Biochemical aspects of cellular antioxidant systems

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Aerobic life is characterized by a steady generation of reactive oxygen species balanced by a similar rate of their consumption by antioxidants. To maintain homeostasis, there is a requirement for the continuous regeneration of antioxidant capacity, and if this is not met, oxidative stress occurs, resulting in pathophysiological events. Cellular protection against oxidative stress is organized at multiple levels. Defense strategies include prevention, interception, replacement, and repair. These mechanisms are coupled to the intermediary metabolism for a continuous supply of energy, reducing equivalents, and precursors, and depend on the dietary supply of metabolic fuels and essential molecules to allow an optimal cellular functioning.

Key words: *antioxidant defense mechanisms, oxidative stress, reactive oxygen species.*

INTRODUCTION

The occurrence of reactive O_2 species, known as prooxidants, is an attribute of normal aerobic life. These include free radicals, peroxides, electronically excited states, and oxidant molecules such as hypochlorous acid, in addition to xenobiotics-derived free radicals produced during their cellular biotransformation (Table I). The interaction of these species with essential biomolecules can lead to substantial changes in their structure and function, when the steady-state production of prooxidants is not adequately balanced by a similar rate of their consumption by cellular antioxidants. This constitutes the molecular basis of the oxidative stress phenomenon (Sies, 1986), that has been considered as one of the major mechanisms involved in the initiation and progression of tissue injury in a number of human diseases (Halliwell *et al*, 1992). Thus, detoxication of reactive species in the cell is a crucial step to maintain homeostasis, through the operation of enzymatic and nonenzymatic systems of prevention and interception, as well as ancillary reactions and replacement and repair mechanisms (Sies, 1993).

ANTIOXIDANT DEFENSE

Several enzymes are available for preventing the damaging effects of reactive O_2 species in biological systems, namely, superoxide dismutases (SOD) and hydroperoxidases such as catalase, glutathione peroxidases, and other hemoprotein peroxidases (Ishikawa *et al*, 1986). Superoxide radicals ($O_2^{\cdot-}$) (Table I) are dismutated into H_2O_2 and O_2 by means of SOD, present in all cell types of mammalian organs, both in the cytosolic compartment (CuZn-SOD) and in the mitochondrial matrix (Mn-SOD), at relatively similar levels, except for liver having a higher activity and adipose tissue a lower activity (Ishikawa *et al*, 1986). Considering the second order rate constant of 1.6 to 2.6 x 10^{-9} M^{-1} x sec^{-1} for $O_2^{\cdot-}$ dismutation by SOD and its high content in the liver, the

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TABLE I

Reactive species related to cellular oxidative stress*

Reactive species	Remarks
Superoxide radical ($O_2^{\bullet-}$)	One-electron reduction state of O_2 formed: a) enzymatically (microsomal, mitochondrial, and peroxisomal redox processes; NADPH oxidase in phagocytes; flavin-dependent oxidations); b) by autoxidation of flavins, hemoglobin, thiols, catecholamines, iron chelates); c) by redox cycling of xenobiotics (adriamycin, paraquat, alloxan, nifurtimox); d) by physical factors (ultraviolet light, X-rays).
Hydrogen peroxide (H_2O_2)	Two-electron reduction state of O_2 formed by dismutation of $O_2^{\bullet-}$ or from O_2 reduction.
Hydroxyl radical ($HO^{\bullet}$)	Three-electron reduction state of O_2 formed by Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet}$) or the iron-catalyzed Haber-Weiss reaction.
Nitric oxide ($NO^{\bullet}$)	Synthesized from arginine in a reaction catalyzed by NO synthase, involving two separate mono-oxygenation steps, which require O_2, NADPH, and tetrahydrobiopterin.
Peroxynitrite ($ONOO^-$)	Produced non-enzymatically by the interaction of $O_2^{\bullet-}$ with $NO^{\bullet}$.
Peroxyl radical ($ROO^{\bullet}$)	Radical species formed by O_2 incorporation into a polyunsaturated fatty acid radical ($R^{\bullet}$) during lipid peroxidation.
Hydroperoxide (ROOH)	Organic compound derived from polyunsaturated fatty acids, cholesterol, thymine.
Alkoxyl radical ($RO^{\bullet}$)	Oxygen-centered organic radical (e.g., polyunsaturated fatty acid-derived radical formed in lipid peroxidation).
Hypochlorous acid (HClO)	Oxidant species formed by phagocytic myeloperoxidase from H_2O_2 and Cl^-.
Xenobiotic-derived radical ($X^{\bullet}$)	Produced in the biotransformation of several chemicals by the microsomal cytochrome P-450 system ($CCl_3^{\bullet}$ from carbon tetrachloride, CH_3-C•H-OH from ethanol).
Singlet oxygen ($^1O_2$)(O_2^*)	First excited state of O_2, 22 Kcal/mol above ground state, with either red (dimol) or infrared (monomol) photoemission. Chemical excitation of O_2 can proceed either by peroxyl radicals interaction or via oxene transfer using heme-Fe^{3+}.
Triplet carbonyls (R·R··CO*)	Excited carbonyl compounds with blue-green photoemission, formed via dioxetane rupture or $ROO^{\bullet}$ disproportionation.

* Adapted from Sies (1986) and Moncada and Higgs (1993).

concentration of $O_2^{\bullet-}$ is kept at 10^{-11} to 10^{-12} M in this organ (Chance *et al*, 1979). H_2O_2 (Table I) is either dismutated into H_2O and O_2 within the peroxisomes by the heme enzyme catalase or it is reduced to H_2O in a glutathione (GSH)-dependent reaction catalyzed by glutathione peroxidases, processes that contribute to maintain its concentration at 10^{-7} to 10^{-9} M (Chance *et al*, 1979). Thus, the generation of secondary radicals (e.g., $HO^{\bullet}$) from $O_2^{\bullet-}$ and H_2O_2 (Table I) is usually kept at a very low rate under normal conditions. It must be pointed out that the operation of SOD and catalase has the advantage, over that of glutathione peroxidases, that essential cofactors are not required for the removal of $O_2^{\bullet-}$ and H_2O_2 in the cell. The subcellular distribution of the Se-dependent glutathione peroxidase in the liver is complementary to that of catalase, being located both in the cytosolic and mitochondrial compartments of the hepatocyte (Chance *et al*, 1979). The Se-dependent glutathione peroxidase also reduces organic hydroperoxides (ROOH) (Table I) to the respective alcohol derivatives, together with the Se-independent glutathione peroxidase identified as the glutathione-S-transferases B and AA, provided that the peroxidized phospholipid is hydrolysed by phospholipase A_2 (Ishikawa *et al*, 1986). In this respect, a Se-dependent phospholipid hydroperoxide glutathione peroxidase has also been described (Ursini *et al*, 1985).

Enzymes catalyzing the removal of $HO^{\bullet}$, $NO^{\bullet}$, and $X^{\bullet}$ or excited states such as singlet oxygen ($^1O_2$) (Table I) have not been found in biological systems. Thus, these as well as other reactive species must be detoxified by reactions using intercepting (free-radical scavengers) and quenching molecules, such as the hydrophilic antioxidants GSH and ascorbate (vitamin C) and the lipophilic antioxidants α-tocopherol (vitamin E) and carotenoids (ß-carotene and lycopene) (Sies, 1986; Ishikawa *et al*, 1986). GSH is linked to the cellular defense mechanisms in multiple ways (Kosower and Kosower, 1979), including the direct nonenzymatic reaction with free radicals and the enzymatic reduc-

tion of peroxides (glutathione peroxidases), processes that lead to its oxidation into glutathione disulfide (GSSG). However, the interaction of GSH with $O_2^{\bullet-}$ has also been shown to generate glutathione sulfonate, probably through O_2 addition to thiyl radicals ($GS^{\bullet}$) yielding a peroxysulphenyl radical intermediate (Wefers and Sies, 1983). In addition, GSH can be used for conjugation with a variety of xenobiotics or their metabolites through the action of the glutathione-S-transferases, which implies a net loss of the tripeptide from the cell (Vos and Van Bladeren, 1990). The highest concentration of GSH is observed in the liver, with hepatocytes in the periportal region containing about twice as much GSH as those in the centrilobular region (Ishikawa *et al*, 1986). Vitamin C is capable of reacting with $O_2^{\bullet-}$, reactive electrophiles and alkylating agents, and, together with GSH, have been proposed to reduce vitamin E radicals, resulting in the regeneration of the active antioxidant form of α-tocopherol (Ishikawa *et al*, 1986; McCay, 1985). Vitamin E is a lipophilic antioxidant able to break the chain reactions of lipid peroxidation by reaction with the lipid peroxyl radicals ($ROO^{\bullet}$) (Table I) produced in the process, as these radicals react with other polyunsaturated fatty acids with rate constants of about 50 $M^{-1} \times sec^{-1}$, but with vitamin E 10^4 to 10^5 times faster (McCay, 1985). The vitamin C content of human organs is about ten times higher than that of vitamin E, with high levels of ascorbate being observed in eye lens, brain and liver, while those of α-tocopherol are more uniformly distributed (Ishikawa *et al*, 1986). $NO^{\bullet}$ (Table I) is oxidized to higher oxides of nitrogen and, as a result, nitrosates molecules containing sulfhydryl groups (GSH, cysteine) and albumin (Moncada and Higgs, 1993). Furthermore, $NO^{\bullet}$ interacts with heme-containing proteins (hemoglobin), leading to the production of nitrate (Moncada and Higgs, 1993).

Singlet oxygen quenching in biological systems can be accomplished by carotenoids, tocopherols and thiols (Di Mascio *et al*, 1991). Carotenoids, the most efficient quenchers of $^1O_2$ (Table I), include ß-carotene and lycopene, the biologically occurring open-chain isomer of ß-carotene, which exhibit quenching constants of 1.4 and 3.1 x 10^{10} $M^{-1} \times sec^{-1}$, respectively. The quenching ability of tocopherols is about 100-fold less than that of carotenoids, while thiols such as cysteine and GSH are the least efficient (10^3 to 10^4 times lower than carotenoids) (Di Mascio *et al*, 1991).

Human extracellular fluids such as plasma contain little of the enzymatic defense mechanisms present in cells. However, plasma has a powerful antioxidant capacity (Lissi *et al*, 1986), that has been ascribed to the presence of micromolar concentrations of the vitamins E and C, carotenoids, GSH and urate, acting as free-radical scavengers and/or quenchers of excited states (Ishikawa *et al*, 1986; Halliwell and Gutteridge, 1986). Furthermore, prevention of metal-ion-dependent free-radical production in plasma is of a major importance. This is elicited by the oxidation of Fe(II) to Fe(III) by the ferroxidase activity of ceruloplasmin, coupled to Fe(III) binding to transferrin, urate or albumin, the latter two components being also able to bind copper (Halliwell and Gutteridge, 1986). In addition, albumin is an effective scavenger of hypochlorous acid (HClO) (Table I), a potent oxidant produced by activated phagocytes (Wasil *et al*, 1987).

ANCILLARY REACTIONS AND REQUIREMENTS

Some of the defense mechanisms described above need ancillary processes for full operation (Sies, 1986; 1993). These include (a) conjugation of reactive xenobiotics or their metabolites capable of inducing oxidative stress [UDP-glucuronyl transferase, sulfotransferase and glutathione-S-tranferases, plus conjugate export systems from cells], (b) regenerative processes [reduction of GSSG into GSH by the NADPH-dependent glutathione reductase; reduction of the semidehydroascorbate radical back to ascorbate by the semidehydroascorbate reductase; supply of NADPH by the pentose phosphate pathway, isocitrate dehydrogenase or malate enzyme; restoration of the thiol/disulfide status of proteins altered by oxidative stress through thioltransferase], and (c) preventing processes [two-electron reduction of quinones by NADPH-quinone oxidoreductase without

production of reactive species; chelation of iron (ferritin) or copper (metallothioneins) ions, promoters of free radical generation and lipid peroxidation] (Sies, 1986; Ishikawa *et al*, 1986; Halliwell and Gutteridge, 1989).

In addition to the ancillary reactions described above, the adequate functioning of the cellular defense mechanisms requires the maintenance of an optimal steady-state level of components. Thus, the content of enzymes and proteins involved in this specialized metabolic activity is maintained by continuous synthesis, which is genetically controlled and susceptible to adaptive changes by oxidative stress (Burdon, 1995). Cellular GSH levels are maintained by reduction of GSSG and by synthesis de novo, catalyzed by the γ-glutamylcysteine synthetase and GSH synthetase system, with supply of the limiting substrate cysteine by the cystathione pathway (Kaplowitz *et al*, 1985). In this respect, enhancement of the sinusoidal ectoactivity of γ-glutamyl transferase of the liver after GSH depletion by oxidative stress has been related to the recovery of hepatic GSH, by supplying the precursors for intracellular synthesis (Speisky and Israel, 1990; Carrión *et al*, 1993). Essential components such as vitamins, minerals (copper, zinc, selenium, manganese, iron), amino acids and unsaturated fatty acids

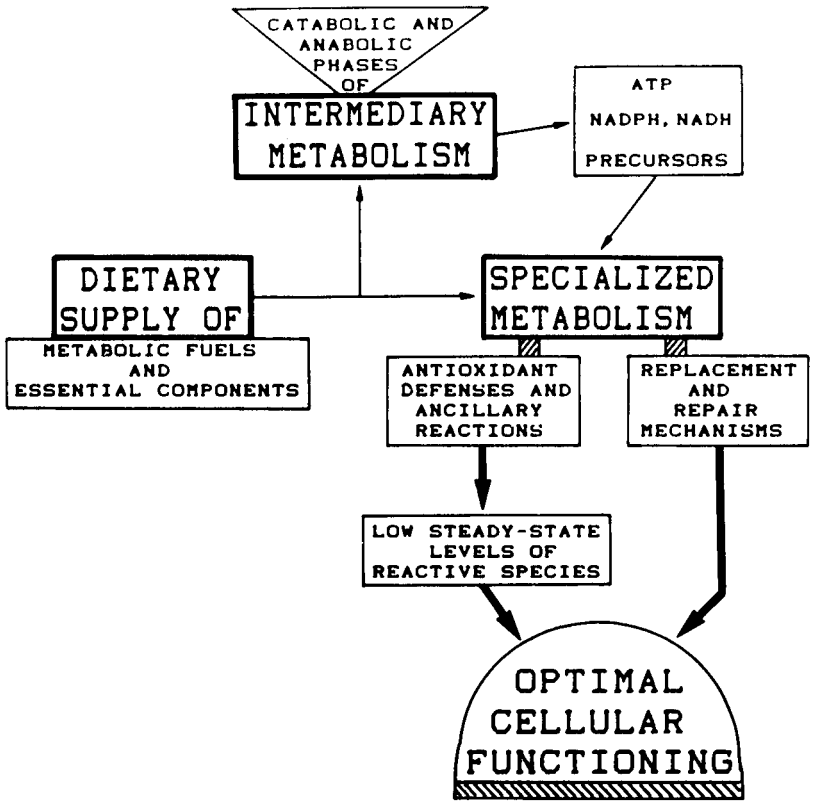

Fig 1. Integration of defense systems, intermediary metabolism, and dietary supply for the adequate performance of living aerobic cells. Cellular aerobic activity generates different reactive species (Table I), which are primarily kept at low steady-state levels by enzymatic processes (superoxide dismutase, catalase, and glutathione peroxidases) as well as by free radical scavengers (glutathione, vitamins E and C) and excited state quenchers (carotenoids). Cellular antioxidant activity requires ancillary reactions for full operation, together with the repair of oxidized biomolecules and replacement by synthesis *de novo* of those irreversibly altered. This specialized type of metabolism necessarily needs to be coupled to the catabolic and anabolic phases of the intermediary metabolism, for an adequate supply of chemical energy (ATP), reducing equivalents (NADPH, NADH), and precursors. In addition, the dietary supply of essential biocomponents (vitamins, trace elements, amino acids, and polyunsaturated fatty acids) and metabolic fuels is crucial, in order to achieve an optimal functioning of both the antioxidant defense mechanisms and the intermediary metabolism.

(linoleic and linolenic acids), must be supplied by the diet, together with metabolic fuels needed for ATP generation (Fig 1).

REPAIR AND REPLACEMENT SYSTEMS

Despite the plethora of defense systems and ancillary reactions in the cell, some damage to biomolecules by active species still occurs under normal or oxidative stress conditions. Thus, cells have evolved specific systems to repair some altered molecules, replacing those that cannot be repaired (Fig 1). Thus, peroxidized polyunsaturated fatty acids are removed from membrane phospholipids by the action of the Ca^{2+}-dependent phospholipase A_2, in order to be reduced in the cytosol by glutathione peroxidase, and the resulting lysophospholipid is then reacylated with a long chain fatty acyl-CoA catalyzed by acyltransferases (Van Kuijk *et al,* 1987). The oxidation of free amino acids or amino acid residues in proteins is a frequent event; in the case of histidine, tryptophan, lysine and tyrosine, the oxidative alterations are irreversible and replacement is necessary. However, oxidation of cysteine and methionine residues in proteins by oxidative stress is a reversible process, as formation of mixed-disulfides in proteins (S-thiolation/dethiolation)(Thomas and Sies, 1991) is strongly dependent on the GSH/GSSG ratio, whereas methionine sulfoxide can be repaired by reduction by a specific NADPH-dependent reductase (Brot and Weissbach, 1982). Finally, oxidized bases and strand breaks in DNA induced by active species can be repaired enzymatically by several mechanisms, including base excision repair and nucleotide excision repair systems (Sies, 1986; Halliwell and Gutteridge, 1989).

OVERVIEW

The metabolic activity of aerobic cells generates reactive species, whose steady-state concentrations are kept at a low level by a specialized phase of their metabolism including prevention, interception and removal of toxic intermediates by antioxidant mechanisms and ancillary reactions, as well as replacement and repair of altered molecules (Fig 1). These mechanisms can effectively perform their functions when coupled to the intermediary metabolism for a continuous supply of energy, reducing equivalents and precursors, with the concomitant dietary supply of metabolic fuels and essential components, thus allowing an optimal cellular functioning (Fig 1).

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