Indian J Physiol Pharmacol 1995; 39(3): 177-196

REVIEW ARTICLE

OXYGEN TOXICITY AND ANTIOXIDANTS: STATE OF THE ART

CHANDAN K. SEN

Membrane Bioenergetics,
Department of Molecular and Cell Biology,
University of California at Berkeley,
CA 94702, California, U.S.A. and

Department of Physiology,
Faculty of Medicine,
University of Kuopio,
P.O. Box 1627, 70211 Kuopio,
Finland

(Received on July 5, 1994)

Abstract: Although the use of oxygen as metabolic fuel allows an attractive harvest of energy rich phosphates per molecule of glucose, a significant fraction of oxygen utilized by the body incompletely reduced and is known to be toxic. Such partially reduced forms of oxygen and some of their derivatives are highly reactive pro-oxidants that are collectively referred to as reactive oxygen species (ROS). A multitude of factors are known to modulate the amount of ROS formation in the body. To escape ROS dependent toxicity biological structures have their protective machinery in the form of physiological antioxidants. It appears that the physiological antioxidants are not independently capable of completely detoxifying the ROS constantly produced by the body. The supply of exogenous antioxidants is thus crucial. Endo- and exo-genous antioxidants act in concert to minimize ROS dependent damage. ROS are involved in the pathogenesis of a large number of clinical disorders. The sensitive balance between the pro- and anti- oxidant forces in the body appears to be very crucial in determining the state of health, well being and longevity. This article presents an introductory overview covering the current concepts related to oxidants and antioxidants with special reference to human health.

Key words: reactive oxygen species free radical oxidative stress exercise therapy disease antioxidant chain reaction

INTRODUCTION

Oxygen toxicity, studied in the early days, usually referred to the toxic effects of oxygen at high pressure (as during diving, hyperbaric oxygen therapy, aerospace travel, etc.). The first work in this area was reported by Paul Bert in 1878, just a century after the discovery of oxygen by Joseph Priestly. In his classic work *La Pression Barometrique* he described the incidence of convulsions in various animal species exposed to oxygen at high pressure (1). Bert's work was proceeded through Michaeli's theoretical considerations, Gerschman's experimental verification and finally attracted the interest of biomedical scientists when in 1969 McCord and Fridovich demonstrated that a metalloenzyme produced hydrogen peroxide (H_2O_2) by combining a toxic metabolite of oxygen known as superoxide ($O_2^{\cdot -}$) with hydrogen (2). Today's concept of oxygen toxicity is not restricted only to hyperbaric oxygen but primarily focusses on the stress caused by the reactive metabolites (oxygen free radicals/reactive oxygen species) of oxygen generated as an integral part of our daily life (3, 4). The area covered in this review, has been rapidly unfolding in the recent years and has already acquired a vast spread. This article presents a concise introductory overview of the salient features of oxidants and antioxidants with special reference to human health.

OXYGEN FREE RADICALS AND REACTIVE OXYGEN SPECIES (ROS)

A free radical is a molecule or molecular fragment containing an unpaired electron in the valence shell (i.e., radical) and capable of existing independently (i.e., free). In 1924 it was established that molecular oxygen has two unpaired electrons in its valence orbit. Ground state O_2 is therefore a 'diradical' (often referred to as 'dioxygen') the two unpaired electrons being accommodated, formally, in the degenerate pair of antibonding π^* orbitals, π^1_x and π^1_y. However, because of quantum-mechanical restrictions O_2 is not extremely reactive. Univalent (stepwise) addition of 4 electrons (tetravalent reduction) to O_2 produces water (Fig. 1). Oxygen radicals and their byproducts that are capable of inciting oxidative tissue damage (reactive oxygen species, ROS) may be produced as a result (Fig. 1). The terms 'ROS' and 'oxygen radical' are not synonymous; unlike the latter, ROS include also non-radical derivatives of oxygen (e.g. H_2O_2, peroxides, singlet oxygen, hydroperoxides, epoxides, etc.) which are capable of causing oxidative tissue damage. In a biological system, oxidative stress refers to a disturbance in the pro- and anti-oxidant balance in favour of the pro-oxidant (Fig. 2). Oxidative stress ensues when ROS evade or overwhelm the antioxidant protective mechanisms of cells and tissues.

Molecular oxygen can be reduced by one electron giving rise to superoxide radicals ($O_2^{\bullet-}$)

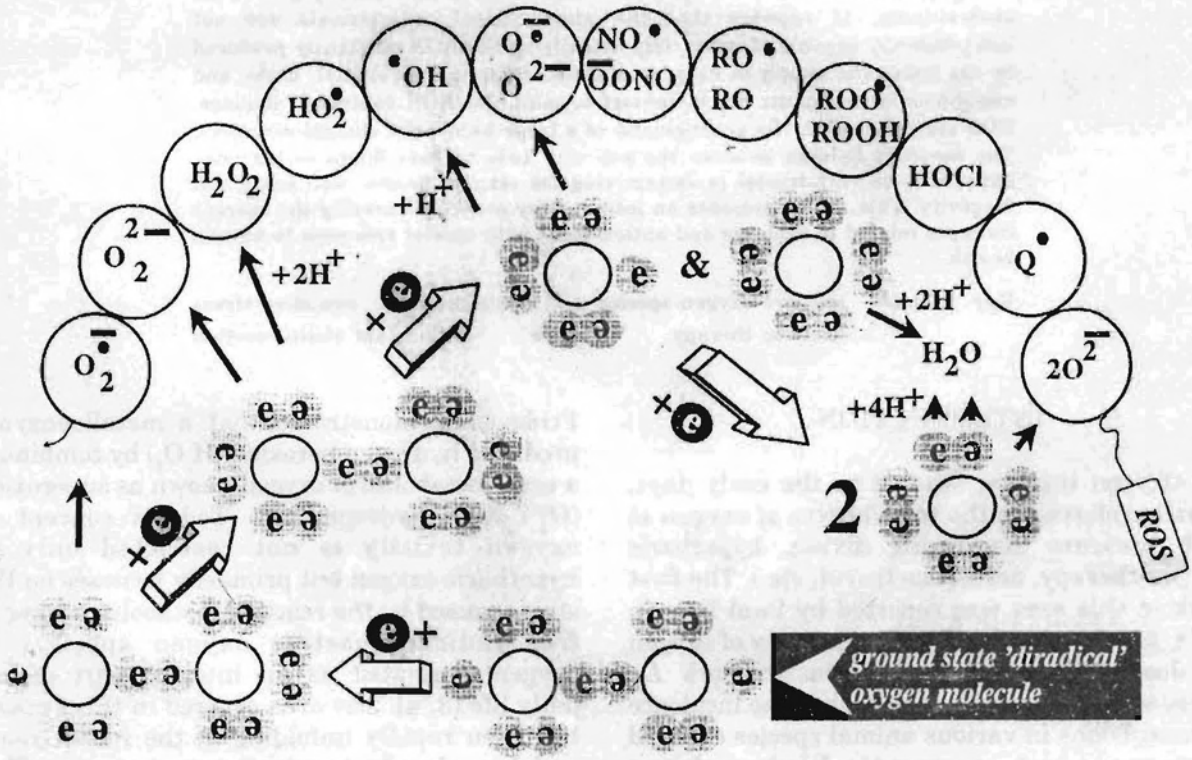

Fig. 1: The four-step univalent reduction of 'diradical' oxygen. Some commonly known members of the reactive oxygen species (ROS) family are: $^{\cdot}OH$, hydroxyl radical; $Q^{\cdot}$, semiquinone; $O_2^{\cdot-}$, superoxide anion radical; $^1O_2$, singlet oxygen; $NO^{\cdot}$, nitric oxide; $ONOO^{\cdot}$, peroxynitrite anion; O_2^{2-}, peroxide ion; $RO^{\bullet}$, alkoxyl radical; $ROO^{\cdot}$, peroxyl radical; ROOH, alkyl hydroperoxide; ROH, alkyl hydroxide; HOCl, hypochlorous; H_2O_2, hydrogen peroxide; $HO_2^{\cdot}$, perhydroxy radical.

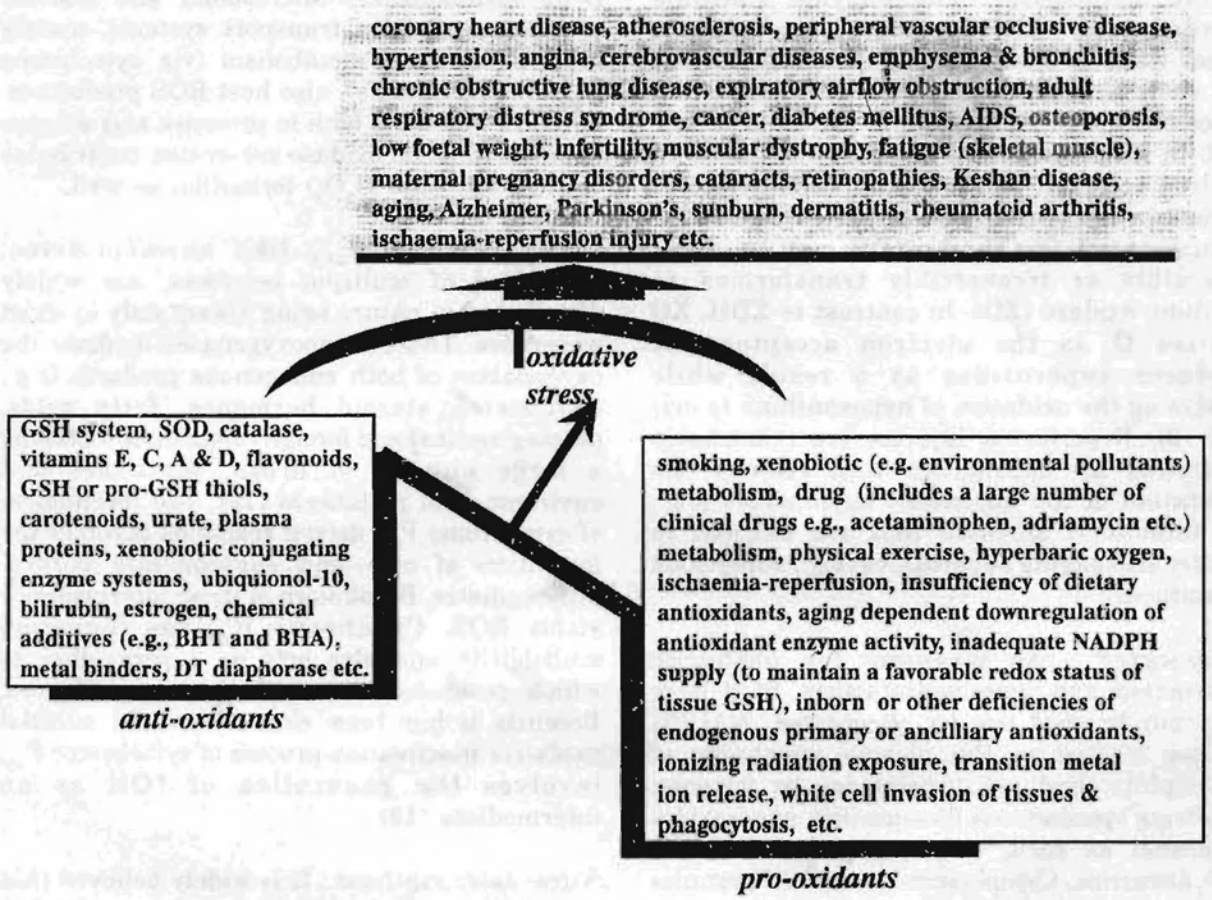

Fig. 2: Oxidative stress: the imbalance between pro- and anti-oxidant forces in favour of the former. *Reprinted with due permission from Sen and Hanninen (4).*

which can be further reduced to hydrogen peroxide and hydroxyl radicals (•OH) and finally to water. Formation of superoxides and hydrogen peroxide can be regulated by either enzymatic or nonenzymatic mechanisms whereas no enzymes are required for the formation of hydroxyl radical. The hydroxyl radical is the most reactive oxygen free radical that may be formed either through transition metal-ion catalysed Fenton ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^•$) or Haber-Weiss ($O_2^- + H_2O_2 + Fe^{2+} \rightarrow O_2 + OH^- + HO^• + Fe^{3+}$) reactions. Hydroxyl radical, thus formed, is highly reactive capable of initiating deleterious reactions such as lipid peroxidation and DNA damage.

Sources of ROS

Mitochondria: In 1973 Boveris and Chance showed that mitochondria can generate H_2O_2 (5). Two years later Dionsi et el. demonstrated the formation of superoxide and H_2O_2 in the organelle from normal and neoplastic tissues (6). Partially reduced oxygen is known to escape as superoxide radical from two specific sites in the electron transport chain. These two sites are: i) ubiquinone→ubisemiquinon→cytochrome c_1 step; ubisemiquinone may reduce oxygen to superoxide (7), and ii) NADH dehydro-genase (8). The superoxides rapidly dismutate to from H_2O_2. Some of the mitochondrial H_2O_2 is suspected to escape to the cytosol.

Purine metabolism: The enzyme xanthine dehydrogenase (XDH), mainly located in the vessel walls of most tissues including cardiac and skeletal muscle, catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid. In its native form, XDH uses NAD^+ as an electron acceptor. Under certain conditions like ischaemia-reperfusion and extreme hypotension as in hemorrhagic shock, XDH may be either reversibly or irreversibly transformed to xanthine oxidase (XO). In contrast to XDH, XO utilizes O_2 as the electron acceptor and produces superoxides as a result while catalyzing the oxidation of hypoxanthine to uric acid (9). Reperfusion injuries are remarkably controlled by approaches that restrict the generation of XO dependent superoxides (e.g., XO inhibitors) or those that are efficient in rapidly scavenging superoxides (e.g., superoxide dismutases).

Phagocytes : As weapons for pathogen destruction and immunoprotection, ROS have been put to good use by phagocytes. NADPH oxidase located in the plasma membrane of neutrophils produces superoxides on purpose. Following spontaneous dismutation, superoxides generated as such, remarkably contribute to H_2O_2 formation, Cytoplasmic azurophilic granules of neutrophils and to a lesser extent monocytes (but not macrophages) contain a hemoprotein peroxidase called myeloperoxidase. When activated by immune-challenge or such other stimuli, neutrophils release myeloperoxidase into the extracellular medium. The released myeloperoxidase complexes with H_2O_2 to form an enzyme-substrate complex with an oxidizing potential. The complex oxidizes chloride (Cl^-) to produce hypochlorous acid (HOCl). $O_2^{\bullet-}$, H_2O_2 and HOCl may be considered as broad spectrum physiological "antibiotics" that eliminate pathogenic infection. Unfortunately, for this, the host cell has to pay a price in the form of inflammation (10). The "oxidant-force" that kills pathogens is also cytotoxic to the host tissue and to the neutrophils themselves. Accelerated generation of ROS by activated (e.g., immune challenged) neutrophils is often referred to as *oxidative burst* (11).

Drug metabolism : Microsomal and nuclear membrane electron transport systems, mainly involved in drug metabolism (via cytochrome P_{450} and b_5 systems), also host ROS production. NADPH oxidation, both in presence and absence of mixed function oxidase substrates, contributes to ROS ($O_2^{\bullet-}$ and H_2O_2) formation as well.

Cytochrome P_{450} like haemoproteins, comprised of multiple isozymes, are widely distributed in nature being absent only in strict anaerobes. These monooxygenases mediate the oxygenation of both endogenous products (e.g., cholesterol, steroid hormones, fatty acids, prostaglandins) and foreign compounds including a large number of drugs, pesticides and environmental pollutants (12). The mechanism of cytochrome P_{450} driven reactions involves the formation of oxy- and subsequently peroxy-intermediates. Breakdown of these intermediates yields ROS. Cytochrome P_{450} has functional multiplicity and also acts as a peroxidase in which peroxides are used as oxygen donors. Recently it has been shown that the suicidal oxidative inactivation process of cytochrome P_{450} involves the generation of $^{\bullet}OH$ as an intermediate (13).

Nitric oxide synthase : It is widely believed that endothelium derived relaxing factor (EDRF) produced by vascular endothelial cells is identical with NO (14). Synthesized in a wide variety of tissues, NO is known to be implicated in a number of crucial physiological functions e.g., control of systemic blood pressure, respiration, digestion, penile erection, platelet aggregation, cerebral blood flow and neuronal synaptic plasticity. NO and its derivatives also contribute to microbicidal and tumoricidal activities of macrophages and neutrophils. The enzyme primarily responsible for the synthesis of NO is tissue specific. NO synthase in the endothelium and neurons is a calmodulin-activated enzyme that oxidizes arginine to citrulline in the presence of biopterin, NADPH and oxygen. Macrophage NO synthase is not regulated by calmodulin and is distinctly different in gene sequences when compared to the brain and endothelial cell variety.

NO has one unpaired electron and is therefore a radical by definition. Cells like macrophages which are capable of producing both NO and superoxides are the likely host of a very powerful deleterious ROS, the peroxynitrite anion ($ONOO^-$). Formed by the reaction of NO with superoxide, peroxynitrite anion is a relatively long lived ROS. In this way, NO may magnify superoxide toxicity remarkably. A recent study demonstrated that $ONOO^-$ may be implicated in the oxidative modification of human low density lipoprotein (15), a process fundamental to the development of atherosclerosis (16).

Transition metals : Conditions (e.g., plasma pH<6.0, haemolysis and ischaemia-reperfusion) that lead to the release of transition metal ions (e.g., that of iron and copper) may remarkably amplify ROS toxicity (17). Iron and copper ions are capable of converting H_2O_2 to $^{\bullet}OH$. The hydroxyl radical is a very powerful ROS which is capable of initiating lipid peroxidation. It has been shown that in the presence of free transition metal ions ascorbic acid, a commonly known antioxidant, functions as a pro-oxidant (18).

Some other possible sources : Some other enzymes known to be responsible for the generation of hydrogen peroxide or superoxide anion are listed below with their respective subsellular localization indicated in parentheses (19): glycolate oxidase (peroxisome), L-α-hydroxyacid oxidase (peroxisome), L-gulonolactone oxidase (cytosol), aldehyde oxidase (cytosol), D-amino-acid oxidase (peroxisome), monoamine oxidase (mitochondrial outer membrane), pyridoxamine oxidase (endoplasmic reticulum), diamine oxidase (endoplasmic reticulum), urate oxidase (peroxisome core), superoxide dismutase (cytosol and mitochondrial matrix). Boveris et al (20) have shown that mitochondria, microsomes, and cytosolic enzymes are effective H_2O_2 generators, contributing in the rat liver, respectively, 15%, 45%, 35% and 5% to the cytosolic H_2O_2 at a PO_2 of 158 mm Hg when fully supplemented by their substrates. PGH synthase dependent arachidonic acid metabolism generate superoxides in the presence of NADH or NADPH (21). Superoxides, generated during the autoxidation of catecholamines, may contribute to oxy-radical toxicity in catecholamine neurones (22). One other plausible biological source of ROS is myoglobin. Oxidation of ferrous myoglobin (MbII) to its hypervalent ferryl from (e.g., MbIV) is suggested to contribute to ischaemia-reperfusion injury in the heart. MbIV, in the presence of H_2O_2 has the potential to act as a peroxidase affecting crucial cellular structures (23). A summary of the the possible physico-chemical mechanisms that may contribute to free radical formation is presented in Table I.

Cigarette smoking and alcoholism enhance oxidative stress risk. Each puff of a cigarette is estimated to contain~10^{14} free radicals in the tar phase and~10^{15} of them in the gas phase. The crucial lipid phase antioxidant vitamin E is consumed at a very high rate in the lungs of smokers, and higher levels of lipid peroxidation, a commonly used index of oxidative stress, have been observed in the plasma of smokers (24). The metabolism of ethanol produces acetaldehyde that is known to consume GSH (25). Ingestion of ethanol is associated with enhanced lipid peroxidation (26). Increased levels of lipid peroxidation by-products were observed in alcohol treated rat cerebral cortex, cerebellum and brain stem (27).

ANTIOXIDANTS

Broadly, the possible mechanisms by which antioxidants may protect against ROS toxicity are; i) prevention of ROS formation, ii) interception of ROS attack by scavenging the reactive metabolites and converting them to less reactive molecules and/or by enhancing the resistivity of sensitive biological targets to ROS attack, iii) facilitating the repair of damage caused by ROS and iv) providing (e.g., as a cofactor or by acting to maintain a suitable redox status) a favourable environment for the effective functioning of other antioxidants. Evolution has favoured the selective development and differentiation of a vast group of oxygen requiring life-forms broadly classified as aerobic organisms. The capacity to detoxify ROS is of critical importance in all aerobes. In

TABLE I : Radical formation : Possible Physico-chemical mechanisms.

Physical

UV light

- A-B + hν (250-400 nm)→A$^{\bullet}$ + B$^{\bullet}$ (photolysis), where A-B is a photosensitive compound
- A-B + hν - (higher energy photons)→ AB$^{\bullet+}$ e- (photoionization)
- Homolytic reaction

  A-B + $h\nu$ →AB* (excited triplet state)
  AB* + R-H → $^{\bullet}$ABH + R$^{\bullet}$ (we get 2 radicals)

Ionizing radiation

- high frequency electromagnetic radiation

  UV-, X-, and γ-rays
  high energy particles e.g.,
  neutron, proton, electron or α-partices (He nuclei)

Water radiolysis

- H_2O --primary ionization → $H_2O^{\bullet+}$ + e-

  $H_2O^{\bullet+} + H_2O \rightarrow {}^{\bullet}OH + H_3O^{+}$

Mechanical

- (stretch, cut, bend, break)-shear → bond homolysis

Ultrasound

- Ultrasonication → thermal fluctuation → bond homolysis
- H$^{\bullet}$ and $^{\bullet}$OH generates when water is ultrasonicated

Chemical

Thermolysis

- if a molecule or ion has one relatively weak bond, precise homolysis may occur on heating

  A-B --heat → A$^{\bullet}$ + B$^{\bullet}$

Redox processes

(significantly contribute to the generation of ROS in biological systems)

- Electron and proton transfer; fast processes

  $A + D \leftrightarrow A^{\bullet-} + D^{\bullet+}$
  $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + {}^{\bullet}OH$

A-B is a hypothecal molecule; A, B, D are elements. $^{\bullet}$denotes a radical.

the human body, a complex combination of enzymatic and non-enzymatic function to minimize the stress induced by ROS. These antioxidants may be classified as: i) endogenous antioxidants-those which are physiological in origin, and ii) exogenous antioxidants-those which cannot be produced by the human body but may protect against pro-oxidant forces when administered as supplements.

Endogenous antioxidants

Although a large number of enzymatic and non-enzymatic physiological substances are known to have "antioxidant-like" functions, the primary contributors are superoxide dismutases, catalase, and the glutathione system. Superoxide dismutases (SOD), superoxide: superoxide oxidoreductase) are enzymes involved in cellular defence against uncontrolled oxidative processed that catalyze the dismutation of the superoxide radical anion and hence diminish toxic effects due to this radical or to other free radicals derived from secondary reactions. In mammalian tissues, two types of SOD have been described: (i) cytosolic cuprozinc-SOD (Cu, Zn-SOD), and (ii) mitochondrial mangano-SOD (Mn-SOD). The clinical significance of endogenous SOD has been discussed in a recent review (28). The principal function of SOD is to catalyze the conversion of superoxides to H_2O_2 — i.e. it catalyzes the conversion of one form of ROS to the other. H_2O_2 thus produced is detoxified either by catalase or reduced glutathione (GSH) dependent reactions. Catalase is present virtually in all mammalian cells and is suggested to play a dual role: (i) a true catalytic role in the decomposition of H_2O_2 ($2H_2O_2 \rightarrow 2H_2O + O_2$); and (ii) a peroxidic role in which the peroxide is utilized to oxidize a range of H donors (AH_2) such as methanol, ethanol and formate, ($AH_2 + H_2O_2 \rightarrow A + 2\ H_2O$). In each case an active enzyme-H_2O_2 complex is formed initially followed

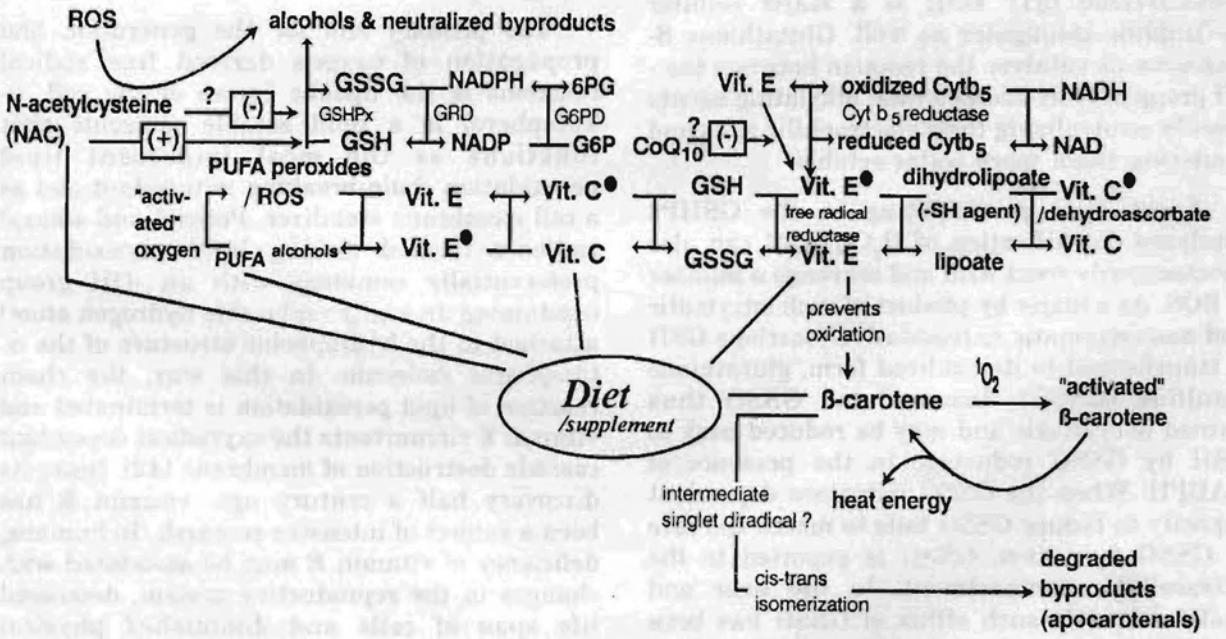

Fig. 3 : The antioxidant chain reaction. PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; a superscript dot symbolises the radical form of the respective compounds; (+), NAC is a pro-GSH drug; ? (-), NAC and ubiquinone is suggested to "spare" the oxidation of GSH to GSSG and vitamin E to tocopheroxyl radical, respectively; GSHPx, glutathione peroxidase; GRD, GSSG reductase; G6PD, glucose 6-phosphate dehydrogenase; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; $Cytb_5$, cytochrome b_5, GSH, reduced glutathione; GSSG, glutathione disulfide. *Reprinted with due permission from Sen and Hanninen (49).*

by an exceedingly rapid second stage in which a second molecule of H_2O_2 serves as a H donor for the enzyme-H_2O_2 complex. The enzyme is mostly localized in the peroxisomes (microbodies) of liver and kidney, and in much smaller aggregates (microperoxisomes) found in other cells.

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is important in the circumvention of cellular oxidative stress, detoxification of electrophiles and maintenance of intracellular thiol redox status (29,30). Glutathione peroxidase (GSHPx) is specific for its hydogen donor, GSH, but may use a wide range of substrates extending from H_2O_2 to organic hydroperoxides. The cytosolic and membrane-bound monomer GSH phospholipid hydroperoxide-GSHPx, and the distinct tetramer plasma GSHPx are able to reduce phospholipid hydroperoxides without the necessity of prior hydrolysis by phospholipase A_2. The protective action of phospholipid hydroperoxide-GSHPx against membrane damaging lipid peroxidation has been directly demonstrated (31). GSH is a major cellular electrophile conjugator as well. Glutathione S-transferases catalyse the reaction between the -SH group of GSH and potential alkylating agents thereby neutralizing their electrophilic sites and rendering them more water-soluble.

Apart from participating in the GSHPx catalyzed detoxification of H_2O_2, GSH can also spontaneously react with and scavenge a number of ROS. As a major by-product of such enzymatic and non-enzymatic antioxidative reactions GSH is transformed to its oxidized form, glutathione disulfide (GSSG). Intracellular GSSG thus formed is cytotoxic and may be reduced back to GSH by GSSG reductase in the presence of NADPH. When the GSSG reductase dependent capacity to reduce GSSG fails to match the rate of GSSG formation, GSSG is exported to the extracellular compartment. In the liver and skeletal muscle such efflux of GSSG has been reported to be energy-linked (32, 33). Therefore, oxidative stress in tissues is often reflected as high GSSG level in the serum (32). As discussed in a latter part of this review, GSH also plays a central role in co-ordinating the synergism of various crucial antioxidants.

The physiological antioxidant defense strategy appears to be a complex process involving a large number of components. In addition to the major physiological antioxidant defense mechanisms mentioned above, a number of other enzyme and non-enzme systems are also known to have ancillary antioxidant properties (Table II).

Exogenous antioxidants

For an effective protection against oxidative insults that we encounter in our daily lives regular consumption of at least some antioxidants, in the diet or as supplements, appears to be very crucial. Among the exogenous antioxidants, vitamins E and C have been recognized to be especially important and deficiency of these may lead to a number of pathophysiological consequences. The term vitamin E is a generic description for all tocol and tocotrienol derivatives that qualitatively exhibit the biological activity of α-tocopherol.

The primary site for the generation and propagation of oxygen derived free radical reactions is the lipoidic layers of the cell. α-Tocopherol is a lipid soluble molecule that functions as the most important lipid peroxidation chain-breaking antioxidant and as a cell membrane stabilizer. Peroxyl and alkoxyl radicals formed during lipid peroxidation preferentially combines with an -OH group (containing an easily replacable hydrogen atom) attached to the hydrophobic structure of the α-tocopherol molecule. In this way, the chain reaction of lipid peroxidation is terminated and vitamin E circumvents the oxyradical dependent cascade destruction of membrane (42). Since its discovery half a century ago, vitamin E has been a subject of intensive research. In humans, deficiency of vitamin E may be associated with changes in the reproductive system, decreased life span of cells and diminished physical capacity.

In the absence of transition metal ions, vitamin C is an outstanding antioxidant in the aqueous phase (43). Vitamin C spontaneously reacts with and scavenges a wide variety of

TABLE II : Endogenous components contributing to antioxidant protection.

Component	*Basic mechanisms*
Superoxide dismutases	• catalyzes $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	• scavanges H_2O_2 • peroxidic (see text)
GSH	• enzymatically (GSHPx) decomposes H_2O_2 and other hydroperoxides • GST may also have "GSHPx-like" (Se independent) function in detoxifying H_2O_2 • spontaneously reacts with and scavenges many forms of ROS • maintains intracellular redox milieu (catalyzes reduction of low molecular weight disulfides) • intracellular reservoir of cysteine (required for protein synthesis; cysteine also scavenges free radicals by donating electron from sulfhydryl groups {-SH}) • replenishes a number of crucial antioxidants (e.g., vitamins E and C) that get oxidized during the course of their antioxidant action • GST and other biotransformation enzymes may contribute to antioxidative protection by catalyzing conjugation reaction of xenobiotics
NADPH supply	• supply NADPH which, together with GRD, reduces GSSG to GSH and helps maintain a favourable intracellular thiol redox status
DNA repair systems, oxidized protein turnover, oxidized phospholipid turnover etc.	• oxidative damage repair
Glucose	• scavenges hydroxyl radical (•OH); glucose in turn becomes oxidized
Oxidative stress regulons	• facilitate "physiological adaptation" processes (see Table 3)
Haem oxygenase	• this has been recently identified as an oxidative stress protein mechanisms by which it may possibly contribute to antioxidative protection are yet unclear
Bcl-2	• this proto-oncogene was shown to intercept the generation of ROS
Ferritin, transferrin, lactoferrin, caeruloplasmin, albumin, metallothioneins, albumin etc.	• iron/copper binding capacity disallows the existence free metal ions that are known to intensify oxidative stress by promoting the generation hydroxyl radical (·OH), a very deletorious ROS • absence of free such transition metal ions allows effective antioxidant action of ascorbic acid. In the presence of such ions, ascorbate looses its antioxidant properties

Urate	• complexes transition metal • scavenges •OH, ROO• • activates prostaglandin synthesis initiate by arachidonate • may spare plasma ascorbate
NADPH-quinone oxidoreductase (DT diaphorase)	• catalyzes the two-electron reduction of quinones and the conjugation reactions of hydroquinones decrease steady-state level of reactive metabolites capable of generating ROS
Ubiquinol-10	• this reduced form of coenzyme Q_{10} is a highly reactive antioxidant effective to protect biological membranes against oxidation. Protects human low density lipoprotein from peroxidative damage more effectively than either α-tocopherol or carotenoids
Adenosine	• this nucleoside (adenine-ribose) decreases superoxide generation by neutrophils and also affects endothelial function • protects against reoxygenation injury
Nicotinamide nucleotides	• following ROS exposure activation of poly (ADP-ribose) synthetase decreases intracellular levels of this • prolonged ingestion of nicotinic acid by humans increase NAD^+ concentrations in circulating lymphocytes making the cells more resistant to oxidative damage
Estrogens	• radical scavengers • suppress peroxidative reactions • especially 2-hydroxyestradiol, was observed to function as a lipid peroxidation chain breaking antioxidant like α-tocopherol • exogenous 17β-estradiol is also known to be associated with elevated levels of erythrocyte glutathione peroxidase
Bile pigments	• lipid peroxidation chain breaking antioxidant • scavenges peroxyl (ROO•) radicals
Vitamin D	• membrane antioxidant capable of inhibiting iron-dependent liposomal lipid peroxidation
Carbonic anhydrase III	• has two reactive - SH that can be S-thiolated by glutathione forming a mixed dusulfide • a recent hypothesis* is that that skeletal muscle CAIII may be effective in protecting the tissue against oxidative stress

GSH, reduced glutathione; GSSG, glutathione disulfide or oxidized glutathione; GSHPx, gluthathione peroxidase; GST, gluthathione S-transferases; GRD, GSSG reductase; ROS, reactive oxygen species; CAIII, carbonic anhydrase III. $^{a}O2^{\cdot -}$ is also known to dismutate spontaneously (rate constant ~2 x 105 M^{-1} $.s^{-1}$) however, the catalytic rate constant of superoxide dismutase is higher by 4 orders of magnitude (i.e. ~2 x 109 M-1 $.s^{-1}$).

*forwarded by Rahkila (34).

TABLE III : Oxidative stress regulons.

Gene / Gene System	*Target / Features / Remarks*
Bacterial	
soxQ	Superoxide (↓ NADPH/NADP?) induced oxidative stress proteins (OSP; the OSP family is yet to be characterized, however, antioxidant enzymes [SOD, GSHPx, catalase, alkyl hydroperoxide reductase], DNA repair enzymes [endo- and exo-nuclease], DNA damage specific gene products, and chaperonins are constituent members of the family). Strains bearing soxQ1 have increased resistance to menadione, TBOOH and multiple antibiotics. A tentative regulon because the natural inducer of the soxQ group of proteins have not been identified.
soxR & soxS	Superoxide (↓ NADPH/NADP?) induced OSP. This E. coli locus controls the expression of 9 of the ~40 proteins induced by superoxide generating drugs (redox-cycling agents). Strains with soxRC (soxR constitutive) mutations are highly resistant to menadione-, organic peroxide-, and bleomycin- induced oxidative stress. Such mutants contain increased levels of several enzymes (e.g., Mn-SOD, endonuclease IV and G6PD) known to be induced by superoxides. Gene regulation primarily occurs at the transcriptional level. soxRC mutants are also remarkably resistant to a variety of antibiotics not usually associated with oxidative stress. This feature is suggested to be dependent on the capacity of the soxR system to affect membrane permeability by diminishing the expression of the outer membrane porin OmpF. soxS cohabits soxR at the soxR locus. soxR and soxS together controls the soxR regulon (most likely soxS is the direct activator of soxRS regulon genes). soxS gene is inducible by paraquat.
oxyR	H_2O_2 induced "early proteins" (classical antioxidant enzymes). This redox sensitive gene controls the expression of 9 H_2O_2 - inducible proteins including catalase, GRD and an alkyl hydroperoxide reductase. Deletion of the oxyR gene causes hypersensitivity to H_2O_2 and increases level of spontaneous mutagenesis during aerobic growth. Oxidation of oxyR proteins brings about a conformational change in the protein. This change, serving as an oxidative stress signal, selectively activates the defence response i.e., DNA transcription for the expression of "early proteins".
oxoR	H_2O_2 induced "late proteins".
Mammalian	
ARE	The antioxidant responsive element has been identified in the 5'-flanking region of the rat glutathione S-transferase Ya subunit and the NAD(P)H:uinone reductase gene. ARE is transcriptionally activated by phenolic antioxidants and metabolizable planar aromatic compounds (e.g., b-napthoflavone and 3-methylcholantrene). ARE is responsive to H_2O_2 and phenolic antioxidants that undergo redox cycling. c-Jun

is possibly involved in the ARE-regulatory protein complex. H_2O_2 exposure induces c-Jun expression.

ORE — The oxygen responsive elements (ORE1 and ORE2) are localized in the 5'-flanking region of the human glutathione peroxidase gene (hgpx1). They are responsive to oxygen tension in culture and trigger transcriptional changes to express antioxidant enzymes. The effect is presumably mediated by some oxygen responsive regulatory factors (ORFF) that activates or represses transcription through its direct or indirect contact with ORE sequences.

TBOOH, tert-butyl hydroperoxide; SOD, superoxide dismutase; GSHPx, glutathione peroxidase; GRD, glutathione reductase; G6PD, glucose 6-phosphate dehydrogenase; (see 35-41). Reproduced with permission from Sen and Hanninen (49).

ROS including $O_2^{\bullet-}$, $^{\bullet}OH$ and various lipid hydroperoxides. However, one feature of this antioxidant that demands special attention is that in the presence of Fe^{3+} or Cu^{2+} excess (~1 mM) vitamin C may act as a strong pro-oxidant and may actually induce lipid peroxidation and oxidative modification of genomic structures. Under such conditions, vitamin C may reduce Fe^{3+} to Fe^{2+} which in turn facilitates the generation of $^{\bullet}OH$.

Another group of antioxidants that may have important lipid protection properties are the carotenoids, especially β-carotene and lycopene. Superoxide radicals can transfer their unpaired electron to carotenoids which can be harmlessly transformed back to normal carotenoids and heat through rotational and vibrational interaction between the solvent and the carotenoid. Comparable to vitamin C, under certain conditions β-carotene may function as a pro-oxidant. Under partial pressures of oxygen below 150 Torr, β-carotene is a very efficient free radical scavenger. However, at higher oxygen pressures, β-carotene exhibits autocatalytic pro-oxidant properties with concomitant loss of its antioxidant capacity (44).

Epidemiological studies have linked high intake of yellow and green vegitables with reduced risk of cancer. Apart from the antioxidants contained in the vegetables, certain compounds in the vegetables may be capable of inducing antioxidant enzymes. Vegetables like broccoli, cauliflower, mustard, cress, brussels sprouts are known to contain compounds that can induce phase II enzymes of xenobiotic metabolism such as quinone reductase, and glutathione S-transferases. Broccoli contains sulforaphane [CH_3-SO-$(CH_2)_4$-NCS], a compound which together with is sulfide and sulfone analogues induces the anticarcinogenic protective enzymes quinone reductase, and glutathione S-transferases (45).

Antioxidant chain reaction

Various endo- and exo-genous antioxidants are known to act in concert as an *antioxidant chain reaction* (46) (Fig. 3). Scavenging of lipid peroxyl radicals and breaking of the lipid peroxidation chain reaction by α-tocopherol is accompanied by the oxidation of vitamin E compound to a radical configuration - the α-tocopheroxyl radical (vitamin E$^{\bullet}$). When accumulated sufficiently, the vitamin E radical may be stressful for other cells (47). In this way, vitamin E in human low density lipoprotein may actually become a pro-oxidant (48). Therefore, to avoid such toxicity and utilize vitamin E in the most beneficial manner it is imperative that the radical form of vitamin E be recycled to its native antioxidant form. The water soluble antioxidants, ascorbic acid and reduced glutathione are suggested to be involved in regenerating α-tocopherol from its radical byproduct (49). Ubiquinol-10 can spare the consumption of α-tocopherol when both antioxidants are present in the same liposomal membrane (50). A recent study reported that

protection against the loss of vitamin E can be provided either by NADH-cytochrome b_5-dependent enzymatic recycling or by a nonenzymatic pathway involving ascorbate and dihydrolipoic acid (51).

The pK_a of ascorbic acid being 4.25, vitamin C in physiological fluids is predominantly in the anionic form (AH^-). While scavenging ROS (i.e., donating its reducing power to ROS) AH^- gets oxidised to a radical structure, the ascorbyl radical (vitamin C•). Reduced glutathione is suggested to regenerate ascorbate from its oxidized byproduct. It is thus evident that apart from contributing to the enzymatic and non-enzymatic decomposition of ROS, reduced glutathione plays a central role in co-ordinating the activities of crucial exogenous antioxidants.

To obtain best results, this synergism should be considered with particular care especially when designing antioxidant therapy protocols.

INTRACELLULAR CHANGES FOLLOWING OXIDATIVE STRESS

Oxidative stress-induced cytotoxic effects appear to be mediated by a perturbation of intracellular free calcium ($[Ca^{2+}]_i$) and thiol homeostasis (52,53). In a flow-cytometric study we observed that when skeletal muscle derived L6 cells were subjected to oxidant challenge, intracellular Ca^{2+} sharply increased immediately following the challenge - such a response was followed by membrane disintegration as detected by propidium iodide staining of DNA (54). An early response to oxidative stress is the depletion (via oxidation or covalent adduct formation) of cellular soluble and protein-bound thiols (e.g., GSH). Such depletion can (i) decrease plasma membrane Ca^{2+} ATPase activity and contribute to plasma membrane blebbing (altered permeability) and to the impairment of the mitochondrial ability to retain Ca^{2+}, (ii) impair Ca^{2+} sequestration capacity of the endoplasmic reticulum (an organelle with high Ca^{2+} affinity in muscle playing a key role in fine-tuning cytosolic levels of the cation), and (iii) perturb microsomal Ca^{2+} homeostasis. We have recently shown that the rate of influx of $[Ca^{2+}]i$ in L6 myoblasts is drastically upregulated by the activation of GTP binding proteins (55). Although various mechanisms have been proposed to explain the possible factors that contibute to impair $[Ca^{2+}]i$ regulation following oxidant challenge, the detail of the whole process are still not clear.

HELTH AND DISEASE

Today, ROS are suggested to be implicated in the pathogenesis of a wide variety of processes that affect our state of health and longevity. The number of clinical disorders expected to be influenced by ROS is rapidly growing with time (56). It is beyond the scope of the present review to thoroughly address the role of ROS in health and disease. Some selected issues have been briefly outlined below.

Physical exercise

In exercise physiology, the traditional approach to assess physical fitness is primarily based on the capacity of an individual to utilize atmospheric oxygen in a given interval of time i.e., the aerobic capacity. Therefore, trainees aim at increasing their aerobic capacity to the highest possible extent. Supply of more and more oxygen to the tissues fuels aerobic metabolism that produces higher amounts of energy rich phosphates (as compared to anaerobic metabolism) and avoids the formation of lactate during the energy supply process. Physical exercise is associated with a 10-15 fold increase in the rate of oxygen uptake by the body. Oxygen flux in the active peripheral skeletal muscle tissue may increase by over 100-fold with an approximately 30-fold increase in blood flow and about three fold increase in arteriovenous O_2 difference. A large number of recent studies indicate that exercise induced increase in O_2 flux through the body is associated with a remarkable increase in the formation of reactive oxygen species (4). This issue has been of particular concern especially because exercising is not only a recreational activity but is also established to have diverse therapeutic value (57). A vivid understanding of the possible mechanisms that contribute to exercise-induced

oxidative stress, and designing of appropriate measures to circumvent/minimize such stress is fundamental to (i) confirming the merit of physical exercise as a therapeutic tool, (ii) controlling exercise-induced reative oxygen species dependent tissue damage, and (iii) enhancing performance capacity in sports. The area of exercise and oxygen toxicity has been comprehensively addressed in a recent multiauthor volume (4).

Exercise-induced oxidative stress has been shown to be implicated in muscular dystrophy as well as in the early onset of oxidative muscle fatigue. A number of studies have shown that endurance training is capable of boosting the physiological antioxidant defense capacity (49). Previously we have shown that the glutathione homeostasis of skeletal muscle and liver is largely influenced by the state of physical activity (58). Glutathione-dependent antioxidative protection was remarkably enhanced following physical training, and chronic inactivity was associated with a significant decrease of the total glutathione pool of skeletal muscle. We had also observed that a single bout of exercise mobilizes hepatic glutathione pool. During exhaustive exercise, hepatic glutathione appeared to be released into the plasma and was thus available to the peripheral tissues in need such as the skeletal muscles, heart and lung. Skeletal muscles are known to be very poor in their ability to uptake GSH from the circulation. The responsible enzyme, γ-glutamyl transpeptidase activity is very poor in the skeletal muscles (58). In a recently published study with cultured skeletal muscle cells we have shown that skeletal muscle cells also possess a γ-glutamyl transpeptidase-independent mechanism for GSH uptake from the extracellular compartment (32). We have also shown for the first time, that such cells had a considerably ability to synthesize GSH (32). Previous studies have reported that a single bout of exhaustive exercise is associated with a remarkable increase in plasma levels of oxidized glutathione (GSSG). However, the source of such GSSG was unclear. Our study suggested that the skeletal muscle cell has an energy-dependent mechanism for rapid expulsion of GSSG from the intracellular space. We hypothesized that during exercise intracellular GSH is oxidised to GSSG. High concentrations of GSSG have been shown to be cytotoxic, but the skeletal muscle cell has an energy-linked mechanism to expel GSSG. In our paper, we therefore suggested that high levels of plasma GSSG may be thought to be mainly coming from the skeletal muscle tissue, and that plasma GSSG concentration may be used as an indicator of exercise-associated oxidative stress in the active skeletal muscle tissue (32). Studies with antioxidant supplementation have indicated a beneficial effect (59). Our recent study with GSH deficient rats provides concrete evidence to support the contention that endogenous GSH is crucial in the circumvention of exercise-associated oxidative stress and maintenance of endurance performance (60). The remarkable contribution of endogenous GSH in protecting against oxidant insult was also verified in another study where GSH deficient muscle cells were challenged with a chemical oxidant and the kinetics of cell viability change was monitored flow-cytometrically (54). In a recently published study with humans we have been able to reveal that exercise-induced blood glutathione oxidation can be effectively controlled by the supplementation of N-acetylcysteine (46, 61), used clinically as a mucolytic agent and also as an antidote for acetaminophen toxicity (62). Assaying immunoreactivity, we have been able to reveal that during exercise a remarkable amount of Mn-SOD (mitochondrial protein) is released, mostly from the heart, to the serum. We suggested that serum levels of Mn-SOD protein may be effectively used to estimate exercise induced mitochondrial membrane damage (63, 64).

Aging

The free radical theory of aging has gained remarkable momemtum in the recent past (65-68). In everyday life we are exposed to a significant number of ROS, much of which are generated as an integral part of the "living process". It is assumed that the antioxidant defense capacity of the cells is insufficient to

provide complete protection. As a result oxidative molecular damage is a continuous process and there are a number of reports demonstratiing that a variety of molecular products of free radical reactions accumulate with age. In 1984 Cutler presented the theory that the life span of an organism depends on its ability to counteract oxidative threat (69). Protein oxidative damage is claimed to be associated with reduced life expectancy. In a very recent stimulating article in *Science* it was shown that simultaneous overexpression of copper-zinc superoxide dismutase and catalase remarkably decrease protein oxidative damage and increase life-span (65).

Cardiovascular disorders

The area of ROS-dependent cardiovascular damage has been throroughly covered in two recent reviews (70,71). The following section summarizes the primary issues. Oxidative modification of low density lipoprotein in the arterial wall is thought to be fundamental to the development of atherosclerosis (16,72,73). Uptake of oxidized low density lipoprotein by monocytes and macrophages results in the formation of lipid-laden foam cells which contribute to the formation of "fatty streaks" on the arterial wall. Biochemical and epidemiological studies strongly support the beneficial role of antioxidants in the control of atherosclerosis (74-77). The phenolic antioxidant probucol which also has cholesterol-lowering effects appears to have a good therapeutic potential as an antiatherogenic drug (77). Chronic heart failure is another disorder where the participation of ROS has been evident. From the reported evidences it may be suggested that the primary mechanisms by which ROS may contribute to heart failure are (i) enhanced production of prostaglandins associated with accelerated generation of ROS through the arachidonic cascade, (ii) auto-oxidation of circulating catecholamines, and (iii) apparently activated polymorphonuclear leukocytes, Antioxidant therapy has proved to be remarkably beneficial in treating a variety of cardiomyopathies (70). Oxidative stress has been also identified as a potential mediator of ischaemic heart disease. Recently it has been shown that levels of lipid peroxidation byproducts were elevated in patients with both stable and unstable angina (78). The current literature authenticates the contention that a large amount of ROS is produced in the post-ischaemic heart upon reperfusion. The beneficial effects of a number of antioxidants in controlling ischaemia-reperfusion injury and myocardial stunning is well established (70). During cardiac surgery, the heart suffers from ischacmia. Revascularization of the heart may thus cause oxidative stress. The protective role of antioxidants in preserving the heart during transplantation has been evident (70).

Central nervous disorders

The following characteristic features of the central nervous system (CNS) make it highly susceptible to oxidative stress: (i) high oxygen consumption, (ii) low antioxidant defense status, (iii) high levels of free iron, and (iv) high concentrations of oxidizable substrates such as polyunsaturated fatty acids (e.g., docosohexanoic acid) and catecholamines (79). Most of the ROS dependent central nervous disorders have been observed to be actually triggered by the presence of free iron (Fe^{2+}). Significant contributors of Fe^{2+} in the central nervous system interstitium are hemoglobin released from extravasated red cells, ferritin and transferrin (80). Free radical generation during brief periods of cerebral ischaemia has been suggested to induce delayed neuronal death (81). In addition to the neurons and glia, ROS induced damage in the vascular elements is of major concern, Such damage may account for oedema formation, loss of autoregulation, hypoperfusion, vasospasm and altered microvascular permeability. Antioxidant therapy, especially that using metal chelators, has proved to be remarkably beneficial (80). The proto-oncogene Bcl-2 inhibits neural death by decreasing ROS generation (82). Oxidative stress has been identified as a possible etiological factor for Pakinson's disease (PD) and Alzheimer's disease (83). In PD high content of iron has been observed in the substantia nigra, especially in the zona compacta.

Diabetes Mellitus

A large number of reports have shown that diabetes is associated with higher oxidative stress (84). ROS induced damage to the insulin-producing pancreatic β-cells induces type I diabetes. Antioxidant therapy involving the use of enzymes and metal chelators has been shown to protect against such damage. Diabetic patients have been found to have higher levels of oxidative stress indices. It has been shown that under physiological conditions glucose may undergo auto-oxidation and contribute to ROS formation (85). ROS are also capable of facilitating glycation (nonenzymatic glycosylation) reactions that are now believed to be responsible for most of the diabetic complications. Glycation reactions are responsible for a remarkable inactivation and fragmentation of copper, zinc-superoxide dismutase thereby causing derpression in the physiological antioxidant defense capacity. Increased glycated copper, zinc-superoxide dismutase has been found to be associated with diabetic retinopathy and cataract (28).

Cancer

ROS induced damage to genomic structures is likely to contribute to the initiation and promotion of carcinogenesis. Cancers caused by radiation or chemical carcinogens have been reasoned to have a free radical dependent etiology (86). One possible mechanism of ROS dependent carcinogenesis is as follows : ROS → activated protein kinases and DNA breakage → induction of immediate early genes c-fos and c-myc → stimulated cell proliferation tumour promotion. Thiol antioxidant e.g., N-acetylcysteine (NAC) has been found to be effective in the chemoprevention of mutation and cancer (87). A number of antioxidants have been shown to inhibit tumour promotion both in vivo and in vitro (88).

Rheumatoid arthritis

Physical movement induced hypoxia-reperfusion in the synovial joint triggers the generation of ROS. During hypoxia, xanthine dehydrogenase located in the endothelium of the synovium is transformed to the oxidase form which is a well established source of superoxides ($O_2^{\bullet -}$). Local bleeding followed by release of iron from haemoglobin magnifies superoxide toxicity. It has been shown that in the joint, ROS (i) oxidatively modifies IgG to produce the "rheumatoid factor", (ii) induces cartilage destruction via the oxidation of α_1-antitrypsin which leads to the increase of both elastase and tumor necrosis factor activity, (iii) oxidises hyaluronan and thereby alters immune function, (iv) produces lipid peroxidation, byproducts of which are toxic and may alter T cell-macrophage interactions, (v) oxidatively modifies lipoproteins thus producing monocyte chemotactic peptides, (vi) stimulates osteoclasts and causes bone erosion and (vi) damages fine pain nerves causing 'typus robustus' synovitis (10).

AIDS

Minor concentrations of ROS induce the expression and replication of HIV-1 in human T cells. The effect was mediated by NF-*k*B transcription factor that was activated by H_2O_2. The thiol antioxidant NAC effectively prevented the ROS dependent activation of NF-*k*B (89). It has been recently reviewed that glutathione status and ROS production modulate HIV (90). HIV infected patients have decreased intracellular GSH levels in their circulating T cells. NAC was found to be effective in anti-HIV therapy (90).

In view of the substantial evidence from laboratory and human population studies supporting the critical role of antioxidants in the maintenance of health and prevention of disease, Packer (91) has called for (i) intensive campaigns to educate the public (especially low income- and ethnic- groups) about the role of antioxidants in nutrition, (ii) recommendation of antioxidant supplementation to high oxidant load groups such as smokers, and (iii) subsidized supply of antioxidant supplementation to low income- and ethnic- groups; this approach, he remarked, would be of considerable economic value for the society as preserving wellness and preventing disease is far less costly than treating diseases once they have developed.

ACKNOWLEDGEMENTS

The author thanks Ms. Marja-Leena Tervo and Pirjo Jaatinen for secretarial help. Part of the text and figures have been obtained from the Ph.D dissertation of the author. This work was supported by research grants from the Finnish Ministry of Education (Dnro 236/722/92 and 28/722/93) and Juho Vainio Foundation, Helsinki to CKS.

Note: A relevant review titled Oxidants and Antioxidants in Exercise by this author will soon appear in the *Journal of Applied Physiology* published by the American Physiological Society.

REFERENCES

1. Bert P.La Pression barometrique (1878). [Eng. trans.: M. Hitchcock and A.Hitchcock]. Columbus, Ohio, College Book Company, 1943.
2. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 1969; 244: 6049-6055.
3. Packer L. Oxygen is a dangerous friend In: Canedo LE, Todd LE, Packer L, Jaz J, eds, Cell function and disease. New York, Plenum Press, 1988: 199-209.
4. Sen CK, Packer L, Hanninen O, eds, Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publisher B.V., 1994: pp 536.
5. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen, *Biochem J* 1973; 134: 707-716.
6. Dionsi D, Galeotti T, Terrehove T, Azzi A. Superoxide radicals and hydrogen peroxide formation in mitochondria from normal and neoplastic. *Biochem Biophys Acta* 1975; 403: 292-303.
7. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 1985; 237: 408-414.
8. Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* 1980; 191: 421-427.
9. Hellsten Y. The role of xanthine oxidase in exercise, In: Sen CK, Packer L, Hanninen O, eds. Exercise and oxygen toxicity. Amsterdam, Elesevier Science Publishers B.V., 1994: 221-234.
10. Edmonds SE, Blake DR. Hypoxia, oxidative stress and exercise in rheumatoid arthritis. In: Sen CK, Packer L, Hanninen O, eds. Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publishers B.V., 1994: 389-422.
11. Lilius EM, Marnila P. Photon emission of phagocytes in relation to stress and disease. *Experientia* 1992; 48: 1082-1091.
12. Otto DME, Lindstrom-Seppa P, Sen CK. Cytochrome P_{450}-dependent enzymes and oxidant-mediated responses in rainbow trout exposed to contaminated sediments. *Ecotox Environ Safe* 1994; 27: 265-280.
13. Karuzina II, Archakov AI. The oxidative inactivation of cytochrome P_{450} in monooxygenase reactions. *Free Rad Biol Med* 1994; 16: 73-97.
14. Beckman JS, Crow JP. Pathological implications of nitric oxide, superoxide and peroxynitrite formation. *Biochem Soc Trans* 1993; 21: 330-334.
15. Hogg N. Darley-Usmar VM, Wilson MT, Moncada S. The oxidation of α-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide. *FEBS Lett* 1993; 326: 199-203.
16. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362: 801-809.
17. Jenkins RR, Halliwell B. Metal binding agents: possible role in exercise. In: Sen CK, Packer L, Hanninen O, eds. Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publishers B.V., 1994: 59-76.
18. Buettner GR. Ascorbate autoxidation in the presence of iron and copper chelates. *Free Radical Res Commun* 1986; 1: 349-353.
19. Sies H. Biochemistry of the peroxisome in the liver cell. *Angew Chem Int Ed Engl* 1974; 13: 706-718.
20. Boveris A, Oshino N, Chance B. The cellular production of hydrogen peroxide. *Biochem J* 1972; 128: 617-630.
21. Kukreja RC, Kontos HA, Hess ML, Ellis EF. PGH synthase and lipoxygenase generate superoxide in the presence of NADH of NADPH. *Circ Res* 1986; 59: 612-619.
22. Cohen G. Oxy-radical toxicity in catecholamine neurones. *Neurotoxicology* 1984; 5:77-82.
23. Arduini A, Eddy L, Hochstein P. Detection of ferryl myoglobin in the isolated ischemic rat heart. *Free Rad Biol Med* 1990; 9: 511-513.
24. Duthie GG, Arthur JR. Cigarette smoking as an inducer of oxidative stress. In: Sen CK, Packer L, Hannine O, eds. Exercise and oxygen toxicity.

Amsterdam, Elsevier Science Publishers B.V., 1994: 297-318.

25. Videla LA, Valenzuela A. Alcohol ingestion, liver glutathione and lipid peroxidation: metabolic interrelations and pathological implication. *Life Sci* 1982; 31: 2395-2407.

26. Nadiger HA, Marcus SR, Chandrakala MV. Lipid peroxidation and ethanol toxicity in rat brain-effect of vitamin E deficiency and supplementation. *Med Sci Res* 1988; 16: 1273-1274.

27. Nadiger HA, Marcus SR, Chandrakala MV, Kulkarni DD. Malonyl dialdehyde levels in different organs of rats subjected to acute alcohol toxicity. *Indian J Clin Biochem* 1986; 1: 133-136.

28. Ohno H, Suzuki K, Fujii, J, Yamashita H, Kizaki T, Oh-ishi S, Taniguchi N. Superoxide dismutases in exercise and disease. In: Sen CK, Packer L, Hanninen O, eds. Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publishers B.V., 1994: 127-162.

29. Meister A. Selective modification of glutathione metabolism. *Science* 1983; 220: 472-477.

30. Meister A. Metabolism and function of glutathione. In: Dolphin D, Poulson R, Avramovic O, eds, Glutathione. Chemical, biochemical and medical aspects. Vol. 3, Part A. New York. John Wiley and Sons, 1989: 367-389.

31. Thomas JP, Maiorino M, Ursini F, Girotti AW. Protective action of phospholipid glutathione peroxidase against membrane-damaging lipid peroxidation. *J Biol Chem* 1990; 265: 454-461.

32. Sen CK, Rahkila P, Hanninen O. Glutathione metabolism in skeletal muscle derived cells of the L6 line. *Acta Physiol Scand* 1993; 148: 21-26.

33. Ishikawa T, Zimmer M, Sies H. Energy-linked cardiac transport system for glutathione disulfide. *FEBS Lett* 1986; 200: 128-132.

34. Rahkila P. Carbonic anhydrase III in skeletal muscle. In: Skeletetal muscle research - Cellular Physiology and Biochemistry. Sen CK, Kankaanpaa MJ (eds), Kuopio, Kuopio University Press, 1993:43.

35. Storz G, Christman MF, Sies H, Ames BN. Spontaneous mutagenesis and oxidative damage to DNA in Salmonella typhimurium. *Proc Natl Acad Sci USA* 1987; 84: 8917-8921.

36. Demple B, Amabile-Cuevas CF. Redox redux: the control of oxidative stress responses. *Cell* 1991; 67: 837-839.

37. Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. *J Biol Chem* 1991; 266:11632-11639.

38. Keyes SM, Emslie EA. Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature* 1992; 359: 644-647.

39. Nguyen T, Pickett CB. Regulation of rat glutathione S-transferase Ya subunit gene expression. *J Biol Chem* 1992; 267: 13536-13539.

40. Abate C, Patel L, Rauscher III FJ, Curran T. Redox regulation of Fos and Jun DNA--binding activity *in vitro*. *Science* 1990; 249: 1157-1161.

41. Cowan DB, Weisel RD, Williams WG, Mickle DAG. Identification of oxygen responsive elements in the 5' -flanking region of the human glutathione peroxidase gene. *J Biol Chem* 1993; 268: 26904-26910.

42. Packer L, Landvik S. Vitamin E in biological systems. In: Emerit I, Packer L, Auclair C, eds. Antioxidants in therapy and preventive medicine. Vol. 264. New York, Plenum Press, 1990: 93-103.

43. Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc Natl Acad Sci USA* 1989; 86: 6377-6391.

44. Burton GW, Ingold KU. b-Carotene is an unusual type of lipid antioxidant. *Science Washington DC* 1984; 224: 569-573.

45. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci USA* 1992; 89: 2399-2403.

46. Sen CK, Rankinen TT, Vaisanen SV, Rauramaa R. Oxidative stress after human exercise: effect of N-acetylcysteine supplementation. *J Appl Physiol* 1994; 76: 2570-2577.

47. Kuzuya M, Naito M, Funaki C, Hayashi T, Asai K, Kuzuya F. Protective role of intracellular glutathione against oxidized low density lipoprotein in cultured endothelial cells. *Biochem Biophys Res Commun* 1989; 163: 1466-1472.

48. Bowry VW, Ingold KU, Stocker R. Vitamin E in human low-density lipoprotein. When and how this becomes a pro-oxidant. *Biochem J* 1992; 288: 341-344.

49. Sen CK, Hanninen O. Physiological antioxidants. In: Sen CK, Packer L, Hanninen O, eds. Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publishers B.V., 1994: 89-126.

50. Frei B, Kim MC, Ames BN. Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc Natl Acad Sci USA* 1990; 87: 4879-4883.

51. Constantinescu A, Han D, Packer L. Vitamin E recycling in human erythrocyte membranes. *J Biol Chem* 1993; 268: 10906-10913.

52. Pascoe GA, Redd DJ. Cell calcium, vitamin E, and the thiol redox system in sytotoxicity. *Free Radical Bio Med* 1989; 6: 209-224.

53. Reed DJ. Status of calcium and thiols hepatocellular

injury by oxidative stress. *Semin Liver Dis* 1990; 10: 285-292.

54. Sen CK, Agrawal YP, Hanninen O. Changes in intracellular Ca^{2+} and membrane disintegration following exposure of myogenic cells to oxidative stress. XXXII Congress of the International Union of Physiological Sciences, Glasgow, Abstract book 284.55/P (Wednesday) 1993: 214.

55. Orolv SN, Sen CK, Kolosova IA, Bernhardt J, Hanninen O, Buhler FR. Evidence for the invlovement of G proteins in the modulation of ^{22}Na and ^{45}Ca cluxes in myogenic L6 and aortic smooth muscle cells. *Biochem Biophys Res Commun* 1993; 191: 802-810.

56. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford, Clarendon Press, 1989.

57. Astrand P-O, Sen CK. The significance of exercise science in the maintenance of general health and enhancement of performance. *Indian J Physiol All Sci* 1994; 48:56-68.

58. Sen CK, Marin E, Eretzschmar M, Hanninen O. Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *J Appl Physiol* 1992; 73: 1265-1272

59. Goldfarb A, Sen CK. Antioxidant supplementation and the control of oxygen toxicity during exercise. In: Sen CK, Packer L, Hanninen O, eds. Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publishers B.V., 1994: 163-190.

60. Sen CK, Atalay M, Hanninen O. Exercise induced oxidative stress: glutathione supplementation and deficiency. *J. Appl. Physiol* 1994; 77:2177-2187.

61. Sen CK, Rankiren T, Vaisanen S, Rauramaa R. N-acetylcysteine atenuates exercise associated blood glutathione oxidation. *Med Sci Sport Exer* 1994; 26: in press.

62. Smilkstein MJ, Knapp GL, Kulig KW, Rumack BH. Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. *N Engl J Med* 1988; 319: 1557-1562.

63. Sen CK, Ookawara T, Ohno H, Taniguchi N, Suzuki K, Hanninen O, Rauramaa R. Mitochondrial superoxide dismutase (SOD) concentration following physical training and exhaustive exercise. *Med Sci Sport Exer* 1993; 25: S30.

64. Sen CK, Ookawara T, Suzuki K, Taniguchi N, Hanninen O, Ohno H. Immunoreactivity and activity of mitochondrial superoxide dismutase following training and exercise. *Pathophysiology* 1994; 1: 165-168.

65. Orr WC, Sohal RS. Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. *Science* 1994; 263 1128-1130.

66. Sohal RS, Orr WC. Relationship between antioxidants, pro-oxidants, and the aging process. *Ann NY Acad Sci* 1992; 663: 74-84.

67. Alessio H. Lipid peroxidation processes in healthy and diseased models. In: Sen CK, Packer L, Hanninen O, eds Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publishers B.V., 1994: 269-296.

68. Ames B, Shigenaga MK. Oxidants are a major contributor to aging. *Ann NY Acad Sci* 1992; 663: 85-96.

69. Cutler RG. Antioxidant and longevity. In: Free radicals in molecular biology, aging and disease. Armstrong D, Sohal RS, Cutler RG, Slater TF (eds.), New York, Raven Press, 1984: 235-266.

70. Das DK, Maulik N. Protection against free radical injury in the heart and cardiac performance. In: Sen CK, Packer L, Hanninen O, eds, Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publishers B.V., 1994: 359-388.

71. Ji LL. Exercise-induced oxidative stress in the heart. In: Sen CK, Packer L, Hanninen O, eds, Exercise and oxygen toxicity. Amsterdam, Elsevier Science Publishers B.V., 1994: 249-268.

72. Esterbauer H, Gebicki J, Puhl H, Jurgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Bio Med* 1992; 13: 341-390.

73. Steinbrecher UP, Zhang H, Lougheed M. Role of oxidatively modified LDL in atherosclerosis. *Free Radical Bio Med* 1990; 9: 155-168.

74. Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willet WC. Vitamin E consumption and the risk of coronary disease in women. *N Engl J Med* 1993: 328: 1444-1449.

75. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC. Vitamin E consumption and the risk coronary heart disease in men. *N Engl J Med* 1993; 328: 1450-1456.

76. Green ESR, Cooper CE, Davies MJ, Rice-Evans C. Antioxidant drugs and the inhibition of low-density lipoprotein oxidation. *Biochem Soc Trans* 1993; 21: 362-366.

77. Kuzuya M, Kuzuya F. Probucol as an antioxidant and antiatherogenic drug. *Free Radical Bio Med* 1993; 14: 67-77.

78. Jayakumari N, Ambikakumari V, Balakrishnan KG, Iyer KS. Antioxidant status in relation to free radical prduction during stable and unstable anginal syndromes. *Atherosclerosis* 1992; 94: 183-190.

79. Jesberger JA, Richardson JS. Oxygen free radicals and brain dysfunction. *Int J Neurosci* 1991; 57: 1-17.

80. Hall ED. Cerebral ischaemia, free radicals and antioxidant protection. *Biochem Soc Trans* 1993; 21: 334-339.

81. Kitagawa K, Matsumoto M, Oda T, Niinobe M, Hata R, Handa N, Fukunaga R, Isaka Y, Kimura K, Maeda H, Mikoshiba K, Kamada T. Free radical generation during brief period of cerebral ischaemia may trigger delayed neuronal death. *Neuroscience* 1990; 35: 551-558.

82. Kane DJ, Sarafian TA, Anton R, Hahn H, Gralla EB, Valentine JS, Ord T, Bredesen JE. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science* 1993; 262: 1274-1277.

83. Ceballos I, Javoy-Agid F, Delacourte A, Defossez A, Nicole A, Sinet PM. Parkinson's disease and Alzheimer's disease: neurodegenerative disorders due to brain antioxidant system deficiency? In: Emerit I, Packer L, Auclair C, eds. Antioxidants in therapy and preventive medicine. Vol. 264. New York, Plenum Press, 1990: 493-498.

84. Oberley LW. Free radicals and diabetes. *Free Radical Biol Med* 1988; 5: 113-124

85. Hunt JV, Wolff SP. The role of histidine residues in the non-enzymatic covalent attachment of glucose and ascorbic and ascorbic acid to protein. *Free Radical Res Commun* 1991; 14: 279-287.

86. Swartz HM. Electron spin resonance studies of carcinogenesis. In: Advances in cancer research. Klein G, Weinhouse S (eds.), New York, Academic Press, 1972: 227-252.

87. De Flora S, Izzotti A, D Agostini F, Cesarone CF. Antioxidant activity and other mechanisms of thiols involved in chemoprevention of mutation and cancer. *AM J Med* 1991; 91: 3C-122S-130S.

88. Kozumbo WJ, Cerutti PA. Antioxidants as anti-tumor promotors. In: Antimutagenesis and anticarcinogenesis mechinsms. Shankel DM, Hartmen PE, Kada T, Hollaender A (eds.), New York Plenum Press. 1986: 491-506.

89. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappaB transcription factor and HIV-1. *EMBO J* 1991; 10: 2247-2258.

90. Roederer M, Ela SW, Staal FJT, Herzenberg LA, Herzenberg LA. N-acetylcysteine: a new approach to anti-HIV therapy. *AIDS Res Hum Retrov* 1992; 8: 209-217.

91. Packer L. Health effects of nutritional antioxidants (letter). *Free Radical Bio Med* 1993; 15: 685-686.