Oxidative Stress and Antioxidants in Exercise

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Abstract: Increased aerobic metabolism during exercise is a potential source of oxidative stress. In muscle, mitochondria are one important source of reactive intermediates that include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and possibly hydroxyl radical ($HO^{\cdot}$). The recent discovery that mitochondria may generate nitric oxide ($NO^{\cdot}$) also has implications for oxidant production and mitochondrial function. In this review, we critically examine the concept that production of reactive intermediates increases during exercise. Because the health benefits of regular exercise are well-documented, we also examine adaptations to exercise that may decrease oxidative stress. These include increased antioxidant defenses, reduced basal production of oxidants, and reduction of radical leak during oxidative phosphorylation.

INTRODUCTION

The well-documented benefits of regular physical exercise include reduced risk of cardiovascular disease, cancer, osteoporosis, and diabetes [1-3]. The complex mechanisms that contribute to these effects include decreased adipose tissue, altered lipid and hormonal profiles, receptor and transport-protein adaptations, improved mitochondrial coupling, and alterations to antioxidant defenses. Such defenses are necessary because aerobic organisms produce reactive oxygen species during normal respiration and inflammatory conditions. Exercise can create an imbalance between oxidant and antioxidant levels, a situation known as oxidative stress. Indeed, oxidative stress resulting from acute exercise in unadapted and adapted subjects has been proposed to damage enzymes, protein receptors, lipid membranes, and DNA [4-8].

In muscle, mitochondria are one source of reactive species that include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and possibly hydroxyl radical ($HO^{\cdot}$) [9-12]. The recent discovery that mitochondria generate nitric oxide ($NO^{\cdot}$) also has implications for oxidant production and mitochondrial function [13] during exercise. In moderate amounts, nitric oxide may regulate respiration [13], but it may also react with radicals such as $O_2^{\cdot -}$ to form peroxynitrite ($ONOO^-$), a powerful oxidant [14].

Phagocytic white blood cells also produce potent oxidants, generating reactive species that kill invading pathogens. However, neutrophils can infiltrate damaged skeletal muscle following strenuous or eccentric exercise, further damaging cells. Activated neutrophils use a membrane-associated NADPH oxidase to generate $O_2^{\cdot -}$, which can directly react with target compounds or dismutate to H_2O_2. Peroxide in turn is converted to hypochlorous acid (HOCl) by myeloperoxidase, a heme protein secreted by neutrophils and monocytes [15,16]. HOCl is an inflammatory mediator and a strongly oxidizing and chlorinating compound that generates other reactive metabolites such as nitryl chloride (NO_2Cl) in the presence of nitrite [17]. Moreover, activated human polymorphonuclear neutrophils can use myeloperoxidase and H_2O_2 to convert nitrite into nitrogen dioxide radical ($NO_2^{\cdot}$), contributing to the formation of potentially harmful compounds [17].

Although endothelial cells, leukocytes, alterations in calcium homeostasis, and liberation of redox-active metals also can contribute to oxidative stress, this review will highlight the possible roles of mitochondria and phagocytes in oxidant production during and after exercise. It will focus on the potentially deleterious effects of reactive oxygen species, reactive nitrogen species, and chlorinating species—referred to here as reactive intermediates.

SOURCES OF REACTIVE INTERMEDIATES DURING EXERCISE

The oxygen molecule in its diatomic ground-state ($^3\Sigma g^-$ O_2) is a highly oxidizing species that is essential for energy production during mitochondrial oxidative phosphorylation. It qualifies as a radical species because it has two unpaired electrons, each located in a different π antibonding orbital. These electrons have parallel spins, i.e., they both share the same spin quantum number. Consequently, ground-state oxygen is sparingly reactive despite its strong oxidation potential: according to Pauli's exclusion principle, O_2 will oxidize another molecule by accepting an electron pair only if both electrons of the pair possess spins that are antiparallel

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to those of its own unpaired electrons. Because this criterion is seldom met, O_2 reacts slowly in the absence of catalysts and tends to accept single electrons during redox chemistry.

In vivo, enzymes generally use one electron at a time to achieve multi-electron reduction of O_2. If a single electron is accepted, it must enter an antibonding orbital and produce $O_2^{\cdot -}$.

$$O_2 + e \rightarrow O_2^{\cdot -} \quad \text{Equation 1}$$

Two-electron reduction of O_2 plus the addition of 2 protons (H^+) generates H_2O_2.

$$O_2 + 2e + 2H^+ \rightarrow H_2O_2 \quad \text{Equation 2}$$

Many oxidases use this mechanism to reduce O_2 directly to H_2O_2. The spontaneous or catalyzed dismutation of $O_2^{\cdot -}$ by superoxide dismutase also produces H_2O_2.

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{Equation 3}$$

Peroxide is a non-radical intermediate that oxidizes a wide variety of biological moieties, though it is a relatively unreactive species.

In the Haber-Weiss reaction (also known as superoxide-driven Fenton chemistry), free or low molecular weight chelates of transition metals such as Fe^{3+} are reduced by $O_2^{\cdot -}$ to Fe^{2+}. The reduced metal ion then reacts with H_2O_2 to generate the extremely reactive $HO^{\cdot}$.

$$Fe^{2+} + H_2O_2 \rightarrow HO^{\cdot} + HO^- + Fe^{3+} \quad \text{Equation 4}$$

This species has been widely postulated to be the major cause of damage to proteins, lipids, carbohydrates, and DNA, but there is little direct evidence that $HO^{\cdot}$ is generated in biological systems. A major unresolved issue regarding the biological relevance of the Haber-Weiss reaction is its requirement for free Fe^{3+} or Cu^{2+} because a wide variety of metal-transporting and metal-binding proteins maintains the concentration of free redox-active metal ions at low levels in normal tissue. However, tissue destruction may liberate redox-active metal ions.

$O_2^{\cdot -}$ reacts extremely rapidly with NO to form $ONOO^-$, a nonradical species that is a potent oxidizing and nitrating intermediate.

$$O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^- \quad \text{Equation 5}$$

This reaction is widely believed to represent a major pathway for generating reactive nitrogen species in vivo.

Much attention has been focused on the production of oxidizing species by $O_2^{\cdot -}$. However, it is important to note that $O_2^{\cdot -}$ is a strong reducing agent. This property accounts for its facile ability to react rapidly with metal ions (M^{n+}).

$$O_2^{\cdot -} + M^{n+} \rightarrow O_2 + M^{(n-1)+} \quad \text{Equation 6}$$

This reaction has been proposed to generate the reduced metals ions necessary for producing $HO^{\cdot}$ by the Haber-Weiss reaction (Equation 4). Recent studies suggest that proteins containing transition metals, such as aconitase, an enzyme of the tricarboxylic acid cycle, are vulnerable to reductive damage by $O_2^{\cdot -}$ [18,19,20], which could be a contributing factor in muscle fatigue during exercise.

Mitochondria and Exercise

Although the main function of mitochondria is energy production, isolated mitochondria generate reactive oxygen species during oxidative phosphorylation. Release of such intermediates accounts for an estimated 1% to 5% of the oxygen consumed during respiration, depending on the substrate and respiration state (see below). However, most studies used isolated mitochondria, and the flux of oxidants was often estimated indirectly. There is surprisingly little direct evidence for the generation of reactive species by mitochondria in intact cells or tissue.

The mitochondrial electron transport chain, which reduces O_2 to H_2O, may be one major source of oxidants (Fig. (**1**)). Electrons from NADH are donated to complex I (NADH-ubiquinone reductase complex), and electrons from succinate are donated to complex II (succinate dehydrogenase complex). Ubiquinone, also known as coenzyme Q, accepts electrons from both complexes and is sequentially reduced, one electron at a time, to ubisemiquinone and ubiquinol. Ultimately, electrons are transferred from ubiquinol to complex III (ubiquinol-cytochrome c reductase), cytochrome c, and complex IV (cytochrome c oxidase) to reduce O_2 to H_2O.

Two kinds of substrates are used experimentally to determine oxygen consumption and the generation of reactive intermediates by complex I and complex II. Pyruvate and malate serve as electron donors for complex I; their oxidation by the tricarboxylic acid cycle generates NADH. Succinate serves as an electron donor for complex II; its oxidation by succinate dehydrogenase generates protein-bound $FADH_2$. Two electrons from $FADH_2$ are then transferred directly to FeS clusters of complex II.

When measuring oxygen consumption and radical production using site II substrates, it is critical to use rotenone (inhibitor of electron transfer within site I) in conjunction with succinate. The reason for this is that rotenone will prevent back flow of electrons to site I and accurate measurements of the electron leak at site II cannot be calculated. In addition, comparison can be made since electrons produced by pyruvate and malate will pass through complex I, III, and IV, whereas with succinate and rotenone they pass through complex II, III and complex IV. Finally, oxygen consumption can be measured under state 4 conditions, where there is no addition of ADP and therefore no active oxidative phosphorylation and therefore a low amount of oxygen consumption. Under state 3 conditions ADP (usually 0.5 mM) is added which actively stimulates oxidative phosphorylation and therefore oxygen consumption. Besides oxygen consumption it is also relevant to measure oxygen radicals under state 3 and state 4 conditions.

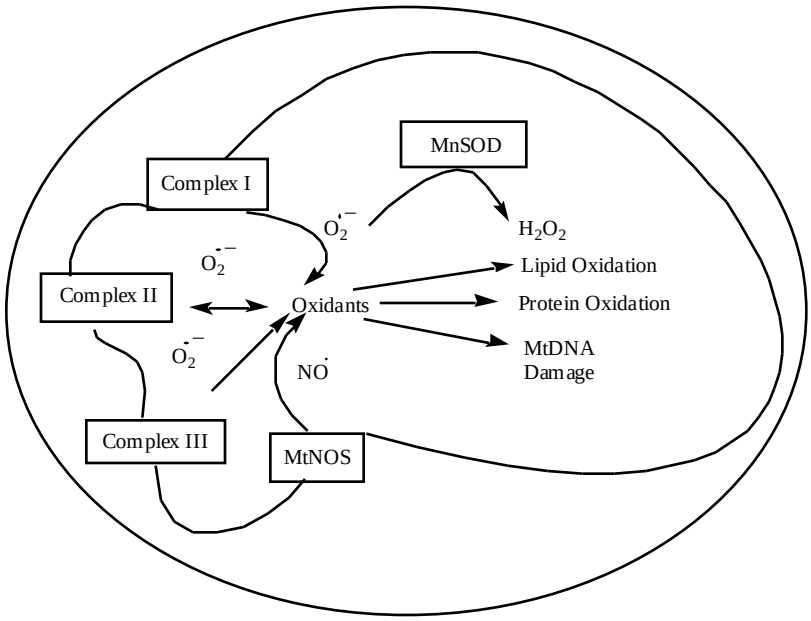

Fig. (1). Mitochondrial oxidant production. Reactive intermediates such as superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite form in mitochondria, where they cause oxidative damage. MtNOS, Mitochondrial nitric oxide synthase. MnSOD, manganese superoxide dismutase.

In vitro studies show that mitochondrial electron transport is imperfect, since it generates $O_2^{\cdot -}$ from the one-electron reduction of O_2. Enzymatic dismutation of $O_2^{\cdot -}$ then produces H_2O_2, another important biological oxidant [9-12,21-24]. This electron is thought to come from the one-electron reduction of ubiquinone, which generates the reactive intermediate ubisemiquinone formed by complex III. Instead of accepting another electron and proton to form ubiquinol, ubisemiquinone may leak its unpaired electron to O_2, forming $O_2^{\cdot -}$. However, recent evidence suggests that complex I is also a major site for the production of reactive intermediates in mitochondria [21]. Site I seems to be mainly responsible for differences in superoxide production with age in different species [21].

If mitochondrial respiration generates both $O_2^{\cdot -}$ and H_2O_2, how much mitochondrial H_2O_2 is derived from dismutation of $O_2^{\cdot -}$? $O_2^{\cdot -}$ production cannot be measured in intact mitochondria because superoxide is dismutated by mitochondrial superoxide dismutase (which contains Mn in its active site). However, sonicating and washing mitochrondria generates submitochondrial fractions that lack superoxide dismutase, making it possible to detect $O_2^{\cdot -}$ production by the electron transport chain. In experiments from the 1970s, submitochondrial particles produced between 4 and 7 nmol $O_2^{\cdot -}$/min per mg of protein, resulting in $O_2^{\cdot -}/H_2O_2$ ratios of 1.5 to 2.1 [9,10,11]. Because two $O_2^{\cdot -}$ anions dismutate to form one molecule of H_2O_2, these results indicate that $O_2^{\cdot -}$ is the likely precursor of mitochondrial H_2O_2.

H_2O_2 production can be determined directly in intact isolated mitochondria by using 4-hydroxy-3-methoxy-phenylacetic acid (homovanillic acid) as substrate [23]. This approach may more accurately reflect $O_2^{\cdot -}$ production because the mitochondria remain intact and are not mechanically or chemically altered. The method used by Barja [23] is specific for H_2O_2 production when intact mitochondria respire with reducing substrate alone (state 4—low rates of oxygen consumption and oxidative phosphorylation) and with addition of ADP (state 3—high rates of oxygen consumption and oxidative phosphorylation). Under these conditions, isolated rat heart mitochondria produce approximately 1 nmol H_2O_2 /min/mg protein in state 4 with complex I- and complex II-linked substrates. In fact, H_2O_2 production is maximal during state 4 respiration, when the electron carriers are highly reduced and the supply of ADP is limiting [9,10,11,21]. The high degree of reduction may partly explain why there is more radical leak during state 4 conditions than during state 3 (see next section). It has been estimated that the release of reactive intermediate accounts for 1% to 5% of the oxygen consumed intermediates during state 4 respiration [9,21,24,25].

It is possible to estimate the total amount of reactive intermediates ($O_2^{\cdot -}$ and H_2O_2) produced by humans at rest. A 70 kg adult utilizes an estimated 3.5 ml O_2/kg/min at rest or ~ 350 L/day (14.7 mole/day). If mitochondria convert about 1% of this O_2 to $O_2^{\cdot -}$, 0.147 mole $O_2^{\cdot -}$ would be produced per day. Because two $O_2^{\cdot -}$ anions dismutate into one H_2O_2 molecule (equation 2), the yield of H_2O_2 would be half that of $O_2^{\cdot -}$.

Does exertion step up rates of oxidant production? Exercise is characterized by a high rate of ADP formation because ATP breakdown increases with workload. High ADP levels activate oxidative phosphorylation and therefore would be considered state 3 respiration in experiments with isolated mitochondria. compelling evidence by Barja *et al.* [12,21,23] shows that isolated heart mitochondria using succinate (complex II-linked substrate) dramatically reduce (almost stop) oxidant production after ADP is added (state 3) and that use of pyruvate and malate (complex I-linked substrates) allows only moderate oxidant production after the

addition of ADP. Therefore, abundant ADP, which equates with high metabolic activity and rapid oxygen consumption, does not necessarily reflect high oxidant production. Thus, the simple notion that production of reactive intermediates increases in proportion to oxygen consumption is not supported by studies of isolated mitochondria. From calculations, one could argue that there is no significant difference in mitochondrial oxidant production between rest (low ADP levels; state 4) and exercise (high ADP levels; state 3).

Because heart mitochondria work continuously, they should have relatively high levels of ADP and approximate state 3, even at rest. Barja [21] has proposed that the lower levels of reactive intermediates seen in state 3 might protect cardiac muscle, preventing massive oxidative stress by improving electron coupling.

Improved coupling of electron transport and oxygen tetravalent reduction to water during state 3 has recently been reported in skeletal muscle mitochondria [22]. Because skeletal muscle can increase its oxygen consumption up to 20-fold between rest and exercise, this could be an important mechanism for controlling reactive intermediate production. When Venditti *et al.* measured H_2O_2 production in mitochondria isolated from skeletal muscle, they found similar levels to those in heart muscle mitochondria. When succinate was the substrate, H_2O_2 production was 50% less in state 3 than in state 4. It was 20% less when the complex I-linked substrates pyruvate and malate were used.

Do mitochondria produce other oxidants, such as nitric oxide, that set the stage for complex chemical interactions at the cellular level? Nitric oxide synthase (NOS), the enzyme that produces the gaseous reactive intermediate NO from L-arginine, has been investigated primarily in endothelial cells. Several lines of evidence suggest that mitochondria express NOS and produce $NO^{\cdot}$ [13]. First, several immunohistochemical studies showed that skeletal muscle expresses endothelial-type nitric oxide synthase (ec-NOS). There also was a strong correlation between ec-NOS expression and mitochondrial content visualized histochemically with succinate dehydrogenase [26]**.** In addition, Bates *et al*. [27,28] localized nitric oxide synthase in mitochondria isolated from heart, skeletal muscle, kidney, and brain using a monoclonal antibody against ec-NOS. Recently, mitochondria have been identified as sources of $NO^{\cdot}$ using electron paramagnetic resonance with spin-trapping techniques. Also, NOS was isolated from Percoll-purified rat liver mitochondria [29]. These findings suggest that mitochondrial NOS and $NO^{\cdot}$ play an important role in oxidative metabolism**.** Furthermore, $NO^{\cdot}$ and $O_2^{\cdot -}$ react to produce another very potent oxidant, $ONOO^-$ (equation 4). This and other reactive intermediates, such as $O_2^{\cdot -}$ and H_2O_2, make mitochondria a potential major source of oxidants during exercise.

Little direct evidence is available to determine whether reactive species generated by mitochondria damage biomolecules in intact tissue. Using gas chromatography/mass spectrometry, however, we recently found that heart mitochondria—but not soluble cellular heart proteins—became oxidatively damaged after untrained rats were acutely exercised (see later section and [4]). It remains possible, therefore, that mitochondrial oxidants can create cellular damage.

Phagocytes and Exercise

A variety of exercises, including treadmill running, downhill running, and eccentric arm muscle movements can induce leukocyte infiltration and provoke inflammation and extensive oxidative damage. Understanding oxidant formation by white blood cells therefore is pertinent to understanding exercise-related oxidative stress. Phagocytes such as activated neutrophils are a major source of oxidants in biology because they use a cocktail of reactive species—including $O_2^{\cdot -}$, H_2O_2 and HOCl—to destroy invading pathogens. These reactants also can destroy surrounding tissue.

Phagocytosis begins after chemotactic signals attract neutrophils to an inflamed site. If a neutrophil's plasma membrane is perturbed by microbes, particulate material, or cytokines, the cell is activated to produce $O_2^{\cdot -}$. The biochemical agent for $O_2^{\cdot -}$ production is the phagocyte NADPH oxidase, a membrane-associated electron transport chain enzyme [15,16]. The integral membrane component of the oxidase, flavocytochrome b_{558}, is composed of a large subunit, gp91-*phox*, and a small subunit, p22-*phox* (*phox* for *ph*agocyte *ox*idase). NADPH oxidase directly reduces O_2 to $O_2^{\cdot -}$, using NADPH ¾ but not NADH ¾ as a cofactor.

$$NADPH + 2\ O_2 \rightarrow 2\ O_2^{\cdot -} + NADP^+ + H^+ \qquad \text{Equation 7}$$

The important role of the NADPH oxidase system in host defenses against microbial pathogens is illustrated by chronic granulomatous disease (CGD). In this genetic disorder, defects in specific components of the oxidase impair $O_2^{\cdot -}$ production, leading to recurrent bacterial and fungal infections.

During phagocytosis, the phagocyte's plasma membrane invaginates around the foreign organism or damaged particulate material, subsequently pinching off to become a phagosome. During this time, primary and secondary granules migrate toward the phagosome, fuse with it, and empty their contents into it. The phagosome thus becomes a phagolysosome. Furthermore, destructive lysosomal enzymes are released to the phagolysosome as well as to the extracellular milieu. These are primarily digestive enzymes such as acid hydrolases, neutral proteases, and alkaline phosphatases. The granules also contain cationic proteins, lipopolysaccharides, lactoferrin, and myeloperoxidase [14,15].

Inside the phagolysosome or in the extracellular environment, myeloperoxidase forms a complex with H_2O_2 called compound I. This complex catalyzes the two-electron oxidation of halides such as Cl^-, Br^-, and I^- by H_2O_2. The reaction, particularly with Cl^-, forms toxic, potent oxidants. For example, Cl^- gives rise to HOCl, which subsequently kills the ingested microorganism but can harm neighboring tissues. The required H_2O_2 is generated in the respiratory burst via dismutation of $O_2^{\cdot -}$ and can be detected in the

phagosome [14,15]. The myeloperoxidase-H_2O_2-Cl^- interaction also generates Cl_2, and human neutrophils use the gas as an oxidant during phagocytosis [30]. Therefore, oxidant produced after an injurious bout of exercise—and almost all forms of exercise cause some degree of damage—may induce an inflammatory response with consequent oxidative stress.

Few studies have determined whether oxidative stress induced by phagocyte activation plays a role after an acute bout of exercise, however. One study investigated lipid peroxidation following downhill running in humans [31], a classic example of eccentric exercise that is characterized by extensive skeletal muscle damage followed by infiltration of neutrophils and consequent inflammation. The concentration of serum thiobarbituric reaction substances, a nonspecific measure of lipid peroxidation, increased after 45 min of downhill treadmill running, with peak values observed at 6 h and 24 h after exercise, and pre-exercise levels returning by 72 h. Other studies have detected increased neutrophil migration into skeletal muscle following several types of exertion, such as eccentric muscle exercises [31,32,33,34].

Therefore, oxidants produced by leukocytes infiltrating damaged muscle may be responsible for post-exercise oxidative damage. Indeed, we recently showed that isoprostanes, (8-150-$PGF_2\alpha$), malondialdehyde, and lipid hydroperoxides, increased dramatically after eccentric arm exercises in humans [34]. Levels peaked 2 and 3 days following the injurious exercise bout. In addition, levels of myeloperoxidase and IL-6—two indicators of inflammation—also rose significantly following exercise. Lipid peroxidation markers and inflammatory markers returned to baseline after 7 days.

Other investigators showed that myeloperoxidase activity became significantly elevated in muscle 24 h after male rats ran on a treadmill; this increase was eliminated by prior administration of estrogen [35]. Female rats experienced no significant post-exercise elevations in muscle myeloperoxidase activity. This later study suggests that estrogen may help diminish post-exercise infiltration of leukocytes and inflammatory response in both male and female muscle.

In summary, a variety of exercises damage tissue and can induce leukocyte infiltration and provoke extensive oxidative damage. Oxidant production by white blood cells is the most likely cause, as indicated by increases in systemic markers.

Endothelial Cells and Exercise

Other sources of reactive species that could contribute to exercise-induced oxidative injury or post-exercise injury are xanthine dehydrogenase and calcium. Xanthine dehydrogenase is found mainly in endothelial cells of blood vessels and in skeletal and cardiac muscle [36,37]. It takes two forms: xanthine dehydrogenase, a dehydrogenase, converts NAD to NADH, whereas xanthine oxidase, an oxidase, utilizes oxygen and xanthine, a purine degradation product, to produce $O_2^{\cdot -}$ and H_2O_2. Conversion of xanthine dehydrogenase to xanthine oxidase apparently involves sulfhydryl oxidation and is accelerated in the presence of calcium [36,37]. Substrates for xanthine oxidase are available especially during exhaustive exercise, when there is extensive purine degradation. Several inflammatory conditions, such as hepatotoxicity, viral infection, and atherosclerosis, also activate xanthine oxidase [36,37]. The xanthine oxidase inhibitor allopurinol reduces the severity of oxidative damage associated with pulmonary inflammation and exercise [36,37]. For example, strenuous exercise in patients suffering from chronic obstructive lung disease increased levels of oxidized glutathione and peroxidized lipids in blood; allopurinol treatment attenuated the oxidative stress, indicating that xanthine oxidase may be an important source of reactive intermediates during exercise in this population [38]. Several recent reviews [36,37] have extensively discussed the role of xanthine oxidase in healthy humans, though evidence that it plays a critical role in oxidant formation in humans remains unclear.

DETECTING OXIDATIVE DAMAGE IN EXERCISED ANIMALS

Although oxidative stress has been widely studied, it is difficult to detect reactive intermediates directly *in vivo* because of their short half-lives (typically 10^{-6}-10^{-12} s). Many studies have used methods such as the thiobarbituric acid reacting substances assay to measure products of lipid peroxidation. A major problem with such methods is their lack of specificity. For example, thiobarbituric acid reacts with a wide variety of compounds, including sugars and DNA. Moreover, oxidation products are typically present at low levels in biological material. Such assays therefore are prone to artifacts and cannot provide strong evidence of oxidative damage *in vivo*.

In contrast, electron spin resonance (ESR) can directly detect free radical species. However, in biological systems where radical species are present at low concentrations, it is generally necessary to capture radicals with a spin-trap compound. Use of a spin-trap can confound the identification of the initial radical species because multiple radicals (and oxidants) can yield the same radical adduct. Gas chromatography-mass spectrometry and other biochemical techniques (such as HPLC with electrochemical or chemiluminescence detection) are other powerful techniques for detecting and analyzing oxidation products. They measure stable end products that betray damage inflicted by reactive intermediates.

Radical Detection by Electron Spin Resonance

As early as 1982, ESR techniques were used to detect reactive intermediates in exercising animals [39]. Subsequently, Ashton *et al.* [40,41] produced the first evidence of reactive intermediates in humans by using ESR spectroscopy in conjunction with the spin-trapping technique. They measured reactive intermediate production in venous blood of healthy humans before and after exhaustive aerobic exercise. They also found that markers of lipid peroxidation (measured using nonspecific assays for malondialdehyde-like substances and lipid hydroperoxides)

increased in plasma but that the total antioxidant capacity after exercise was unchanged. The same group determined that ascorbic acid supplementation prevented the post-exercise increase in electron spin resonance signal intensity. Markers of oxidative stress and inflammation—lipid hydroperoxides or malondialdehyde—indicated that acute ascorbic acid supplementation prevented the exercise-induced oxidative stress [41]. Using similar techniques, others showed that ESR signals from pulmonary tissue increased when animals deficient in vitamin E (an inhibitor of lipid peroxidation) and selenium (a cofactor for glutathione peroxidase, a scavenger of hydroperoxides) were exercised acutely [42]. Moreover, vitamin E and selenium supplementation prior to exercise blunted the ESR signal [42]. Therefore, ESR suggests that reactive intermediates are generated during exercise. It is important to note that these studies did not define the exact nature of the oxidizing intermediates and that the methods used to assess lipid peroxidation were nonspecific and prone to artifacts when used with biological material.

Detection of Oxidized Amino Acids by Gas Chromatography-Mass Spectrometry

Isotope dilution gas chromatography-mass spectrometry (GC/MS) also has produced evidence for oxidant formation during exercise [4]. This combination of techniques monitors protein oxidation by quantifying specific unnatural amino acids that appear when oxidants modify the corresponding natural amino acids. These oxidized amino acids are stable and can be used for *in vivo* studies. GC/MS detects trace amounts of end-products such as *o*-tyrosine, *m*-tyrosine, *o,o'*-dityrosine, 3-chlorotyrosine, and 3-nitrotyrosine (Fig. (**2**) [4, 43-46].

Fig. (**2**) depicts several oxidized amino acids and their pathways of origin. For example, hydroxyl radical ($HO^{\cdot}$) converts phenylalanine to *o*-tyrosine and *m*-tyrosine [43]. In the presence of H_2O_2 and tyrosine, tyrosyl radical is generated by hydroxyl radical as well as by peroxidases and other heme proteins [43,46]. Tyrosyl radical can cross-link

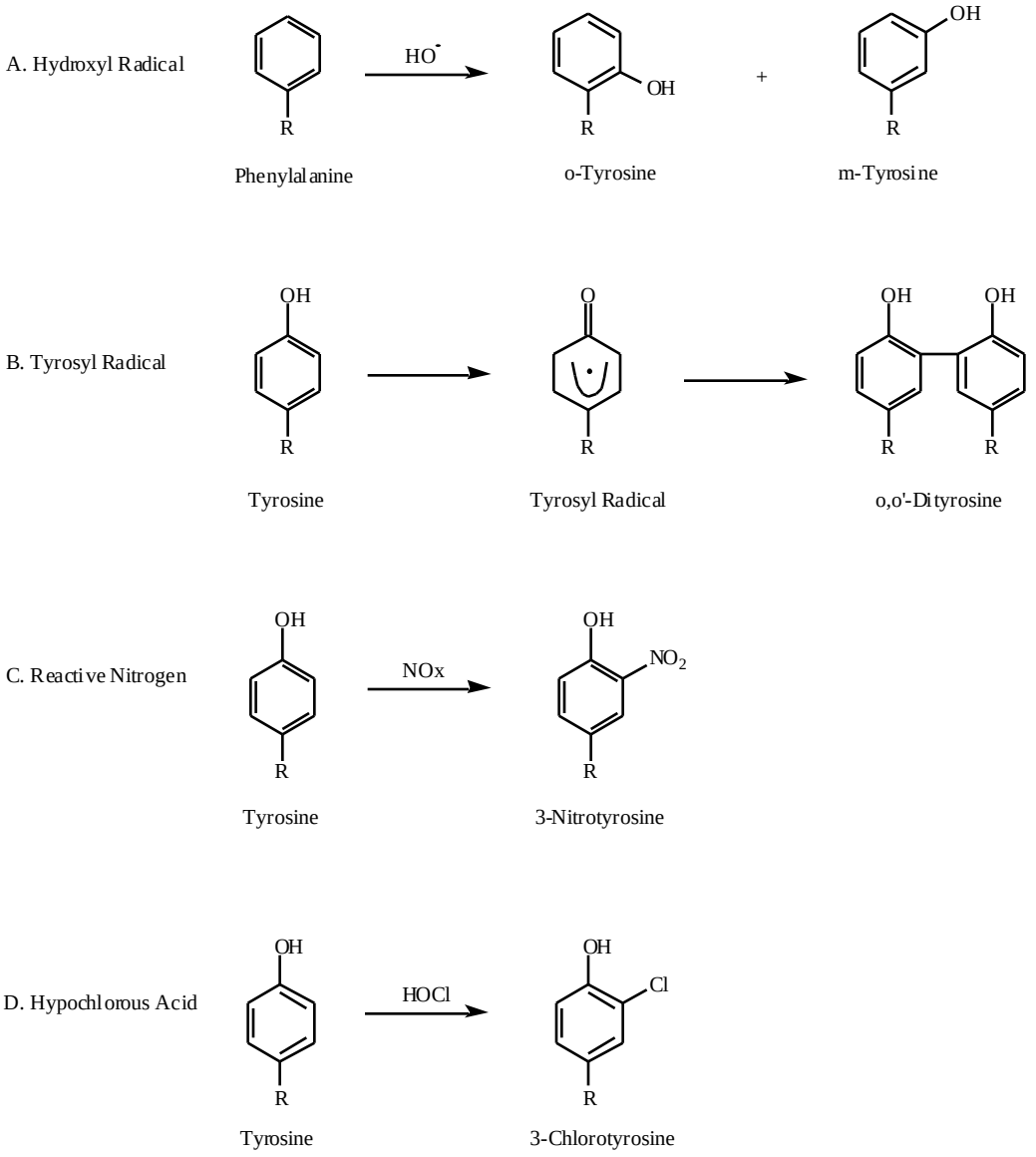

Fig (2). Pathways that produce oxidized amino acids.

tyrosine residues on proteins to form protein-bound *o,o'*-dityrosine, an unnatural isomer and stable end-product of protein oxidation. Another potential agent for protein oxidation is $NO^{\cdot}$, a long-lived radical that plays a critical role in cellular signaling and cytotoxic defense mechanisms [47,48]. Its interaction with $O_2^{\cdot -}$ yields $ONOO^-$. Protonated peroxynitrite rapidly decomposes into several other reactive nitrogen species, including the nitronium ion (NO_2^+) and nitrogen dioxide ($NO_2^{\cdot}$) [48]. Reactive nitrogen species derived from NO generate 3-nitrotyrosine *in vitro* and *in vivo.* Moreover, physiological concentrations of nitrite and HOCl (a product of the phagocyte protein myeloperoxidase) generate several nitrating and chlorinating species (ref 17; Fig. (**2**)). These *in vitro* studies demonstrate that an intermediate, nitryl chloride (NO_2Cl), may also contribute to protein modification [17]. Moreover, carbon dioxide-catalyzed oxidation of tyrosine by $ONOO^-$ may be relevant to oxidative injury as well [49].

In a recent study, we used isotope dilution GC/MS to measure three specific "fingerprints" of oxidative damage in heart mitochondrial proteins of exercising rats [4]. We found that levels of *o*-tyrosine and *m*-tyrosine (markers for protein oxidation by hydroxyl radical) and dityrosine (a marker for oxidation by hydroxyl radical or tyrosyl radical) were elevated in mitochondrial proteins of acutely exercised animals. Levels of these abnormal amino acids increased by 50% in mitochondrial but not soluble proteins of heart muscle [4]. They returned to normal after the animals were allowed to rest. There also was a transient increase in the concentration of *o,o´*-dityrosine in the urine of exercised rats. This relationship between mitochondrial and urine levels of *o,o´*-dityrosine suggests that assaying this oxidized amino acid in urine might provide a noninvasive measure of oxidative stress. Our observations also provide direct evidence that mitochondria in exercising heart muscle generate a $HO^{\cdot}$-like species that promotes protein oxidation *in vivo* [4].

The appearance of oxidized amino acids in urine raises the possibility that specific proteolytic systems can recognize and degrade oxidatively damaged proteins. Indeed, investigators have found that mitochondria have their own proteolytic system, perhaps because their inner and outer membranes prevent access by cytosolic proteases and peptidases [50]. The presence of such a system indicates the need to remove unnatural oxidized proteins and amino acids from mitochondria. Little is known about this process, but it is feasible that products such as o,o'-dityrosine are recognized, proteolyzed, released into plasma, and excreted into urine. Indeed, Guiliviv and Davies [51] found that o,o'-dityrosine – a specific marker of oxidation [52,53] – was released after red blood cells were exposed to a continuous flux of H_2O_2. In this system, the proteasome macroxyproteinase appeared to be responsible for o,o'-dityrosine release during the selective degradation of oxidatively modified proteins [51-53].

Another question is whether oxidized amino acids incorporated into proteins can interfere with function. These unnatural amino acids are very similar in structure to their native forms, and they have been detected in biological tissues under normal and pathophysiological conditions. For example, Leeuwenburgh *et al.* detected an increase in the dityrosine content of skeletal and heart muscle in aged mice. Caloric restriction, which prolongs life in many different animal species, attenuated this increase, raising the possibility that protein oxidation is causally involved in the aging process [46], perhaps by interfering with protein function. Two other unnatural amino acids, 3-nitrotyrosine and 3-chlorotyrosine, have been detected in various disease states, including cardiovascular and neurodegenerative diseases [44,54,55,56]. These findings indicate that oxidative reactions catalyzed by the myeloperoxidase system of phagocytes provide likely pathways for protein oxidation *in vivo* [56]. Thus, these markers should be useful for further studies of oxidative damage by reactive intermediates during and after physical exertion.

ADAPTING TO EXERCISE

Regular physical exercise has well-documented health benefits and can prolong mean life span in animals. It reduces signs of aging and increases average life expectancy by approximately 10% in rats [3,57]. Maximum life span remains unchanged, however. Certain forms of exercise also improve skeletal muscle function [58,59] and properties such as vascular adaptation and maintenance of muscle mass [60,61]. On the other hand, acute exercise in untrained subjects may increase oxidative stress [4-8], though consecutive bouts over a given period induce adaptations that prevent massive oxidative damage. This paradox will be discussed in the following sections. We hypothesize that some of the health benefits of exercise stem from upregulation of antioxidant enzymes and reduction of oxidant production.

Reduced Radical Production at Rest Follows Chronic Exercise Training

One important adaptation that accompanies regular endurance training is a decrease in the basal level of H_2O_2 generated by isolated mitochondria. Venditti *et al.* [22] produced the first evidence for this phenomenon by demonstrating that endurance training decreased H_2O_2 production in State 4 (no ADP) by ~40% with succinate and ~20% with pyruvate and malate. This change also could have direct implications for mitochondrial DNA damage because $O_2^{\cdot -}$ and H_2O_2 are believed to be the major reactive intermediates that damage the mitochondrial genome. It is currently unclear what mechanism(s) improves electron coupling and which inner mitochondrial membrane proteins reduce the leak of reactive intermediates. The greatest documented leak occurs at Site I, and damage to this protein has not been investigated during exercise training.

However, Venditti *et al.* [22] detected a slight elevation in the total amount of H_2O_2 per gm of tissue after animals underwent training. The increase resulted from an increase in both mitochondrial content and mitochondrial numbers. Holloszy *et al.* [62,63] documented both these changes in rats in the early 1970s. However, reducing the amount of H_2O_2 per mg of mitochrondrial protein potentially could

reduce oxidant oxidative stress within a given mitochondrion.

We believe the Venditti *et al.* [22] study is critical in explaining the increase in mean life span seen in voluntary wheel-running animals. It also could explain why maximum life span increases with caloric restriction but not with regular exercise: caloric restriction dramatically reduces oxidant production without increasing the total number of mitochondria in heart muscle. In addition, it is possible that caloric restriction lowers oxidant production more than regular exercise does, a hypothesis that remains to be tested.

Increased Antioxidant Defenses

Endurance exercise training protects rats from exercise-induced oxidative stress, raising levels of antioxidants and antioxidant enzymes in both skeletal and cardiac muscle [64,65,66,67]. Leeuwenburgh *et al.* found that a 10-week exercise program increased glutathione peroxidase and superoxide dismutase activities in the deep portion of vastus lateralis muscle [64]. In another study, they detected a 33% increase in the glutathione content of this muscle in endurance-trained rats [65]. The rats also had 62% more glutathione peroxidase activity and 27% more superoxide dismutase activity than untrained sibling controls [65]. Moreover, Powers *et al.* [66] found that increases in muscle antioxidant enzymes induced by exercise training were muscle-specific. They also showed that high-intensity and moderate-intensity exercise upregulated superoxide dismutase activity in the ventricular myocardium [67]. In addition, we recently demonstrated that old rats that voluntarily ran on a wheel all their lives had higher levels of several skeletal muscle antioxidant enzymes than their sedentary counterparts. The exercising animals also had lower levels of markers of oxidative stress in muscle and urine [45]. They continued to be active into old age, though they decreased their running time. This study also detected lower levels of o,o´-dityrosine in skeletal and heart muscle of the exercising animals. This difference may reflect a decrease in the overall rate of oxidant generation or an increase in antioxidant defenses.

To begin to distinguish between these two possibilities, we assayed the activities of antioxidant enzymes in rat skeletal muscle samples. The activities of two major antioxidant enzymes, mitochondrial superoxide dismutase and cytosolic glutathione peroxidase, were significantly higher in exercising animals than in sedentary animals. There was little difference in catalase and cytosolic superoxide dismutase activities, however. These results indicate that increased activities of glutathione peroxidase and mitochondrial superoxide dismutase might account in part for the decline in protein oxidation in exercise-trained animals. Importantly, the decline in o,o'-dityrosine levels in the urine of exercising animals may reflect an increase in antioxidant enzyme protection in skeletal muscle and/or a decrease in the basal production of species that oxidize skeletal muscle proteins. Furthermore, other studies found that exercise training decreased malondialdehyde levels (measured using a nonspecific assay) in the soleus muscle of old rats. Moreover, the trained animals lost less muscle as they aged than their sedentary counterparts, suggesting that exercise training attenuates both muscle loss and oxidative damage [45,64].

Additional beneficial effects of exercise training have been proposed. For example, one study examined the effect of exercise on the tendons of aging male Sprague-Dawley rats. The tail tendon collagen of a group that trained on a treadmill showed greater thermal stability and was better able to resist mechanical stress than that of a sedentary group. Thus, physical exercise appeared to deter aging of rat connective tissue [68].

In summary, acute exercise increases oxidant levels and oxidative stress in untrained animals [4,5], but long-term exercise may counter this effect by increasing the activity of antioxidant enzymes and reducing oxidant production [64-67,21,22]. These defenses may be critical for preventing chronic oxidative damage to muscle during exercise and even at rest.

FUTURE DIRECTIONS

Detecting specific oxidation products in isolated mitochondria has provided direct evidence for a link between exercise and oxidative damage. However, this approach cannot establish the relevance of oxidation chemistry to tissue injury. The use of animal models that over-express or lack enzymes and proteins that promote oxidation *in vitro* should provide powerful evidence regarding the roles of these pathways in increasing oxidative stress *in vivo*. Human genetic studies offer another important approach for identifying biologically relevant oxidation mechanisms. If such studies can identify the pathways that promote oxidative damage during exercise, it may be possible to develop specific inhibitors to prevent tissue damage during aging.

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ABBREVIATIONS

$NO^{\cdot}$	=	Nitric oxide
$ONOO^{-}$	=	Peroxynitrite
NO_2Cl	=	Nitryl chloride
$HO^{\cdot}$	=	Hydroxyl radical
HOCl	=	Hypochlorous acid
$O_2^{\cdot -}$	=	Superoxide

H_2O_2 = Hydrogen peroxide

GC/MS = Gas chromatography-mass spectrometry

MDA = Malondialdehyde

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