



OXIDATIVE STRESS IN ATHLETES: FROM THE SCIENTIFIC BASIS TO PRACTICAL ASPECTS

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Abstract Oxidative stress, which occurs as a result of physical exercise, has been intensively investigated in the last two decades. A large number of studies have been done on humans and in animal models, which resulted in clarification of the mechanism of free radical generation during exercise. Free radicals, unstable and reactive molecules that can be involved in cellular damage, are mainly generated by the mitochondria, xanthine oxidase, and neutrophils and other phagocytes. Increased production of free radicals during exercise is followed by the enhanced activity of the endogenous antioxidant defense system, which consists of enzymes and non-enzymatic substances. Very important results of previous studies have been related to adaptation mechanisms activated in response to increased oxidative stress during exercise. In addition to mitochondrial biogenesis and synthesis of new components of the respiratory chain to match higher energy demands, adaptation to regular physical exercise involves an increased expression of genes responsible for antioxidant enzyme synthesis. Also, great attention has been paid to the use of antioxidant supplements. The importance and effect of supplementation with dietary antioxidants depend on several factors such as intensity, frequency and mode of exercise as well as on the type and dose of the antioxidants used. Applying the results of previous research and monitoring the parameters of oxidative stress and antioxidative defense could help health professionals in understanding the medical condition of the athlete.

Key words: Free radicals, antioxidative defense, exercise, adaptive responses, dietary antioxidants

INTRODUCTION

There are many known health benefits of exercise in general population. Regular physical exercise has not only been proven to increase mean life span but it is also the first nonpharmacological treatment for certain conditions, such as obesity, mild hypertension and diabetes [76, 77]. The results of previous research, including the most recent studies, have led to the inclusion of exercise training in rehabilitation programs for persons with disabilities and with other forms of limitations [11, 40]. However, regarding elite performance levels, athletes, coaches and health professionals are faced with various challenges.

It is well known that physiological mechanisms that determine the speed or power of performance could be improved by training [50]. During exercise, human respiratory and cardiovascular systems alter the velocity of blood flow between the heart and the lungs in order to adapt to the increased metabolic demand and to improve maximal oxygen uptake (VO_{2max}). A successive increase in training intensity over time leads to enhancing the lactate threshold. Also, training adaptations cause physiological and biochemical changes in the body [10, 26, 88]. Although the whole body adapts to increased physical activity, it comes at a price. Training-induced adaptations include some changes in metabolic pathways and, consequently, in the increased production of free radicals, unstable and reactive molecules that can be involved in cellular damage [12]. There are numerous mechanisms for the production of reactive oxygen species (ROS) in the human body; notably, they are produced during normal aerobic metabolism. Despite the fact that the overall conditions and reactions that produce free radicals during exercise have not yet been fully elucidated and substantiated, it is believed that these molecules are generated by three main sources: the mitochondria, xanthine oxidase, and neutrophils and other phagocytes [3]. Regardless of the origin of free radicals, their damaging effect can be interrupted by endogenous or exogenous antioxidants. Antioxidants are a heterogeneous group of molecules, whose main role is to convert ROS into harmless derivatives. The antioxidant defense system involves two complex internal protective mechanisms:

enzymatic (superoxide dismutase, catalase, paraoxonase and glutathione peroxidase) and non-enzymatic (vitamin C, vitamin E, -SH groups and reduced glutathione) [19]. The prolonged exposure to reactive oxygen species common in elite athletes can lead to oxidative stress-related injury and health-threatening states. Although there is general consensus that increased reactive oxygen species are detrimental, it should be noted that in low doses free radicals could promote cell proliferation and upregulation of cell signaling [42, 45]. In addition to mitochondrial biogenesis and synthesis of new components of the respiratory chain to match higher energy demands, adaptation to regular physical exercise involves an increased expression of genes responsible for antioxidant enzyme synthesis.

There is some controversy over whether supplementation with antioxidants is helpful in preventing antioxidant deficiency and subsequent oxidative damage [22, 41], as studies conducted on athletes and untrained subjects have varied in the intensity, duration and type of physical activity. Also, there has been some disagreement concerning assays used to assess the antioxidative status and the level of oxidative stress. While trying to estimate the extent of free radical effects on cellular components, researchers have mainly determined by-products of radical-induced reactions because of their high reactivity and short half-lives [71]. Many studies have found evidence of increased products of lipid peroxidation, protein oxidative damage and DNA oxidation in athletes [1, 23, 46, 49, 84], but the best method for oxidative stress assessment in athletes remains elusive.

A substantial part of the discussion in the present article relates to the recent studies demonstrating underlying mechanisms and evidence of exercise-induced free radical generation. In addition, the article analyzes adaptation to the oxidative stress of exercise training and usefulness of antioxidant protection.

SOURCES OF REACTIVE OXYGEN SPECIES PRODUCTION IN EXERCISE

FREE RADICAL GENERATION BY THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

The electron transport chain is the final pathway of cellular respiration and the place where the greatest amount of energy is produced and stored in ATP molecules. It consists of four cytochrome complexes immersed in the inner membrane of the mitochondria. Furthermore, electron transport chain has two mobile electron carriers, coenzyme Q and cytochrome c. The electrons from NADH and FADH₂, reduced coenzymes generated during glycolysis and the citric acid cycle, are moved to molecular oxygen through cytochrome complexes and carriers. Electron transport along the chain causes vectorial transport of protons from the mitochondrial matrix to the intermembrane space. This proton translocation generates an electrochemical gradient that directly powers ATP synthesis [58].

The side reaction of this transfer system is electron leakage and molecular oxygen reduction to the superoxide anion radical. The production of superoxide anion by electron leaks from the transport chain is greatest during the electron flow between Complex I and ubiquinone and between ubiquinone and Complex III [7].

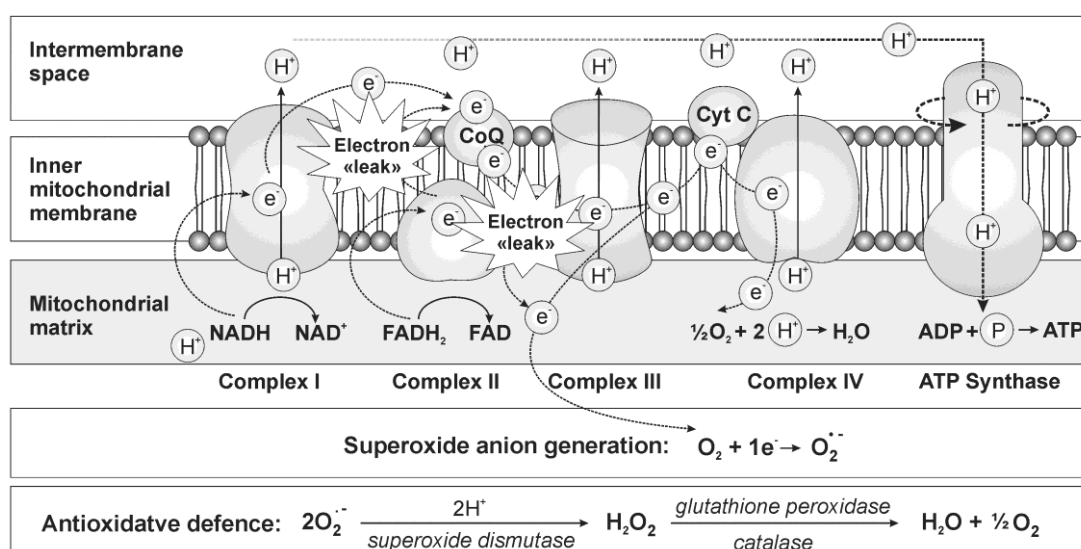


Figure 1. The electron transport chain in the mitochondrion is the site not only of major energy production but also of origin of a large amount of free radicals. The electron leaks between Complex I and ubiquinone, and between ubiquinone and Complex III result in the production of superoxide anions during exercise. Unavoidably, even during resting state, small amounts of reactive oxygen species are formed. During exercise, oxygen utilization is enhanced by 10 to 20 times, thus greatly increasing the generation of free

radicals. It is estimated that some 1–4% of the oxygen consumed is reduced to superoxide anion by mitochondria. Because of acidic pH in the intermembrane space, a small amount of the formed superoxide anion will exist in the protonated form but any superoxide generated at the inner surface of the inner mitochondrial membrane will remain in the unprotonated form. In earlier studies, some researchers have proposed that the temperature rise causes mitochondrial uncoupling and decreases the stability of ubisemiquinone species [5, 73]. According to this theory, mitochondrial uncoupling along with the increased oxygen consumption during exercise increases the electron transfer to oxygen and, subsequently, superoxide anion formation.

FREE RADICAL FORMATION BY XANTHINE OXIDASE

Another site for free radical production during exercise is the enzyme xanthine oxidase (XO). Under normal physiological conditions, the main form of xanthine oxidase is xanthine dehydrogenase, which catalyzes the oxidation of hypoxanthine to xanthine and further oxidation of xanthine to uric acid with concomitant reduction of NAD^+ to NADH. However, during exhaustive exercise, certain tissues may be affected by ischemia or hypoxia, causing intracellular xanthine dehydrogenase conversion to xanthine oxidase. Under such conditions, this conversion is the result of reversible cysteine residue modification or partial but irreversible proteolysis.

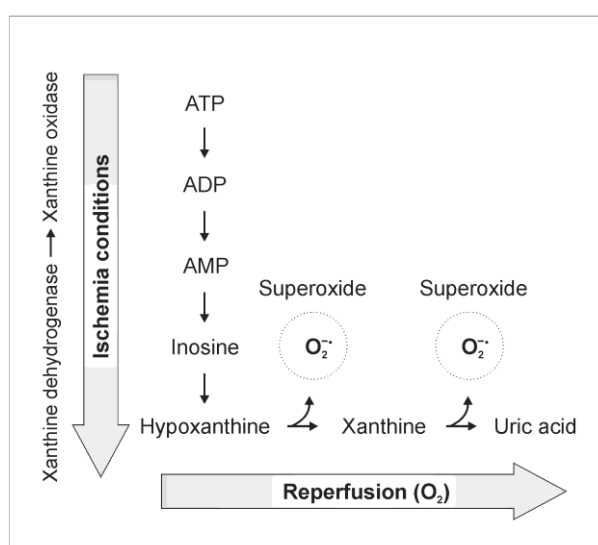


Figure 2. The mechanism of superoxide generation after ischemia and subsequent reperfusion

On the other hand, ATP catabolism results in the production of hypoxanthine, which is a substrate for xanthine oxidase. Thus, the simultaneous rise in the concentration of xanthine oxidase and hypoxanthine occurs at low oxygen availability. When oxygen is finally reintroduced (reperfusion), xanthine oxidase still converts hypoxanthine to uric acid, but preferentially utilizes oxygen as the electron acceptor instead of NAD^+ and forms superoxide [16, 72].

The main question is how the free radicals formed by xanthine oxidase contribute to the overall oxidative stress in athletes. Most researchers used the allopurinol (a xanthine oxidase inhibitor) to investigate the impact of free radicals generated in hypoxia- and ischemia-like conditions to oxidative damage of molecules. Indeed, most studies confirmed that xanthine oxidase was responsible for the production of free radicals and subsequent tissue damage. Viña and collaborators examined lipid peroxidation and oxidation of glutathione in rats and humans, with and without administration of allopurinol [89]. In addition to finding a positive correlation between exercise and oxidative damage (in control rats), they found significantly reduced levels of oxidative damage in all animal and human subjects that were administered allopurinol. Their study showed a positive correlation between exercise and oxidative damage as well as significantly lower levels of oxidative stress in humans and animals that were treated with allopurinol. Very similar results were observed when patients with chronic obstructive pulmonary disease were subjected to exercise with or without allopurinol treatment [27]. Gomez-Cabrera and associates showed that although allopurinol prevented free radical production, it also inhibited important signaling pathways involved in upregulating the expression of enzymes important for cell defense and adaptation to exercise [21].

NEUTROPHILS AND PHAGOCYTES AS A SOURCE OF FREE RADICALS

Table 1. Enzymatic and non-enzymatic antioxidants and their localization in the cell and plasma

| Enzymatic antioxidative defense | | |
|--|------------------------------|--|
| Enzyme | Localization | Mechanism of protection |
| Superoxid dismutase | Mitochondria - MnSOD | Acts on superoxide anion to form oxygen and the less reactive H ₂ O ₂ |
| | Cytosol - CuZnSOD | |
| | Extracellular form - CuZnSOD | |
| Glutathion peroxidase | Mitochondria | Reduces H ₂ O ₂ and other hydroperoxides using reduced glutathione as the electron donor |
| | Cell membrane | |
| | Cytosol | |
| Catalase | Mitochondria | Reduces H ₂ O ₂ and other hydroperoxides |
| | Peroxisomes | |
| Non-enzymatic antioxidants | | |
| Antioxidants | Localization | Mechanism of protection |
| Glutathione | Cytosol | Tripeptide that acts as an electron acceptor |
| Vitamin C | Aqueous | Scavenger of free radicals |
| Vitamin E | Cell membrane | Reduce exercise-induced lipid peroxidation |
| Carotenes | Cell membrane | Scavengers of singlet oxygen and peroxy radicals |
| Coenzyme Q | Cell membrane | Acts as an electron donor and prevents lipid peroxidation |

Most antioxidant enzymes are localized in the mitochondria, the cell compartments with the highest free radical production, but some of them have isoforms whose role is to protect cell membranes and structures in the cytoplasm. Also, non enzymatic antioxidants contribute significantly to the protection of the cytoplasm and the cell membrane (Table 1).

ANTIOXIDANT ENZYMES

Superoxide dismutase. The first line of defense against superoxide produced in the electron transport chain is an enzyme, superoxide dismutase (SOD). There are two isoforms of SOD in humans: CuZn SOD, located mostly in the cytosol, and MnSOD, found primarily in the mitochondria. There is also a third isoform of the enzyme, extracellular SOD (ECSOD), found in both plasma and tissues. In the mitochondria, the dismutation of O₂⁻ catalyzed by superoxide dismutase into hydrogen peroxide, H₂O₂, which is not a free radical itself; however, in Fenton and Haber-Weiss reactions (with transition metals, mainly free iron), it is capable of forming the highly reactive hydroxyl radical (OH[·]), probably the most powerful radical produced by the human body [58]. This is the reason for the defense mechanisms against free radical-mediated oxidative damage to include iron binding proteins such as transferrin and ferritin, and copper binding proteins such as albumin and caeruloplasmin. Ferritin and transferrin can restrict the availability of iron to participate in the conversion of hydrogen peroxide to toxic hydroxyl radicals by Fenton and Haber-Weiss reactions. Also, albumin and caeruloplasmin reduce reactive oxygen species as they participate in copper transport [43].

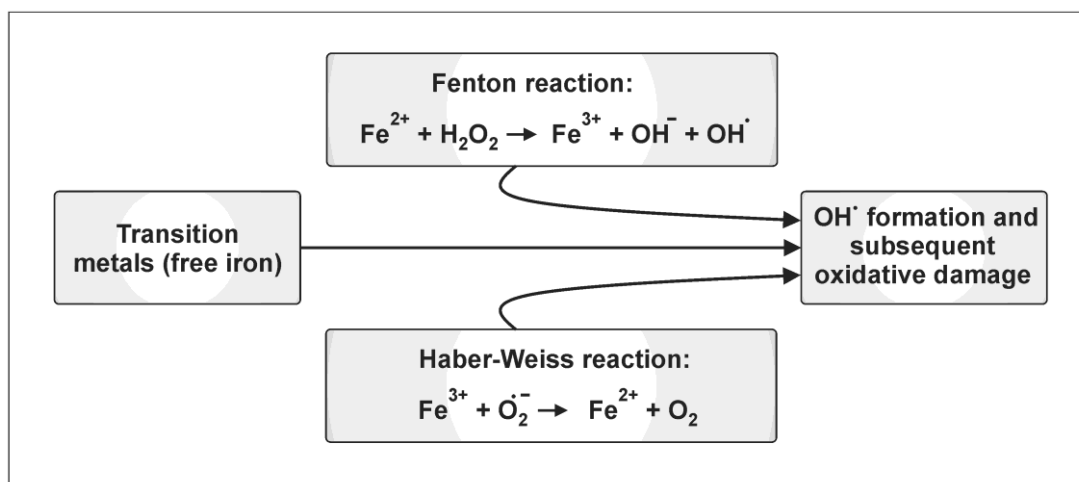


Figure 4. The mechanism of free radical formation through Fenton and Haber-Weiss reactions

In relation to exercise and training, SOD has been shown to correlate positively with enhanced resistance to oxidative stress [18]. For instance, resting SOD activity was higher in soccer players than in untrained subjects [8], while Melikoglu et al observed a similar relationship with higher SOD activity in basketball players compared to sedentary controls [52]. Also, the increase in SOD enzyme activity corresponds to years of training experience: in our previous research we found that approximately ten years of training permitted superoxide-dismutase activity to reach its maximum in elite female volleyball players [45]. Thus, the above studies lead to the conclusion that the increased SOD activity is more common in trained individuals when compared to the untrained subjects. The majority of studies have shown that SOD activity increases following short-term and prolonged exercise [31, 35]; however, a significant number have reported no change [15,82], whereas a few have found a decrease [28, 81]. Under conditions of increased O₂ consumption during exercise, glutathione peroxidase is activated in order to decompose hydrogen peroxide and organic hydroperoxides to less harmful products.

Glutathione peroxidase. This enzyme, localized in the mitochondria, the cytosol and the cell membranes, reduces H₂O₂ and organic hydroperoxides while glutathione (GSH) is oxidized to glutathione disulfide (GSSG). Although the amounts of GSH in a cell are limited, the constant protection from H₂O₂ is maintained by glutathione reductase (GR), which reduces GSSG into GSH by using electrons from the NADH. This enzyme activity is selenium dependent and its activity is highest in type I muscle fibers (highly oxidative fibers) [29, 64]. Similarly to SOD, several researchers have reported higher resting GPx activity in trained subjects compared to untrained individuals, particularly in muscle fibers with a high oxidative capacity [9, 30, 36]. As a response to overload training, Palazzetti et al [60] found an increase in plasma but not erythrocyte GSH-Px activity in triathletes. Turgay et al. observed that endurance-trained athletes had a higher erythrocyte GPx activity than anaerobic-trained athletes [85] but when the activity of this enzyme was compared in endurance-trained athletes and sprinters, it was higher in the latter [48]. In contrast, the comparison between before and after marathon and cycling races showed little or no difference in erythrocyte GPx activity [69, 82].

Catalase. Similar to GPx, catalase is distributed among muscle cells, blood cells (erythrocytes, lymphocytes and neutrophils) and plasma. In a cell, catalase is mainly located in the peroxisomes and the mitochondria, its main role being to dissociate hydrogen peroxide and to form water and oxygen. The activity of antioxidative enzymes in the muscle is highest in high oxidative muscle fibers (types I and IIa). As a part of muscle adaptation to exercise, it involves mitochondrial biogenesis and synthesis of new components of the respiratory transport chain to match the increased energy demands [2, 87]. Endurance training did not result in any change in the activity of catalase measured after marathon running [70]; conversely, a study by Aguilo et al showed a decrease of nearly 20% in erythrocyte catalase activity. Miyazaki et al. reported that the resting erythrocyte catalase activity did not increase in human subjects following intense endurance training despite increases in SOD and GPx [55].

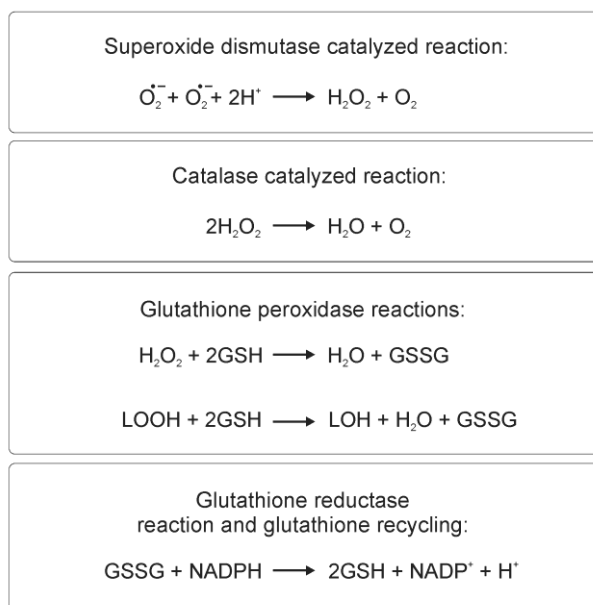


Figure 5. The biochemical reactions involving ROS and antioxidant enzymes

NON-ENZYMATIC ANTIOXIDANTS

There are a number of low molecular weight substances that are considered to be antioxidants of biological importance. The following five non-enzymatic antioxidants were investigated particularly in the studies related to exercise:

- Glutathione, one of the most important intracellular antioxidant and a very important factor in preventing damage that reactive oxygen species could cause. Glutathione is a tripeptide: L-γ-glutamyl-L-cysteinyl-glycine (GSH) and its active group is the sulfhydryl (-SH) group of cysteine. This peptide is synthesized in the cytosol by the sequential action of glutamate-cysteine ligase and glutathione synthetase, and its mitochondrial presence requires inner membrane transport. The dicarboxylate carrier protein and the 2-oxoglutarate carrier protein are two mitochondrial antiport carrier proteins that have been shown to participate in GSH transport. The ratio of oxidized glutathione (glutathione disulphide - GSSG) to total glutathione in blood, known as the glutathione redox ratio (GRR), has been identified as a sensitive measure of oxidative stress [36, 51].
 - The lipid-soluble vitamin E, considered to be the most efficient among antioxidants because it contributes to membrane stability and fluidity by preventing lipid peroxidation. Vitamin E (α-tocopherol) is localized in virtually all cell membranes; however, the major amount of membrane-bound vitamin E is found where it is most needed, in the inner mitochondrial membrane [20]. The α-tocopherol reacts with peroxy radicals much faster than with polyunsaturated fatty acids and a small amount of vitamin E is able to protect a large amount of polyunsaturated fatty acids [6].
 - Biosynthesis of coenzyme Q occurs in all tissues and cells and this lipid-soluble molecule is present in all membranes. It is a member of the mitochondrial respiratory chain and has several other regulatory functions of great importance for the cellular metabolism, such as the regulation of mitochondrial permeability transition pores and physicochemical properties of membranes [86]. The reduced form of coenzyme Q (ubiquinol) acts as antioxidant and inhibits lipid peroxidation. Ubiquinol effectiveness is based on prevention of lipid peroxide radicals generation during first step of lipid peroxidation, known as initiation. Coenzyme Q is also effective in preventing DNA and protein oxidation [4].
 - The physiological role of vitamin A (retinol) is to maintain the differentiated epithelia, mucus secretion, reproduction and vision. Beta-carotene, the major carotenoid precursor of vitamin A, is also an antioxidant and the most efficient “quencher” of singlet oxygen [80].
 - Ascorbic acid or vitamin C (water-soluble) is the predominant non-enzymatic antioxidant in plasma and in cytosol compartments. It also appears that vitamin C participates in recycling vitamin E radicals. Physiologically, vitamin C participates as a cosubstrate in the synthesis of procollagen, catecholamines and carnitine. Also, a significant number of different ROS in aqueous phase are scavenged by this antioxidant [59]. Some studies have shown that vitamin C can also act as a pro-oxidant under certain circumstances [63].

The results of human studies on the effects of antioxidant supplementation on exercise-induced oxidative stress have been controversial. The majority of studies have proved that antioxidants reduce the exercise-induced oxidative stress. Generally, the studies used antioxidant cocktails because antioxidant substances exhibit a synergistic effect, especially vitamin A and C [17]. Some studies suggested that in situations characterized by free radical overload, exogenous antioxidants should be administered in order to minimize muscle function decrement [74, 75] whereas others failed to demonstrate such positive effects [32, 83]. The importance and effects of antioxidant supplementation depend on several factors such as the intensity, frequency and mode of exercise as well as on the type and dose of antioxidants used. Several studies demonstrated that a considerable number of biochemical, hormonal, hematological and oxidative stress parameters differed with respect to the duration and type of exercise as well as its energy expenditure [44, 54, 56]. Thus, it has been shown that oxidative stress varies between athletes engaged in aerobic, anaerobic or combined type of physical exercise [13]. In our previous research we also found that certain conditions associated with exercise, e.g. iron deficiency, can significantly affect the level of oxidative stress [14, 47]. In their excellent recent review of this subject, Viña et al. proposed that the critical factor for oxidative damage was exhaustive exercise rather than intense exercise. Supplementation with dietary antioxidants could partially prevent muscle damage caused by exhaustive exercise. Regardless of recognizing the benefit in the prevention of oxidative damage, many studies have been unable to prove that antioxidant supplementation may increase athletic performance [90]. Bearing these facts in mind, before antioxidants are used, all aspects that lead to increased oxidative stress should be considered as well as the effects of antioxidant substances on the adaptation processes.

ADAPTIVE RESPONSES TO FREE RADICAL GENERATION IN PHYSICAL EXERCISE

Despite the complexity in the mechanisms responsible for exercise-induced adaptive responses, basic mechanisms leading to adaptation have been delineated. Muscle adaptation to regular physical exercise involves mitochondrial biogenesis and synthesis of new components of the respiratory chain to match increased energy demands. It has been found that exercise is a potent activator of gene expression, with expression patterns varying considerably depending on the mode of exercise [78].

During exercise, skeletal muscle energy turnover increases and introduces a large energetic challenge with an extensive oxygen flow to the mitochondria. Therefore, it is not surprising that the increase in the muscle mitochondria is largely responsible for the increased endurance capacity. Free radicals, which are thereby increased during exercise, have been suggested as the possible stimulus for mitochondrial biogenesis [10].

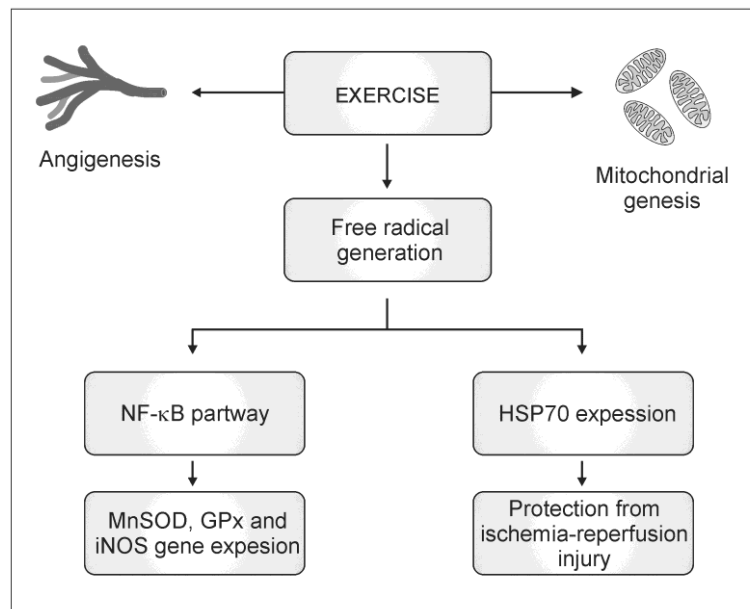


Figure 6. Physical exercise and the accompanying adaptive processes

The idea of free radical-induced adaptive response begins with research on drosophila, where upregulation of certain stress genes and proteins was observed by Ritossa [68]. The regulatory response to high temperatures that this scientist observed became known as the heat shock response and the proteins were termed the heat shock proteins (HSPs). Within the HSP family, containing a series of proteins with different

molecular weights, HSP70 with the molecular weight of 72 was determined as one of the prominent inducible HSPs in humans [37]. Exercise was indicated as one of the stressors that could cause HSP70 production [65] and the protein was marked as a transporter that removed polypeptides from the nucleus to the mitochondria to be incorporated into complete mitochondrial proteins. This mechanism was suggested as a possible link between exercise-induced oxidative stress and mitochondrial biogenesis [73]. In the studies that followed, it was concluded that HSP70 was associated with the protection of the muscle from ischemia-reperfusion injury [61] and that the production of this shock protein depended on the intensity and/or duration of exercise [39]. Physical exercise was also found to stimulate arteriogenesis and angiogenesis, as evidenced by the increased collateral-dependent blood flow and greater muscle capillarity after training [25]. Upregulation of angiogenic factors in response to increased muscle activity has been studied in detail by a considerable number of investigators and it is not discussed here. Gustafsson and Kraus provided a recommendable comprehensive review of this subject [24].

Another important way of adapting to an increased production of free radicals is the NF- κ B pathway. The major contribution to this issue was made by Gomez-Cabrera and co-workers. The researchers showed that the free radicals generated during exercise activated the MAPKs (mitogen-activated protein kinase) [21]. The MAP kinases consist of cytoplasmic protein serine/threonine kinases that participate in the transduction of signals from the surface to the interior of the cell. The ERK1/ERK2 and p38 are specific kinase families whose phosphorylation increases due to contractile activity. Phosphorylation of these kinases in turn activates NF- κ B, a transcription factor that resides in an inactive form in the cytosol, bound to the inhibitor protein I κ B. Activation of NF- κ B results in an increased expression of important enzymes associated with cell defense (such as MnSOD and GPx), and causes the expression of nitric oxide synthases (endothelial and inducible form), which is another contribution to the adaptation to physical exercise. Also, direct interaction of reactive oxygen species with NF- κ B leads to its translocation to the nucleus and binding to target gene promoters through its DNA binding subunits, p50 and p65. This gene is responsible for the mitochondrial form of superoxide dismutase or Mn-SOD [53].

The majority of studies have focused on muscles because of contractions, which are primarily responsible for all the force required to support movement and contractile activity. Amounts of reactive oxygen species are different across tissues at rest and, as expected, during exercise. Moreover, different frequency and intensity of exercise lead to different levels of oxidative stress depending on organ or tissue types. Liu et al. proved that responses to free radical formation in the heart and muscle caused by chronic or acute exercise were quite different from those in the brain and liver [38]. These two organs with high metabolic rates also undergo oxidative challenge-related adaptive processes very similar to skeletal muscle adaptation [67].

CONCLUSION & PRACTICAL APPLICATION

The practical application of knowledge related to oxidative stress resulting from exercise is a major challenge to most health professionals. Previous research clearly shows that there is a high risk of oxidative damage, especially in excessive exercise. On the other hand, the significant contribution of exercise to the development of natural endogenous antioxidant protection should not be overlooked. Understanding the mechanisms of free radical generation during exercise and ensuing adaptive processes suggests that the gradual increase in oxidative stress leads to the resistance to new stress. Therefore, recreational and moderate exercise improves antioxidant status by itself whereas in elite athletes, especially at the professional level, the use of antioxidant supplements can reduce the effects of fatigue, exhaustion and, potentially, the symptoms of overtraining. Therefore, their use should be rational and adapted to the intensity and duration of exercise as well as to the years spent practicing a certain sport. Also, it is important to monitor the parameters of antioxidative defense and to analyze the results in line with the current medical condition of the athlete.

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