PROTECTION AGAINST OXIDATIVE STRESS

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Contents

Summary

Oxidative stress plays a large role in the disease process and natural aging. It has been defined as an imbalance of the pro-oxidant/antioxidant equilibrium in favor of the pro-oxidants. In biological systems, cells respond to mild oxidative stress by inducing their antioxidant defenses and other protective systems, but severe oxidative stress can cause permanent damage to DNA, proteins and lipids.

In healthy organisms, protection against deleterious effects of reactive oxygen species (ROS) is achieved by maintaining a delicate balance between oxidants and antioxidants. Therefore, the continuous production of ROS in aerobic organisms has to be matched with a similar rate of consumption by antioxidants. Either enzymatic or non-enzymatic, antioxidants are substances that prevent the formation of ROS, scavenge them, or repair the damage they cause. Protection against oxidative damage and related chronic disease

is best served by the variety of both endo- and exogenous antioxidants. The balance between not only oxidants and antioxidants, but also between various antioxidants, may be of major importance in the protection against ROS-mediated injury. Dietary antioxidants serve as potential protective agents that can counteract ROS and potentially re-establish a healthy cellular redox balance. Antioxidants can, however, also promote oxidative stress at excessive concentrations.

Aging can adversely influence antioxidant enzyme capacity in tissues including skeletal muscle, but regular exercise training can preserve such protective function. While regular physical exercise has been presented as a recommended activity for health reasons, exhaustive acute exercise may, however, induce the production of ROS and may end up with increased oxidative stress. It has been well demonstrated that glutathione-dependent antioxidant protection in the skeletal muscle is influenced by the state of physical activity: endurance training enhances and restriction of chronic activity diminishes such protection, although the training effects are highly tissue specific.

1. Introduction and General Considerations

Oxygen toxicity has been of scientific interest for over a century, but it was not until the 1960s that it was suggested that the damaging effects of oxygen could be attributed to the formation of reactive oxygen species (ROS). In the 1970s the discovery of superoxide dismutase strengthened the theory of free radical mechanism of cell injury.

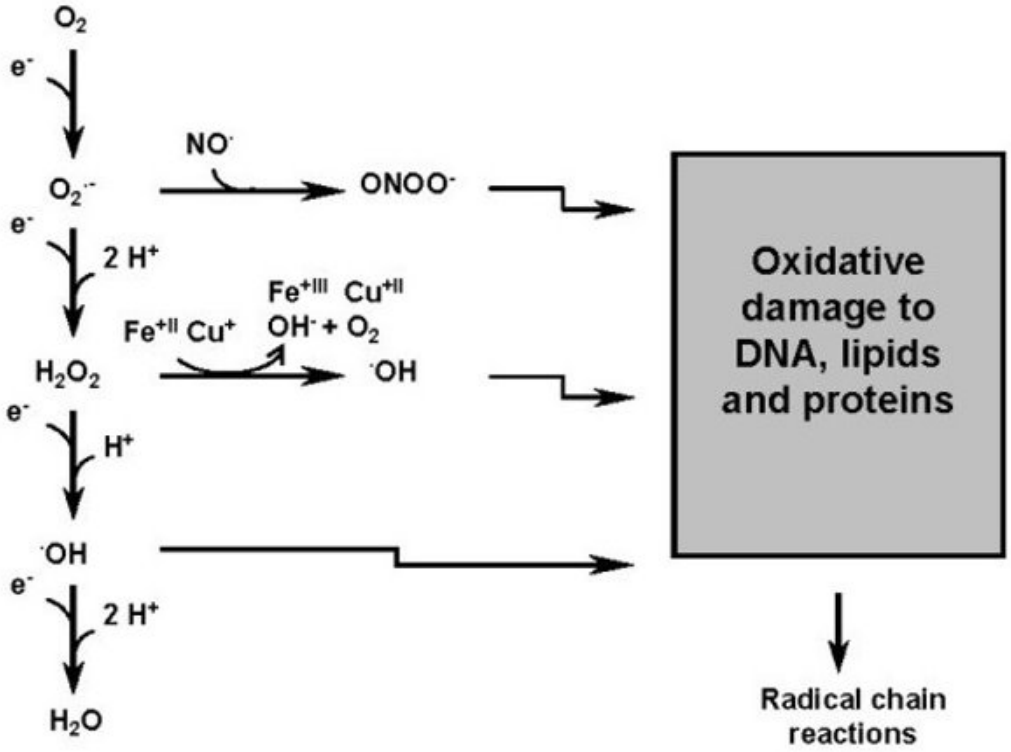

Figure 1. Generation and harmful effects of reactive oxygen species from tetravalent reduction of molecular oxygen to water. OH: hydroxyl radical; $O_2^{\cdot-}$: superoxide; H_2O_2: hydrogen peroxide; $NO^{\cdot}$: nitrogen radical; $ONOO^-$: peroxynitrite. Source: modified from

Freeman and Crapo (1982). Biology of disease: free radicals and tissue injury. *Laboratory investigation* 47, 412-426.

Occasionally, under certain biological conditions, oxygen does manage to steal away electrons from other molecules by non-enzymatic autoxidation. Because oxygen cannot accommodate a spin-matched pair, it must settle for stealing electrons one at a time. This breaking-up of electron pairs results in free radical formation. Generally, a free radical is defined as a molecule or a single atom containing an unpaired electron. Unpaired electron or spins result in the instability of these molecules and usually make them highly reactive. Molecular oxygen (O_2) has two unpaired electrons in its outer orbit. In steady state O_2 is therefore a "diradical" (often named as "dioxygen"). However, because of quantum-mechanical restrictions, O_2 is not extremely reactive. Under normal resting conditions, approximately 95 to 98% of all oxygen consumed by the mammalian cells is reduced by addition of four electrons (tetravalent reduction, see Figure 1) to O_2 yielding two molecules of water and energy. The remaining proportion of oxygen consumed at rest can be utilized in an alternative stepwise addition of electrons (called a univalent reduction) resulting in ROS production.

Approximately 98% of the oxygen metabolized is handled by a single enzyme, cytochrome oxidase in mitochondria, which transfers four electrons to oxygen in a concerted reaction to produce two molecules of water as the product. This enzyme is structurally quite complex, containing four redox centers (two hemes and two copper ions), each of which can store a single electron. When all centers are reduced, the simultaneous transfer of four electrons to an oxygen molecule occurs with no detectable intermediate steps. Reduction-oxidation (or redox) reactions are at the core of human metabolic machinery. Redox reactions involve the transfer of electrons or hydrogen atoms from one reactant to another.

In biological systems, the term oxidative stress refers to an imbalance favoring pro-oxidants over antioxidants. Increased oxidative stress can lead to widespread lipid, protein and DNA damage. Oxidative stress has been proposed to be implicated in the aging and in the pathogenesis of a number of diseases including atherosclerosis, cancer, diabetes, cerebrovascular diseases, Parkinson's and Alzheimer's diseases, cataractogenesis, rheumatoid arthritis and muscular dystrophy. Uncontrolled production of ROS during exercise has been also suggested as inducing muscle damage and fatigue. In this way exercise-induced oxidative stress may compromise the overall health benefits of regular exercise. In tissues, a variety of antioxidant defence agents have evolved to cope with oxidative stress, acting in concert to detoxify ROS. The study of redox regulation of gene expression has exploded in recent years and clearly suggests that oxidants are major determinants of gene expression. Reactive oxygen intermediates have been implicated in the activation of a variety of kinases and transcriptional factors, and therefore, it is evident that free radicals can serve as signals that stimulate adaptive processes.

2. Reactive Oxygen Species and their Formation

About 2-5% of oxygen consumed escapes from the normal metabolic routes to water and other products and ends up in profound generation of different forms of oxygen, including its reactive metabolites. The one-electron reduction product of oxygen is the

superoxide radical (O_2^-). If two electrons are transferred, the product is hydrogen peroxide (H_2O_2), which is not a radical. It is nonetheless still eagerly receptive of two more electrons, causing H_2O_2 to be a cytotoxic oxidant. Certain chelates of ferrous iron and cuprous copper are capable of transferring a third electron to hydrogen peroxide, causing lysis of the O-O bond. One fragment is reduced to the state of water; the other fragment is the hydroxyl free radical (HO^-) that is one of the most potent oxidants known. It can initiate lipid peroxidation, cause DNA strand breaks, and indiscriminately oxidize virtually any organic molecule.

There are multiple locations and sources for ROS formation in the body, as well as certain exogenous sources (see Table 1). In skeletal muscle mitochondria represent a major site of ROS production (see *Muscle Energy Metabolism*). As stated earlier, ROS are formed in the mitochondrial electron transfer chain through univalent reduction of O_2. The rate of O_2^- formation appears to be directly proportional to the rate of mitochondrial oxygen utilization. During exposure to hyperoxia, the rates of leakage in lung mitochondria are believed to increase in direct proportion to the increased oxygen tension. Hence, healthy adult rats will die within 72 hours if placed in an atmosphere of 100% oxygen, only five times the normal concentration of oxygen at sea level. Most species can tolerate sublethal concentrations of oxygen (i.e. 50 to 85%) for several weeks. Although animals exposed to this extent of hyperoxia survive, they nevertheless develop a severe lung injury.

Primary localization	**Endogenous source**
Cytosol	Xanthine oxidoreductases, transition metals (Fe^{+II}, Cu^{+II}), auto-oxidation of small molecules, riboflavin
Endoplasmic reticulum	Cytochromes P-450 and b5
Intracellular granules	Myeloperoxidase (neutrophils)
Mitochondria	Electron transport chain
Peroxisomes	Various oxidases
Plasma membrane	NADPH oxidase (phagocytic cells), cyclo-oxygenase and lipoxygenase
Primary localization	Exogenous sources
Lung	Cigarette smoke
Systemic	Cytotoxic chemicals
Lung	Hyperoxia
Skin	Irradiation (UV-A and ionizing)

Table 1. Sources of reactive oxygen species in cells
Source: modified from Freeman and Crapo (1982). Biology of disease: free radicals and tissue injury. Laboratory investigation 47, 412-426.

The ultraviolet A component of sunlight (UV-A, wavelength 320-400 nm) has the potential to generate oxidative stress in cells and tissue so that both endogenous and

exogenous antioxidants strongly influence the biological effects of UVA. The expression of several genes (including heme oxygenase-1, collagenase and the nuclear oncogenes *c-fos* and *c-jun*) is induced following physiological doses of UV-A to cells, and this effect can be strongly enhanced by removing intracellular glutathione (GSH) or enhancing singlet oxygen lifetime. Repeated exposure of human skin to UV radiation leads to not only skin carcinogenesis but also photo-aging through DNA damage. Ionizing radiation causes decomposition of H_2O, resulting in formation of $HO^{\cdot}$ and hydrogen atoms which influence the cellular antioxidant status.

It has been universally accepted that the production of O_2^- by activated polymorphonuclear leukocytes and other phagocytes is an essential component of their bactericidal armamentarium. NADPH oxidase is the enzyme which reduces oxygen to O_2^-. Because superoxide is cytotoxic, it serves as an extremely broad-spectrum antibiotic, being also a mediator of inflammation. NADPH oxidase equips phagocytes, which have evolved mechanisms to detect and engulf invading microorganisms, with a way to destroy chemically the ingested microbes. NADPH oxidase is present also in other tissues having various functions. In B-lymphocytes NADPH oxidase provides cell-cell communications, and in fibroblasts and vascular endothelial cells it promotes fibroblast proliferation. In many cell lines, low levels of ROS actually stimulate cell proliferation whereas higher levels have shown inhibitory and even cytotoxic effects.

Xanthine oxidase (XO) is an important source of both O_2^- and H_2O_2, but XO varies widely in abundance among cell types, organs, and species. XO normally occurs *in vivo* as a NAD^+-dependent dehydrogenase, xanthine dehydrogenase (XD), incapable of ROS production. XD is an enzyme which converts xanthine and hypoxanthine to uric acid, and its activity converts by sulfhydryl oxidation or limited proteolysis (conditions that exist during reoxygenation) to an oxidase that produces both O_2^- and H_2O_2. XO is phosphorylated in hypoxic microvascular endothelial cells through a mechanism involving p38 MAPK and casein kinase II, and phosphorylation appears to be necessary for hypoxia-induced enzyme activation. However, reoxygenation injury also unquestionably occurs in cells in which XO activity is exceedingly low or absent. Under normal conditions, XO accounts for only a minor proportion of total ROS production. ROS production by XO has been implicated as a major source of ROS leading to increased oxidative stress under certain pathological conditions such as ischemia and reperfusion, and recently it has been implicated as having a pathophysiological role in congestive heart failure. Ischemia and reperfusion can lead to tissue injury and are serious complications in organ transplantation, myocardial infarction, and stroke. Massive ROS production has been identified as an important causative factor.

Prostaglandins are important mediators in host defense mechanisms and inflammation. Cycloxygenase (COX), also known as prostaglandin H synthase (PGHS), is the rate-limiting enzyme in prostaglandin biosynthesis. COX catalyzes prostaglandin formation from arachidonic acid and their involvement in inflammation processes is well known. Free radical and COX-mediated oxidation of arachidonic acid products are intimately associated with experimental hepatotoxicity. It has been suggested that there is a link between initial involvement of oxidative stress and subsequent induction of the COX-mediated inflammatory process, which may have an eminent role in the pathogenesis of diseases related to oxidative stress.

Lipoxygenases (LOX) form a family of lipid peroxidation enzymes that oxygenate free and esterified polyenoic fatty acids to the corresponding hydroperoxy derivatives. The conversion of iron from the ferrous (Fe^{+II}) to the ferric (Fe^{+III}) oxidation state is necessary for the activation of LOX. Therefore, the activity of LOX might be regulated by a small amount of hydroperoxy lipids, acting as essential activators of the enzyme. Lipid-peroxidizing and lipid peroxide-reducing enzymes are inversely regulated in various mammalian cells. Upregulation of the 12/15-LOX and simultaneous downregulation of certain glutathione peroxidases may lead to an increased oxidizing potential, which is reflected by an augmented intracellular peroxide tone. Arachidonic acid itself, and both its COX and LOX metabolites, have been reported to modulate apoptosis signaling. However, the involvements of COX and LOX in apoptosis signaling are somewhat controversial and uncertain.

Cytochrome P450 (CYP) family of enzymes are membrane-bound, terminal oxidases that exist in a multi-enzyme system that also includes a NADPH-cytochrome P450 reductase and cytochrome b5. Members of this enzyme superfamily oxidize, peroxidize, and/or reduce cholesterol, steroids, arachidonic acid, bradykinin, vitamins, xenobiotics, and numerous therapeutic substances in an oxygen- and NADPH-dependent manner. It is has become increasingly clear that CYP enzymes play a key role in the modulation of vascular homeostasis. CYP represent a substantial source of ROS and also generate arachidonic acid metabolites with diverse effects on protein kinases, ion channels and mitochondria.

Dietary factors are also determinants of oxidative stress. A high degree of unsaturation of the (*n*-3) fatty acids in the diet induces oxidative stress via peroxidation of membrane lipids, increased oxidation low-density lipoproteins (LDL) and peroxisomal beta-oxidation. Fish oils rich in (*n*-3) fatty acids increase the proportion of membrane polyunsaturated fatty acids and also induce peroxisomal beta-oxidation in the liver (see *n-3, n-6 Fatty Acids and Cholesterol in Metabolism*).

3. Oxidative Damage and Physiological Significance of Reactive Oxygen Species

In the pathogenesis of acute or chronic tissue injury, a key player is the oxidative damage of cellular membranes. Unsaturated fatty acids present in membrane (phopholipids, sterols, glycolipids, and glycerides) and transmembrane proteins containing oxidizable amino acids are particularly susceptible to free radical damage. Generally, $HO^{\cdot}$ has the ability to initiate membrane peroxidation, whereas lipid peroxidation can result in the formation of more lipid alkoxyl ($LO^{\cdot}$) and peroxyl ($LOO^{\cdot}$) radicals. These radicals can abstract hydrogen atoms from adjacent polyunsaturated fatty acids and propagate the chain reaction of lipid peroxidation. Produced LO and LOO also react with proteins at their amino acid side chains and attack the peptide bonds. In addition, oxidatively modified proteins are more susceptible to degradation by proteases. Oxidative modification of membrane lipids and proteins alters membrane permeability and disturbs maintenance of ionic gradients, receptor and transport functions resulting in cellular dysfunction.

Among cellular macromolecules, polyunsaturated fatty acids (PUFA) exhibit the highest sensitivity to ROS-induced damage, their sensitivity to oxidation exponentially

increasing as a function of the number of double bonds per fatty acid molecule. A low degree of fatty acid unsaturation in cellular membranes, and particularly in the inner mitochondrial membrane, may be advantageous by decreasing their sensitivity to lipid peroxidation. This would also protect other molecules against lipoxidation-derived damage. In agreement with this, it has been found that long-lived animals have a lower degree of total tissue and mitochondrial fatty acid unsaturation (low double-bond index) than short-lived ones. Oxidation of PUFAs leads to the formation of hydroperoxides and endoperoxides, which undergo fragmentation to yield a broad range of reactive intermediates, including alkanals, alkenals, hydroxyalkenals, glyoxal, and malondialdehyde with the final formation of advanced *Maillard* products. These carbonyl compounds, and possibly their peroxide precursors, react with nucleophilic groups present in proteins, resulting in indirect chemical modification of the protein. Proteins are also directly modified by ROS, causing the formation of oxidatively modified amino acids. As a consequence, the fundamental findings related to this protein damage and aging are as follows:

- The accumulation of protein damage during aging in extracellular matrix and tissues;
- The concentration and the rate of accumulation of protein damage is higher in short- than in long-lived animal species;
- The concentration and rate of accumulation of protein damage is generally lower in food-restricted than in *ad libitum*-fed animals, most importantly the oxidative breakdown of peptide backbone and oxidation of side chains of glutamyl, aspartyl, lysyl, arginyl, prolyl and threonyl produces carbonyl derivatives

Oxidative stress may also cause irreversible modifications of DNA. In humans, oxidative hits to the DNA of a cell is estimated to occur approximately ten thousand times in one day, and oxidative damage is known to accumulate with aging. In addition to HO and ionizing and ultraviolet radiation, also oxidizing lipids such as lipid LO and LOO radicals can cause DNA damage. The chemical reactions of these radicals are slower than that of HO, and because of the longer half-life and faster transportation of these substances, oxidizing lipids have recently been suggested to be important causes of DNA damage and other biological damage. With relevance to oxidative damage in DNA level, the apparent inconsistency between the uncontrolled cell growth in ROS-producing malignant cells and the ROS-induced senescence in normal cells suggests that ROS production may be necessary but not sufficient to induce malignant cell growth.

High concentrations of ROS are hazardous for living organisms and they damage all major cellular constituents. At moderate concentrations, however, ROS and other ROS-related reactive molecules such as nitric oxide (NO), play an important role in the regulation of signaling processes. ROS act as secondary messengers to control a variety of physiological responses. The regulation of vascular smooth muscle relaxation, the monitoring of oxygen concentration in respiratory ventilation and erythropoietin production, and the enhancement of signaling cascades from various membrane receptors are prominent examples. Furthermore, in physiological conditions, hormones, cytokines, or other inducing mechanisms precisely regulate the production of ROS and

related products. Finally many of the ROS-mediated responses, indeed, protect the cells against oxidative stress and re-establish the "redox homeostasis".

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Biographical Sketches

Jani Lappalainen was born in 1975 in Helsinki, Finland. He studied at the University of Kuopio, Finland, where he obtained the Master's degree in Exercise Medicine in 2003, and since then has continued his postgraduate studies on exercise physiology at the Department of Physiology, University of Kuopio. One of his research interests is exercise-induced oxidative stress. He has also studied medicine and biochemistry at the University of Helsinki, Finland.

Mustafa Atalay was born in 1963 in Ankara, Turkey, and received his M.D. degree in the University of Ankara School of Medicine in 1986. He specialized in family practice in the State Hospital of Ankara in 1992, and continued his postgraduate studies on exercise physiology and sports medicine in Kuopio, Finland, from the beginning of 1993. In 1995 he received a Master of Public Health degree from the Department of Public Health, University of Kuopio. In 1998 he defended his Ph.D. thesis on "Tissue Antioxidant Responses to Physical Exercise-Induced Oxidative Stress" in the department of Physiology, University of Kuopio, and he received the degree of "Docent of Sports Medicine" from the National Board of High Education of Turkey in 1999. He was selected as a Fellow of the American College of Sports Medicine same year. He completed his postdoctoral fellowship at Ohio State University Medical Center and Laboratory of Molecular Medicine between 2000 and 2001. In 2003 he received the degree of "Docent of Exercise Physiology" from the Faculty of Medicine, University of Kuopio. His research interest is in exercise-induced oxidative stress and antioxidant defenses. Currently he is working in the University of Kuopio, Finland, as a senior lecturer and researcher.