. NO). Any attenuation of the H_2O_2 signal could have ramifications for superoxide generation since NADPH oxidases are, in part, activated by H_2O_2 [135]. However, Nox4 is a NADPH oxidase expressed in skeletal muscle that can generate H_2O_2 directly [63; 136]. It is extremely unlikely that ascorbate diminishes Nox4 mediated H_2O_2 generation. Any indirect inhibition is not possible for α-tocopherol owing to lack of appreciable reaction with superoxide [69]. Second, the reaction of hydrogen peroxide with transition metal centres can yield superoxide and/or hydroxyl radical [69]. It is possible that these radicals could then transmit a local signal that could be scavenged. However, there are two major problems with this hypothesis (1) the random nature precludes specific signalling and (2) the reaction of either antioxidant with hydroxyl radical is biologically meaningless, since hydroxyl radical reacts with the first biomolecule it encounters [137-138]. Overall, we do not exclude indirect interference with H_2O_2 signalling, probably via reaction of ascorbate with superoxide, but emphasise that experimental support in an exercise setting is required.

Removal of the cysteine modification once formed: S-Nitrosylation as an exemplar paradigm

Ascorbate and α-tocopherol might remove redox modifications once formed and this could interfere with exercise-induced redox signalling. S-Nitrosylation (S-NO) is considered as an exemplar paradigm. S-NO defines the attachment of NO to cysteine [139]. NO is a weak nitrating agent and cannot generate S-NO directly [140]. Indeed, the precise reactions involved in S-NO formation *in vivo* are ill-defined [141]. It is suggested that transition metal catalysed pathways, formation of dinitrogen trioxide and thiyl radical species contribute to S-NO generation [142-143]. Knowledge of exercise-induced S-NO events are limited but the following observations support a role (1) protein kinases and phosphatases are S-nitrosylated [139] (2) transcription factors implicated in exercise adaptations are S-nitrosylated, including

HIF-α [144], p53 [145] and NF-κB [52] and (3) the ryanodine receptor type I is S-nitrosylated with attendant implications for Ca^{2+} signalling and muscle function [146]. Ascorbate can denitrosylate proteins indeed this property forms the basis of the biotin-switch assay, a S-NO analytical tool [147-148]. Denitrosylation can proceed in a copper dependent or independent manner [149]. The former is unlikely *in vivo* given the chelation of transition metals whilst the latter is associated with high ascorbate concentrations (5-50 mM), and even then only partial denitrosylation of a sample occurs [27]. Whether ascorbate dependent denitrosylation occurs at physiological concentrations and in the relevant cellular microdomains is debatable but should not be discounted at this stage. The literature appertaining to denitrosylation reactions involving α-tocopherol is limited and hence its feasibility and relevance in vivo is an open question. Nevertheless, similar concentration, localisation and specificity concerns apply. Further, it is unlikely that exogenous antioxidants exert an effect greater than the existing endogenous denitrosylation system [139]. This system includes the S-nitrosoglutathione and thioredoxin pathway and enzymes such as: protein disulphide isomerase, SOD isoforms and xanthine oxidase [150]. Taken together, two observations are apparent (1) S-NO modifications relevant to the adaptive exercise response require investigation (2) the effect of ascorbate and α-tocopherol on the skeletal muscle S-NO proteome is not known. Ascorbate and a-tocopherol are unlikely to interfere with other modifications (e.g. S-glutathionylation) once formed as there is limited chemical basis for any direct interference.

Alternate mechanisms

Reduction of potentially bioactive oxidised macromolecule adducts

Direct signalling by indiscriminately reactive one electron oxidants, notably hydroxyl radical, is limited by lack of specificity, precluding signalling via conventional mechanisms (e.g. protein post-translational modifications [26-27]). Indirect signalling might be afforded by the generation of oxidised lipid, DNA and protein adducts [151-152]. In particular, pre-treatment of cells with low-doses of lipid peroxidation products (e.g. 4-hydroxynonenal) inducts favourable responses, notably activation of the Nrf-2-KEAP1 pathway, that protect against the stress imposed by a subsequent oxidative challenge [153-154]. Nrf-2-KEAP1 pathway activation is likely to proceed via S-alkylation of KEAP1 and subsequent inactivation, an event that promotes the nuclear translocation of Nrf-2 [66, 155]. Interestingly, S-alkylation also regulates NADPH oxidase activity [156], facilitating a putative negative feedback loop. The sensing of damaged proteins and DNA adducts by chaperones and repair enzymes, respectively, could provoke an adaptive response. Cell signalling processes are subject to intricate spatiotemporal regulation [20-22, 80-85]. Macromolecule oxidation, secondary to hydroxyl radical attack, fails to satisfy this fundamental signalling requirement, being inherently random and non-specific [137-138, 157]. Whether levels of oxidised macromolecules serve as a general non-specific redox rheostat that informs signalling responses is an open question. Nevertheless, this is unlikely on a global level owing to the compartmentalised and specific nature of cell signalling [20-22].

Acute exercise bouts are usually, but not always [see 158], associated with an increase in oxidised macromolecule adducts [159]. If these products were acting in a signalling fashion, this postulate requires investigation in an exercise setting, then an ascorbate and α-tocopherol mediated reduction in oxidised macromolecule adducts might blunt this potentially favourable response (see figure 2). Although, both antioxidants scavenge radicals implicated in the initiation of macromolecule oxidation the effects of antioxidant supplementation on oxidised adduct levels are variable [137-138]. This is best exemplified in pathological contexts wherein global levels of oxidised macromolecule adducts are constitutively elevated [160], possibly reflecting deregulated redox signalling. In these settings, ascorbate and α-tocopherol supplementation does not decrease disease incidence and generally only marginally decreases macromolecule oxidation [137-138, 161-164]. This might reflect a failure of ascorbate and α-tocopherol to accumulate in redox signalling compartments and effect a reduction in the levels of a reactive species or indeed a failure to react appreciably with the relevant species [161-164]. Further, positive effects are generally evident in individuals presenting with ascorbate and α-tocopherol deficiency at baseline [165]. Of course, the nature of macromolecule oxidation at rest compared to exercise are likely different. In an exercise setting, ascorbate and α-tocopherol afford limited protection against exercise-induced macromolecule damage [166]. Indeed, a recent meta-analysis concluded that α-tocopherol does not reduce exercise-induced lipid peroxidation [166]. Overall, a signalling role of oxidised macromolecules is speculative in an exercise setting and neither antioxidant consistently protects against exercise-induced macromolecule oxidation. Reduction of potentially bioactive oxidised macromolecule adducts does not likely explain the attenuation of favourable cell signalling responses to exercise training following ascorbate and α-tocopherol supplementation.

Pro-oxidant potential

The oxidation of ascorbate results in the formation of an ascorbyl radical [93]. Ascorbyl radical is unlikely to exert pro-oxidant effects *in vivo* owing to its poor reactivity and existence of glutathione and NADPH dependent recycling systems [167]. Ascorbate has well-documented pro-oxidant properties *in vitro* when free transition metal are present [76]. Ascorbate can reduce Fe^{3+} to Fe^{2+}, and Fe^{2+} can then in turn react with O_2 to generate superoxide [176]. Ascorbate can also generate hydroxyl radical and H_2O_2 via classical Fenton chemistry [177]. Indeed, this is the basis for the use of pharmacological intravenous ascorbate administration as a cancer treatment owing to the toxicity of H_2O_2 to certain cancer cells [177-178]. This treatment paradigm bypasses gut metabolism removing the absorption constraints that restrict peak plasma ascorbate concentrations to ~200 μM following even high-dose oral supplementation [178]. The relevance of these pro-oxidant effects *in vivo* is highly debated, and indeed controversial, especially in non-pathological contexts [178]. Any pro-oxidant action is likely dependent on the availability of transition metals. It is emphasised that these are largely sequestered by the metallothionein family, transferrin and ferritin [170]. Despite the intracellular sequestration of certain transition metals, cells still contain small (~20 μM) un-sequestered pools of free iron that could participate in pro-oxidation reactions [171]. Interestingly, microarray analysis has revealed that metallothionein mRNA abundance

is significantly enriched following acute endurance exercise [172]. This could reflect a stress response to exercise-induced perturbations in intracellular transition metal handling. Such perturbations are likely to be greater following exercise that evokes muscle damage, given that muscle injury increases labile iron levels in skeletal muscle [173] possibly owing to increased hemolysis [174]. The aforementioned scenarios would permit increased free transition metal availability and pro-oxidant ascorbate potential. Any pro-oxidant actions could elevate the 'redox' signal from an adaptive to maladaptive threshold. This supposition is, however, speculative at present. Some species (e.g. mice and rodents) retain the capacity to endogenously manufacture ascorbate from glucose owing to expression of gulonolactone oxidase [175]. Humans harbour a defunct gulonolactone oxidase gene and hence need to acquire ascorbate exogenously, via dietary sources. Disruption of ascorbate homeostasis in lower order species with large dose supplementation could favour pro-oxidant and cytotoxic effects that contribute to blunted training adaptations.

Similar to ascorbate, any pro-oxidant effect of α-tocopherol could elevate the 'redox' signal from an adaptive to maladaptive threshold. The oxidation of α-tocopherol yields α-tocopherol radical [75]. Although, α-tocopherol radical is capable of inducting lipid peroxidation *in vitro*, this has not been consistently been documented *in vivo* [93, 176]. Toxicity of α-tocopherol radical is thought to be limited by ascorbate mediated recycling of α-tocopherol radical to α-tocopherol [93]. Indeed, this reason is often cited as a justification for α-tocopherol and ascorbate co-supplementation [16]. Ascorbate mediated recycling of α-tocopherol radical is well documented *in vitro* but evidence for this interaction *in vivo*, particularly in humans, is often inconsistent [69]. Recycling can also be achieved by glutathione [177], which could be an important contributor *in vivo*. Analogous to ascorbate, tocopherol isoforms can exert transition metal dependent pro-oxidation effects *in vitro* but their sequestration and localisation is likely to limit this possibility *in vivo* [75]. Overall, it is unlikely that α-tocopherol is acting in a pro-oxidant fashion to diminish exercise-induced redox signalling.

Perspectives

Beyond theory and speculation there is a paucity of evidence supporting the notion that ascorbate and α-tocopherol supplementation interferes with exercise-induced redox signalling via a redox-dependent 'scavenging' mechanism. Unfortunately, obtaining supporting evidence is hampered by several analytical limitations. Electron spin resonance and fluorescent based probe technology are not readily applicable to the *in vivo* human situation and many fluorescent probes are prone to experimental artefact, that is, spurious side-reactions that artificially amplify the signal [178-180]. Interpretation of these techniques in animal and cell culture models is complicated by interspecies differences (e.g. rodents can manufacture ascorbate) and the oxidative stress that cell culture can impose [181-182]. This has fostered a reliance on biochemical footprints, such as lipid peroxidation biomarkers (e.g. malondialdehyde [44, 157]. A change in a biochemical footprint does not necessarily reflect a redox-dependent scavenging effect of exogenous antioxidants it could simply reflect differential repair or dietary changes [69, 133]. Redox signalling occurs in specific cellular compartments hence altered macromolecule oxidation levels do not necessarily reflect the incidence of redox signalling [80-86]. That is, redox signalling does not require global

changes in oxidised macromolecule adducts to occur [80-82]. Instead, specific, reversible and compartmentalised signals define redox signalling [80-86]. Whether assaying global levels of oxidised macromolecule adducts provides any useful information on the interference of ascorbate and α-tocopherol supplementation with exercise-induced redox signalling is therefore debatable.

In considering possible technical solutions, redox proteomics enables quantitative and unbiased analysis of redox-regulated post-translational modifications implicated in cell signalling [183-187]. However, signalling proteins might be masked by the abundance of metabolic and contractile proteins in skeletal muscle [183-187]. Further, determining the functionality of novel modifications would require further experimentation [188]. Application of redox proteomics to the study of exercise-induced redox signalling is strongly encouraged. Another way might be to analyse redox regulated end-points, such as activity and abundance of antioxidant enzymes and heat shock proteins [46]. Ascorbate and α-tocopherol supplementation did not interfere with antioxidant enzyme and heat shock protein abundance when this approach was recently applied [8]. This might suggest a lack of a redox dependent mode of action since these outcome markers are one principal end-point of exercise-induced redox signalling. However, this approach provides limited mechanistic information being unable to identify the nature of any possible interference [189]. Overall, it is clear that further mechanistic research is required and that redox proteomics represents an admiral starting point.

Ascorbate and α-tocopherol could act in a redox independent manner to attenuate favourable cell signalling responses to exercise training. Ascorbate is a co-factor for α-ketoglutarate dependent dioxygenases (e.g. prolyl 4-hydroxylase [93,169,175]) and also promotes HIF-α repression via proline hydroxylation [190-191]. This is particularly relevant to exercise given the role of HIF-α in the regulation of angiogenesis, growth, apoptosis and metabolism [192-193]. Of interest, ascorbate can regulate the activity of enzymes implicated in the regulation of histone methylation [194-195], an epigenetic process that regulates exercise adaptations [196]. Similarly, α-tocopherol can inhibit 5-LOX, protein kinase C isoforms and phospholipase A_2 which could influence exercise-induced cell signalling [197-199]. Inhibition of these enzymes is suggested to be redox independent and appears to be related to the interaction of α-tocopherol with signalling proteins [197-199]. This could explain the observation that several genes (e.g. tropomyosin) are regulated by α-tocopherol [197]. Altogether, it is possible that redox-independent actions contribute and this is worthy of further investigation.

Irrespective of the mechanism, redox dependent or independent, blunted cell signalling responses following ascorbate and α-tocopherol supplementation have seldom translated to impaired whole-body exercise adaptations (e.g. diminished increases in aerobic capacity [1]). There are several possible explanations for this however, two are particularly cogent. First, changes at the whole-body level are a product of peripheral and central adaptations hence any peripheral impairment can be compensated for [15]. Second, the molecular processes measured are often stress responses and have rarely been shown to be either essential to adaptation and/or predict the magnitude of adaptation [200]. Further, signalling processes

have an in built reserve capacity, therefore, suppression of an upstream signal does not always translate to blunted downstream responses [20-22]. When it is considered that a whole-body response is the reflection of highly regulated processes across several cell types it is unsurprising that blunted activation of one or two regulatory proteins fails to impact adaptation. The physiological relevance of an impaired molecular response to functional end-points is, therefore, debatable.

Conclusion

Current paradigms posit that ascorbate and α-tocopherol supplementation act as antioxidants to diminish global superoxide, NO, peroxynitrite and H_2O_2 levels and thus affect an attenuation of exercise-induced redox signalling. For this to be possible, it is contended here that the criteria outlined in box 1 must be satisfied. Our largely theoretical analysis reveals that all of assumptions implicit in a redox dependent mechanism of action are not met for any of the aforementioned species. The best candidate for a scavenging effect represents the reaction of ascorbate with superoxide, with attendant implications for H_2O_2 signalling. Even in this case, it is unclear whether the requisite chemical (out-competing other reactants) and spatiotemporal (co-localisation with relevant targets) concerns are satisfied. It is readily acknowledged that the present analysis is limited by knowledge of the mechanisms underpinning exercise-induced redox signalling being fragmentary. It is also emphasised that a nuanced view of kinetics in space, time and context is warranted. That is, kinetic information is usually derived from *in vitro* experiments that do not faithfully mimic the *in vivo* situation. A situation characterised by compartment specific redox potentials and pH characteristics, all of which could influence the reaction of ascorbate and α-tocopherol with a given species and thus our conclusions. Despite the aforementioned caveats, a clear challenge to the current interpretational framework is presented. It cannot be assumed that just because a molecule has ‘antioxidant properties’ that it is acting as an antioxidant to attenuate exercise-induced redox signalling *in vivo*. Further, in the current context altered global levels of oxidised macromolecules should not be used to evidence an attenuation of exercise-induced redox signalling. Indeed, it is our view that redox signalling networks that are insulated from nutritional antioxidants have evolved. Whilst ascorbate and α-tocopherol could scavenge reactive species that diffuse out of signalling microdomains the insulation could protect against any major interference. This observation may be novel in an exercise setting but is consistent with the failure of nutritional antioxidant therapy to modify diseases associated with oxidative stress and pathological disruption of redox signalling. It is hoped that the present dialogue stimulates investigations into the molecular mechanisms underpinning the blunting of exercise-induced redox signalling following ascorbate and α-tocopherol supplementation. It is emphasised that this discourse applies only to the antioxidants discussed and should not be extrapolated to other antioxidants, since antioxidants are not biochemically and functionally homogenous [133]. In this regard, it might be worthwhile exploring alternate antioxidant paradigms, such as N-acetyl-cysteine [201].

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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Figure Legends

Figure 1: A) A current general scheme. In this generic model, exercise increases ROS/RNS generation and this is associated with kinase activation. Ascorbate and α-tocopherol are proposed to reduce ROS/RNS generation to interfere with phosphatase inactivation. Note that in this general model the specific species are not identified underscoring a significant limitation of this generic model. From this scheme it is not possible to appraise whether this

redox dependent mode of action is feasible. B) Proposed specific scheme. In this model, exercise activates NADPH oxidases resulting in increased superoxide production. Superoxide is then dismutated to hydrogen peroxide in a reaction catalysed by SOD isoforms. Hydrogen peroxide then reacts, in a two electron reaction, with the phosphatase PTP1B, possibly relieving kinase inhibition. Whether this is possible given the peroxiredoxin kinetic bottleneck is discussed in text. Nevertheless, ascorbate could inhibit this signalling response by competing with SOD isoforms and NO (not shown for clarity) for reaction with superoxide.

Figure 2: Reduction of potentially bioactive oxidised macromolecule adducts. In this model, exercise increases superoxide, NO, peroxynitrite and H_2O_2 generation resulting in the generation of bioactive oxidised adducts, such as 4-hydroxynoneneal. This could lead to Nrf-2 activation and the induction of a cyto-protective response via S-alkylation of KEAP1, a negative regulator of Nrf-2. Any ascorbate and α-tocopherol mediated reduction in bioactive oxidised macromolecule adducts could attenuate Nrf-2 activation. However, this possibility is speculative for several reasons that are discussed in text.

Figure 3: Summary of the limited reaction of ascorbate and α-tocopherol with specific reactive species implicated in exercise-induced redox signalling. Of note, ascorbate can react with superoxide ($O_2^{\cdot -}$) and this could have implications for exercise-induced redox signalling. The existence of kinetically favourable out-competing reactions for nitric oxide, hydrogen peroxide and peroxynitrite might restrict any interference via a scavenging mechanism at least for these species. It is possible for nitrogen dioxide and carbonate radical, but the roles of these radicals in redox signalling is not well established.

Box

Box 1. Assumptions implicit in a redox dependent mechanism of action.

Assumptions implicit in a redox dependent mechanism of action.
1. Specific ROS/RNS are involved in redox signalling. 2. Ascorbate and α-tocopherol react chemically with the relevant ROS/RNS. 3. The localisation of ascorbate and α-tocopherol makes interference in cellular microdomains implicated in redox signalling likely (e.g. lipid rafts). 4. Ascorbate and α-tocopherol out-compete enzymes and/or other ROS/RNS for reaction with the relevant ROS/RNS.