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Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology

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Abstract

Reactive Oxygen Species (ROS) are produced during normal cellular function. ROS include hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide. They are very transient species due to their high chemical reactivity that leads to lipid peroxidation and oxidation of DNA and proteins. Under normal conditions, antioxidant systems of the cell minimize the perturbations caused by ROS. When ROS generation is increased to an extent that overcomes the cellular antioxidants, the result is oxidative stress. It is now clear that several biological molecules, which are involved in cell signaling and gene regulation systems are very sensitive to redox statue of the cell. Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. The various antioxidants exert their effect by scavenging superoxide, or by activating of a battery of detoxifying/defensive proteins. The prevention of oxidation is an essential process in all the aerobic organisms, as decreased antioxidant protection may lead to cytotoxicity, mutagenicity and/or carcinogenicity. This article also focuses on the mechanisms by which antioxidants and xenobiotics induce the gene expression of detoxifying enzymes. On the other hand, small molecules that mimic antioxidant enzymes are becoming new tools for the treatment of many diseases.

Keywords: Antioxidants; Apoptosis; Catalase; Glutathione peroxidase; Reactive oxygen species; Superoxide dismutase

1. Introduction

Reactive Oxygen Species (ROS) are produced during normal cellular function. ROS include hydroxyl radicals ($^{\cdot}OH$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO). They are very transient species due to their high chemical reactivity that leads to lipid peroxidation and oxidation of some enzymes, and a massive protein oxidation and degradation (Matés et al., 1999a). The role of oxygen-derived species in causing cell injury or death is increasingly recognized: superoxide and hydroxil radicals are involved in a large number of degenerative changes, often associated with an increase in peroxidative processes and linked to low antioxidant concentration (Tamagno et al., 1998).

The prevention of lipid peroxidation is an essential process in all the aerobic organisms, as

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lipid peroxidation products can cause DNA damage. Increased lipid peroxidation and decreased antioxidant protection frequently occurs: epoxides may spontaneously react with nucleophilic centers in the cell and thereby covalently bind to DNA, RNA and protein (Matés and Sánchez-Jiménez, 1999). Such a reaction may lead to cytotoxicity, allergy, mutagenicity and/or carcinogenicity, depending of the properties of the epoxide in question. Moreover, oxidative events may play an important role in the mechanism of action of ether lipids, and oxidizability may contribute to cellular drug sensitivity (Wagner et al., 1998).

On the other hand, hydrogen peroxide has been implicated recently as an intracellular messenger that affects cellular processes including protein phosphorylation, transcription and apoptosis (Choi et al., 1998).

2. ROS neurotoxicity

Brain is especially susceptible to oxidative damages. In spite of the high rate of ROS production, due to high rate of oxidative metabolism and abundance of polyunsaturated fatty acids in cell membrane, brain has a relatively low antioxidant defense system. Among the different ROS scavengers, the glutathione (GSH) dependent system is of great importance. This system not only work as peroxide scavengers, but also to regulate the redox state of the cells.

Oxygen species are key participants in damage caused, among others, by neurodegenerative processes, including cell death, motor neuron diseases and axonal injury. On the other hand, antioxidant enzymes dysfunctions have been associated to amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease and Parkinson's disease (Sun and Chen, 1998; Matés et al., 1999a).

Among the neural cells, astrocytes are more resistant to oxidative stress. They provide a protective role for neurons. It has been shown that their higher GSH content is a major reason for this ability (Thorburne and Juurlink, 1996). During scavenging the ROS, GSH is oxidized and forms glutathione-protein mixed disulfides; hence, the cell's ability to reduce or synthesize GSH is the key to how effectively the cell can manage the oxidative stress.

The rate-limiting enzyme for GSH synthesis is L-γ-glutamyl-cysteine synthase (GCS) (Meister, 1989). GCS is a heterodimer comprised of a catalytic and a regulatory subunit with both subunits being upregulated by phase II enzyme inducers. Electrophilic compounds known as phase II enzyme inducers (Prestera et al., 1993; Talalay et al., 1995) activate transcription factor complexes that promote transcription of phase II enzyme genes.

Mammalian cells have evolved elaborate mechanisms for protection against the neoplastic and neurotoxic effects of electrophilic metabolites of carcinogens and reactive oxygen species. Phase II enzymes (e.g. glutathione transferase, NAD(P)H:quinone reductase, UDP-glucuronosyltransferases) and high intracellular levels of glutathione play a prominent role in providing such protection. Phase II enzymes are transcriptionally induced by low concentrations of a wide variety of chemical agents and such induction blocks chemical carcinogenesis. The inducers belong to many chemical classes including phenolic antioxidants, Michael reaction acceptors, isothiocyanates, 1,2-dithiole-3-thiones, trivalent arsenicals, $HgCl_2$, organomercurials, hydroperoxides, and vicinal dimercaptans. Induction by all classes of inducers involves the antioxidant/electrophile response element (ARE/EpRE). Inducers are widely, but unequally, distributed among edible plants. Search for such inducer activity in broccoli led to the isolation of sulforaphane, an isothiocyanate that is a very potent Phase II enzyme inducer and blocks mammary tumor formation in rats (Ahlgren-Beckendorf et al., 1999). Induction of these enzymes have been reported to prevent glutamate toxicity (Murphy et al., 1991). Phase II enzyme induction can also prevent cell death in astrocytes exposed to high concentration of peroxides.

2.1. Cytotoxic effect of oxidized lipoproteins

Recent studies have demonstrated the involvement of oxidative stress in the pathogenesis of Alzheimer's disease and the amyloid beta peptide (Aβ) and the apolipoprotein E (apoE) have been

implicated as the key factors contributing to these oxidative events (Lyras et al., 1997; Sayre et al., 1997; Mattson and Pedersen, 1998; Behl, 1999). ApoE are found in amyloid plaques, neurofibrillary tangles and vasculatures of autopsied Alzheimer's disease (AD) brain. Lipoproteins (LP) in the central nervous system, particularly those associated with apoE, are known to play important roles in support of many brain functions. For example, they are known for their ability to mediate intercellular lipid transport, promote neurite outgrowth, maintenance of cholesterol homeostasis and repair of membrane during injury (Ignatius et al., 1986, 1987; Poirier et al., 1993; Holtzman et al., 1995; Fagan-Niven et al., 1996; Beffert et al., 1998). Lipoproteins released from astrocytes contain high levels of phospholipids and cholesterol but low levels of triglycerides and cholesterylesters (LaDu et al., 1998).

In AD brain, the oxidative environment results in LP oxidation and exacerbates the progression of the disease. In recent studies, it has been observed the ability of oxidized low-density lipoproteins (LDL) in serum to enhance oxidative stress and apoptotic cell death in PC12 cells (Draczynska-Lusiak et al., 1998a,b; Li and Sun, 1999). Oxidized LDL has also been implicated to cause cell differentiation, inflammation and cytotoxicity in embryonic neuronal cells (Keller et al., 1999). Due to the high content of polyunsaturated fatty acids (PUFA) in brain membranes (Sun and Sun, 1976), it is possible that the phospholipids in brain LPs also contain high PUFA and are more susceptible to oxidative stress. In agreement with the increased oxidative stress in AD brain, there is evidence that LPs in cerebral spinal fluid of AD patients are more vulnerable to oxidation (Bassett et al., 1999).

Accumulation of neuritic plaques and amyloid beta peptides are important pathological landmarks of AD (Strittmatter et al., 1993a,b; Strittmatter and Roses, 1996; Price et al., 1998). Aβ with 39–43 amino acid residues are derived from the amyloid precursor protein (APP) through cleavage by secretases. APP is a transmembrane protein present in both neurons and glial cells in the brain. While the cellular function of APP is still unknown, accumulation of Aβ, especially in their aggregated form, is known to cause a number of cytotoxic events and exacerbate oxidative stress in the brain (Mattson and Pedersen, 1998). Under normal conditions, soluble Aβ can be detected in the cerebral spinal fluid and plasma at levels between 10^{-8}–10^{-10} M. However, when Aβ peptides are converted to their fibrillar form, these peptides can enhance the production of ROS, resulting in protein carbonyl formation and lipid peroxidation, and subsequent alteration of cellular functions (Huang et al., 1999a,b; Yatin et al., 1999).

3. Antioxidants against molecular toxicology

Antioxidants are substances that either directly or indirectly protect cells against adverse effects of xenobiotics, drugs, carcinogens and toxic radical reactions (Halliwell, 1995). Several biologically important compounds have been reported to have antioxidant functions. These include vitamin C (ascorbic acid), vitamin E (α-tocopherol), vitamin A, β-carotene, metallothionein, polyamines, melatonin, NADPH, adenosine, coenzyme Q-10, urate, ubiquinol, polyphenols, flavonoids, phytoestrogens, cysteine, homocysteine, taurine, methionine, *s*-adenosyl-L-methionine, resveratrol, nitroxides, GSH, glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), thioredoxin reductase, nitric oxide sintase (NOS), heme oxygenase-1 (HO-1) and eosinophil peroxidase (EPO) (Krishna et al., 1996; Chanvitayapongs et al., 1997; Evans et al., 1997; Beyer et al., 1998; Devamanoharan et al., 1998; Jourd'heuil et al., 1998; McKenzie et al., 1998; Nohl et al., 1998; Halliwell, 1999; Fremont, 2000).

The antioxidant role of phenolic compounds including 3 - (2) - *tert* - butyl - 4 - hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), and *t*-butyl hydroquinone (*t*-BHQ) in prevention of oxidative stress have been well documented (Kahl, 1984). The various antioxidants either scavenge superoxide and free radicals or stimulate the detoxification mechanisms within cells resulting in increased detoxification of free radicals formation and thus in prevention of many patho-

physiologic processes (Table 1). Glutathione, superoxide dismutase and catalase directly scavenge superoxide, whereas BHA, BHT and *t*-BHQ coordinately induce the expression of a battery of genes, the products of which protect cells against oxidative stress and related consequences (Radjendirane et al., 1997). Other compounds as glutamine, specifically induces heat shock protein 72 (hsp72) in intestinal epithelial cells, which mediates cell protection against inflammation-induced stress such as oxidants (Musch et al., 1998).

3.1. Antioxidant defense in aflatoxin toxicity

Aflatoxins are a group of toxic metabolites produced by Aspergillus flavus (AFB1 and AFB2) and Aspergillus parasiticus (AFB1, B2, G1, G2). These mycotoxins contaminates the food stuffs through various means such as through water, air or improper storage. Amongst these toxins AFB1 is the most potent naturally occurring carcinogens and is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC). Exposure to the most potent mycotoxin AFB1 has also been suggested to increase primary hepato cellular carcinoma risk (IARC, 1987).

The mechanism of action of mycotoxins on the cell is mediated through the production of free radicals and ROS. Nakae et al., (1990) reported that killing of rat hepatocytes by AFB1 (or) dimethylnitrosamine was prevented by CAT, SOD, mannitol (or) deferoxamine. The results of his experiment indicated the important role of active oxygen species in the cytotoxicity of hepatocarcinogens and suggested the possible existence of free radical metabolites.

A number of reports are available on the ability of antioxidants to defend chemical carcinogenesis when they are administered prior to (or) concomittantly with the carcinogen. Aflatoxin toxicity in Salmonella has been found to be partially suppressed by the antioxidants vitamin A, C and E (Raina and Gurtoo, 1985). Antioxidants such as BHA, BHT and ethoxyquin also inhibit carcinogenesis caused by AFB1 (Williams et al., 1986). The dietary intake of other antioxidants such as β-carotene, ascorbic acid, selenium, uric acid and vitamin-E could reduce AFB1 induced liver cancer in rats (Nyandieka et al., 1990). Most recently *Amrita Bindu* (a salt-spice herbal mixture formulated by Shanmugasundaram et al., 1994)

Table 1
Some examples of diseases and its relationship to antioxidant molecules as putative inhibitors of such pathophysiologic states

Disease	Cell/tissue	Antioxidant	Reference
Male infertility	Spermatozoa	Zn	Gavella et al., 1999
Female infertility	Luteinizing hormone (brain)	SOD-1	Al-Gubory and Locatelli, 1999
Diabetes	Beta cells (mice)	EC-SOD	Sentman et al., 1999
HIV	Plasma	SOD	Edeas et al., 1997
Cancer	Bladder, blood, bowel, breast, colorectum, liver, lung, kidney, oesophagus, skin, ovary, prostate	SOD, GPX, catalase	Burdon et al., 1990; Mulder et al., 1995; Janssen et al., 1998; Matés and Sánchez-Jiménez, 2000
ALS	Motor neurons	SOD-1	Estévez et al., 1999
Alzheimer Huntington Parkinson	Neurons	SOD, GPX Catalase Polyphenols	Price et al., 1998; Sun and Chen, 1998; Bassett et al., 1999
Brain injury Heart diseases	Artery occlusion Arteries	SOD, GPX SOD	Baker et al., 1998; Petyaev et al., 1998
Postischemic injury	Brain	Catalase SOD-1	Weisbrot-Lefkowitz et al., 1998; Wang et al., 1998
Crohn's disease Ulcerative colitis	Gastrointestinal tract	SOD, GPX, catalase	Niwa, 1999; Blau et al., 1999
Allergy to pollen and house dust mite	Blood cells	SOD, GPX, catalase	Matés et al., 1999b
Hearing loss	Cochlea	SOD-1	McFadden et al., 1999

has been used as a food supplement to prevent nitrosamine mediated degenerative changes in the body. *Amrita Bindu* supplementation in the diet prevented *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) induced depletion of the antioxidant enzymes and scavengers SOD, CAT, GPX, GSH, vitamins A, C in liver, kidney, intestines and other tissues, while lipid peroxidation was under control. The effectiveness of *Amrita Bindu* in reducing or preventing AFB1 induced toxicity was investigated in experimental model Carps (*Labeo rohita*) of acute toxicity.

4. Main detoxifying enzymes

4.1. Superoxide dismutase

Superoxide dismutase (EC 1.15.1.1) destroys the free radical superoxide by converting it to peroxide that can in turn be destroyed by catalase or GPX reactions. A low level of superoxide is constantly generated by aerobic respiration. The electron-transport chain of mitochondria, which is meant to escort four electrons to molecular oxygen to form water, occasionally leaks a single electron. Superoxide reduces Fe(III) to Fe(II), releasing the iron from storage sites so that it can react with hydrogen peroxide and produce hydroxyl radicals. SOD converts superoxide to hydrogen peroxide and molecular oxygen.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2.$$

Another function of superoxide dismutase is to protect dehydratases (dihydroxy acid dehydratase, aconitase, 6-phosphogluconate dehydratase and fumarases A and B) against inactivation by the free radical superoxide (Benov and Fridovich, 1998).

Four classes of SOD have been identified, containing either a dinuclear Cu, Zn or mononuclear Fe, Mn or Ni cofactors (Whittaker and Whittaker, 1998). Fe-SODs and Mn-SODs show homology and posses identical metal chelating residues at the active site, sharing substantial sequence and three dimensional structural homology, while the other superoxide dismutases are structurally unrelated. In humans, there are three forms of SOD: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extracellular-SOD (EC-SOD) (Majima et al., 1998). SOD catalyses the dismutation of by successive oxidation and reduction of the transition metal ion at the active site in a Ping Pong type mechanism with remarkably high reaction rates (Hsieh et al., 1998).

4.1.1. Manganese superoxide dismutase

Mn-SOD is a homotetramer (96 kDa) containing one manganese atom per subunit that cycles from Mn(III)–Mn(II) and back to Mn(III) during the two step dismutation of superoxide. The respiratory chain in mitochondria is a major source of oxygen radicals. Mn-SOD is a nuclear-encoded primary antioxidant enzyme that functions to remove these superoxide radical (Guan et al., 1998). The biological importance of Mn-SOD is demonstrated among others by the following (Matés and Sánchez-Jiménez, 1999): (1) inactivation of Mn-SOD genes in *E. coli* increases mutation frequency when grown under aerobic conditions; (2) elimination of the gene in *Saccharomyces cerevisiae* increases its sensitivity to oxygen; (3) lack of expression in Mn-SOD knock-out mice results in dilated cardiomyopathy and neonatal lethality; (4) tumor necrosis factor (TNF) selectively induces Mn-SOD, but not Cu, Zn-SOD, catalase or GPX mRNA in various mouse tissues and cultured cells; (5) transfection of Mn-SOD cDNA into cultured cells rendered the cells resistant to paraquat, TNF and adriamycin-induced cytotoxicity, and radiation induced-neoplastic transformation; (6) expression of human Mn-SOD genes in transgenic mice protects against oxygen-induced pulmonary injury and adriamycin-induced cardiac toxicity. Thus, the expression of Mn-SOD is essential for the survival of aerobic life and the development of cellular resistance to oxygen radical-mediated toxicity.

Mn-SOD (SOD-2) has been proposed as a tumour suppressor gene (Bravard et al., 1998). St. Clair's group is now studying the identification of promoter/enhancer elements, the role of critical transcription regulators, and the mechanisms by which these transcription regulators cooperate to synergistically induce the expression of the human Mn-SOD gene.

4.1.2. Copper, zinc superoxide dismutase

Cu, Zn-SOD (SOD-1) are another class of enzyme conserved throughout evolution, which usually have two identical subunits of about 32 kDa, each containing a metal cluster, the active site, constituted by a copper and a zinc atom bridged by a common ligand: His 61 (Banci et al., 1998). Inactivation of copper- and zinc-containing SOD by H_2O_2 is the consequence of several sequential reactions. First, reduction of the active site Cu(II) to Cu(I) by H_2O_2; then oxidation of the Cu(I) by a second H_2O_2, thus generating a powerful oxidant, which may be Cu(I)O, Cu(II)OH or Cu(III); and finally oxidation of the histidine, causing loss of SOD activity (Liochev et al., 1998).

Whereas Mn-SOD was found in all tumors, and the ratio between the activities of Cu, Zn-SOD and Mn-SOD was not different from that of the normal tissues, tumors posses less Cu, Zn-SOD than did the more metabolically active tissues (Westman and Marklund, 1981). Cu, Zn-SOD is believed to play a major role in the first line of antioxidant defense by catalyzing the dismutation of superoxide anion radicals, to form hydrogen peroxide and molecular oxygen. Mice lacking this enzyme exhibited a pronounced susceptibility to paraquat toxicity. Most surprisingly, female homozygous knock-out mice showed a markedly reduced fertility compared with that of wild-type and heterozygous knock-out mice. They exhibited a marked increase in embryonic lethality. These data suggest a role of oxygen free radicals in causing abnormality of female reproduction in mammals (Ho et al., 1998). Other recent reports involving SOD knock-outs have revealed that Mn-SOD is essential for life whereas Cu, Zn-SOD is not. Cu, Zn-SOD knock-out mice appear normal and exhibit differences only after traumatic injury, whereas Mn-SOD knockouts do not survive past three weeks of age (Reaume et al., 1996).

It is being now discussed the induction mechanisms of SOD-1 gene by natural tonics (ginsenosides), cytokine (interferon gamma), and environmental factors; heat, hydrogen peroxide, heavy metals (Cd, Cu, Zn), xenobiotics (β-naphthoflavone), herbicides (paraquat), and dioxin. From these results and difficult uptake of SOD-1 by cells, SOD-1 induction appears as a very important enzyme for the prevention of aging and mutation by oxidative stresses and hazardous effects from environmental factors (Seo et al., 1997).

4.1.3. Extracellular superoxide dismutase

EC-SOD is a secretory, tetrameric, copper and zinc containig glycoprotein (with a high affinity for certain glycosaminogycans such as heparin and heparan sulfate) found in the intersticial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity of plasma, lymph, and synovial fluid (Adachi and Wang, 1998). It is the major antioxidant in the blood vessel wall interstitium (Sentman et al., 1999) and it is the only known extracellular enzyme designed to scavenge the superoxide anion (Enghild et al., 1999). EC-SOD, is not induced by its substrate or other oxidants (xanthine oxidase plus hypoxanthine, paraquat, pyrogallol, α-naphthoflavone, hydroquinone, catechol, Fe^{2+} ions, Cu^{2+} ions, buthionine sulphoximine, diethylmaleate, *t*-butyl hydroperoxide, cumene hydroperoxide, selenite, citiolone and high oxygen partial pressure) and its regulation in mammalian tissues primarily occurs in a manner coordinated by cytokines, rather than as a response of individual cells to oxidants (Buschfort et al., 1997). EC-SOD controls the availability of extracellular superoxide, which is important for a variety of physiological pathways, including means of inactivating nitric oxide. EC-SOD has primary control over the inactivation of NO and its role in neurobehavioral function has been recently established (Levin et al., 1998).

4.1.4. Nickel superoxide dismutase

Ni–SOD has been purified from the cytosolic fraction of Streptomyces sp. and Streptomyces coelicolor. It is composed of four identical subunits of 13.4 kDa, stable at pH 4.0–8.0, and up to 70°C. It is inhibited by cyanide and H_2O_2 but little inhibited by azide. Amino acid composition is different from iron, manganese and zinc-copper SODs. The apoenzyme, lacking in nickel, had no ability to mediate the conversion of superoxide anion to hydrogen peroxide, strongly indicating that Ni^{III} plays a main role in the activity (Young et al., 1996).

4.2. Catalase

Catalase (EC 1.11.1.6) is a tetrameric haemin-enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa. Therefore, it contains four ferriprotoporphyrin groups per molecule, and its molecular mass is about 240 kDa. Catalase is one of the most efficient enzymes known. It is so efficient that it cannot be saturated by H_2O_2 at any concentration (Lledías et al., 1998).

Catalase reacts with H_2O_2 to form water and molecular oxygen; and with H donors (methanol, ethanol, formic acid, phenol…) using 1 mole of peroxide in a kind of peroxidase activity:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2,$$

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A.$$

H_2O_2 is enzymically catabolized in aerobic organism by catalase and several peroxidases. In animals, catalase and GPX detoxify H_2O_2. Catalase protects cells from hydrogen peroxide generated within them. Even though catalase is not essential for some cells type under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Hunt et al., 1998). The increased sensitivity of transfected enriched catalase cells to adriamycin, bleomycin and paraquat is attributed to the ability of catalase in cells to prevent the drug-induced consumption of O_2. Thus, capturing H_2O_2 before it can escape the cell and converting it to O_2. In this way, catalase can maintain the concentration of O_2 either for repeated rounds of chemical reduction or for direct interaction with the toxin (Speranza et al., 1993).

4.3. Glutathione peroxidase

The selenium-containing peroxidases, being the more important example glutathione peroxidase (EC 1.11.1.19), catalyze the reduction of a variety of hydroperoxides (ROOH and H_2O_2) using GSH, thereby protecting mammalian cells against oxidative damage.

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSG + H_2O$$

There are at least five GPX isoenzymes found in mammals. Although their expression is ubiquitous, the levels of each isoform vary depending on the tissue type. Cytosolic and mitochondrial glutathione peroxidase (cGPX or GPX1) reduces fatty acid hydroperoxides and H_2O_2 at the expense of glutathione. GPX1 and the phospholipid hydroperoxide glutathione peroxidase GPX4 (or PHGPX) are found in most tissues. GPX4 is located in both the cytosol and the membrane fraction. PHGPX can directly reduce the phospholipid hydroperoxides, fatty acid hydroperoxides, and cholesterol hydroperoxides that are produced in peroxidized membranes and oxidized lipoproteins (Imai et al., 1998). GPX1 is predominantly present in erythrocytes, kidney, and liver, and GPX4 is highly expressed in renal epithelial cells and testes. Cytosolic GPX2 (or GPX-G1) and extracellular GPX3 (or GPX-P) are poorly detected in most tissues except for the gastrointestinal tract and kidney, respectively. Recently, a new member, GPX5, expressed specifically in mouse epididymis, is interestingly selenium-independent (De Haan et al., 1998).

GPX1 (80 kDa) contains one selenocysteine (Sec) residue in each of the four identical subunits, which is essential for enzyme activity (Ding et al., 1998). Although GPX shares the substrate, H_2O_2, with catalase, it alone can react effectively with lipid and other organic hydroperoxides. The glutathione redox cycle is a major source of protection against low levels of oxidant stress, whereas catalase becomes more significant in protecting against severe oxidant stress (Yan and Harding, 1997). In animals cells, and specially in human erythrocytes, the principal antioxidant enzyme for the detoxification of H_2O_2 has for a long time been considered to be GPX, as catalase has much lower affinity for H_2O_2 than GPX (Izawa et al., 1996).

Cells depleted of glutathione peroxidase were more sensitive to the toxicity of paraquat and adriamycin than untransfected parental cells from which they derived but not more sensitive to bleomycin, menadione, or phenazine methosulfate. In fact that the mildly increased sensitivity to paraquat and adriamycin was the consequence of the diminished cellular content of glutathione per-

oxidase was confirmed by the increase in sensitivity of untransfected cells after treatment with buthionine sulfoximine, an agent which depletes cells of glutathione. These and other data strongly suggest that the enzymatic action of GPX protects cells from the toxicity of paraquat and adriamycin. The toxin that these agents engender is likely to be hydrogen peroxide or another hydroperoxide upon which glutathione peroxidase acts (Taylor et al., 1993).

GPX equally protects against the oxidation of dihydrorhodamine 123 (an indicator dye) by peroxynitrite ($OONO^-$), requiring GSH as reductant. Thus, there is also a function of GPX and potentially of other selenoproteins containing selenocysteine or selenomethionine, in the GSH-dependent maintenance of a defense line against peroxynitrite-mediated oxidations, as a peroxynitrite reductase (Sies et al., 1997).

5. Induction and expression of other detoxifying enzymes genes

Detoxifying enzymes also including NAD(P)H:quinone oxidoreductases (NQO1 and NQO2) and glutathione *S*-transferases (GSTs) that catalyze metabolic detoxification of xenobiotics, drugs and carcinogens and, thus, protect the cells against redox cycling and oxidative stress. Genes encoding the various detoxifying enzymes are ubiquitiosly expressed and coordinately induced in response to antioxidants and xenobiotics (Radjendirane et al., 1997; Rushmore and Pickett, 1993). Deletion mutagenesis and transfection assays have identified antioxidant response element (ARE) in the promoters of the various detoxifying enzyme genes, which regulate the expression and coordinated induction of detoxifying enzyme genes. Band and supershift assays have been used to demonstrate that nuclear transcription factors Nrf1, Nrf2, Jun, Fos, and Fra bind to the NQO1 and GST genes ARE. Overexpression of Nrf1 and Nrf2 individually in human hepatoblastoma (Hep-G2) cells significantly increased the ARE-mediated genes expression and induction by beta-naphthoflavone (β-NF) and *t*-BHQ. Nrf2 containing one mutated leucine in its leucine zipper region was more efficient in upregulation of ARE-mediated gene expression, as compared to Nrf1 with two mutated leucines. Immunoprecipitation assays demonstrated that Nrf1 and Nrf2 heterodimerize with Jun (c-Jun, Jun-B and Jun-D) proteins that bind to the ARE.

Interestingly, many xenobiotics, oxidants, peroxides, UV light, and heavy metals also coordinately induce the expression of similar genes as observed with antioxidants. Both, antioxidants and xenobiotics are metabolized by cellular enzymes to generate superoxide and electrophiles. It is believed that this initial generation of superoxide is to activate a battery of genes for cellular protection. Failure in this mechanism leads to the accumulation of superoxide and other free radicals. As stated above, the accumulation of superoxide and other free radicals is known to cause oxidative stress, DNA and membrane damage, mutagenicity, degeneration of tissues, premature aging, apoptotic cell death, cellular transformation and cancer (Talalay et al., 1995; Matés and Sánchez-Jiménez, 1999).

5.1. Genes encoding enzymes that detoxify xenobiotics and carcinogens

Expression and induction of enzymes that metabolize xenobiotics, drugs and carcinogens play an important role in determining the risk of cancer in human (Talalay et al., 1995; Matés et al., 1999a; Matés and Sánchez-Jiménez, 2000). In other words, the development of chemically induced neoplasia is regulated by a balance between phase I (cytochromes P450, cytochrome P450 reductase, hydroxylases, lipoxygenases, peroxidases and oxidases), which activate carcinogens, and phase II (detoxifying/chemopreventive) enzymes, which detoxify them. As previously described, detoxifying enzymes include NQOs, which catalyze obligatory two-electron reduction of quinones and their derivatives thus preventing their participation in redox cycling and oxidative stress (Talalay et al., 1995; Radjendirane et al., 1997); glutathione *S*-transferases (GSTs), which conjugate hydrophobic electrophiles and reactive oxygen species with GSH (Rushmore and Pickett, 1993); UDP-glucuronosyl transferases (UDP-GT),

which catalyze the conjugation of glucuronic acid with xenobiotics and drugs for their excretion (Tephly and Burchell, 1990)epoxide hydrolase (EH), which inactivates epoxides (Oesch et al., 1991); γ-glutamylcysteine synthetase (γ-GCS), which plays a key role in the regulation of glutathione metabolism (Meister, 1994) and so on.

Among the various detoxifying enzymes, the NQOs and the GSTs have been extensively studied. The NQO gene family contains two members designated as NQO1 and NQO2 (Radjendirane et al., 1997). Mutations in the NQO1 gene resulting in the loss of NQO1 enzyme activity have been reported in certain types of cancers (Rauth et al., 1997). Various GSTs including the GST Ya and GST P genes are encoded by five gene families (Rushmore and Pickett, 1993). Loss of GSTs are associated with several kinds of cancer. This includes prostate, urothelial, lung and colorectal cancer (Mulder et al., 1995).

In addition to these enzymes, several other enzymes also protect cells from oxidative stress by preventing the generation of superoxide or by scavenging superoxide. These enzymes include HO-1, SOD-1 and catalase. HO-1 increases the intracellular levels of ferritin (Vile et al., 1994). The increase in ferritin limits the availability of iron to catalyze harmful reactions, such as the peroxidation of lipids and the Fenton and Haber-Weiss reactions producing hydroxyl radicals thus, protecting the cells against UV induced oxidative stress (Matés and Sánchez-Jiménez, 2000).

$$Fe^{2+} + H_2O \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH \text{ (Fenton reaction)} O_2^{\bullet-} + H_2O_2$$

$$\rightarrow O_2 + {}^{\bullet}OH + {}^{-}OH \text{ (Haber-Weiss reaction)}$$

Studies have revealed that the capacity of many diverse chemicals to block carcinogenesis correlates with their capacity to induce NQO1 and other enzymes including GSTs (Talalay et al., 1995). Induction of NQO1 and GST by Sulforaphane from *Saga broccoli* blocks the formation of mammary tumors in Sprague-Dawley rats treated with single dose of 9,10 dimethyl-1,2-benzanthracene (Zhang et al., 1994).

The genes encoding the human and rat NQO1, human NQO2, rat GST Ya and rat GST P have been cloned and sequenced (Jaiswal, 1994b). The NQO1 and GST Ya genes are expressed at higher levels in liver tumors and tumor cells as compared to normal liver and liver hepatocytes (Radjendirane et al., 1997).

5.2. Antioxidant response element

Deletion mutagenesis studies of the human NQO1 gene promoter identified several *cis*-elements that are essential for the expression and induction of the NQO1 gene (Xie and Jaiswal, 1996). One of these elements was 24 base pairs of the antioxidant response element (ARE) that are required for basal expression as well as induction of NQO1 gene in response to β-NF, BHA, *t*BHQ and hydrogen peroxide. Other element include an AP2 element essential for cAMP induced expression of the NQO1 gene. ARE-like elements have also been found in the promoter regions of the rat NQO1 gene (Rushmore et al., 1990); the human NQO2 gene (Jaiswal, 1994b); the rat and mouse glutathione *S*-transferase Ya subunit genes (Friling et al., 1992), the rat glutathione *S*-transferase P gene (Okuda et al., 1990) and γ-glutamyl cysteinyl synthetase gene (Mulkahy et al., 1997). The conservation of the ARE in the genes of many detoxifying enzymes indicated that these genes may be coordinately regulated by a single mechanism involving ARE (Jaiswal, 1994a). The NQO1 gene ARE contains one perfect and one imperfect TRE [12-*O*-tetradecanoylphorbol-13-acetate-(TPA) response element] arranged as inverse repeats separated by three base pairs followed by a GC box. The human NQO1 gene ARE and other detoxifying enzyme gene AREs are unique *cis*-elements even though they contain TRE and TRE-like elements. This is because it is ARE, rather than TRE, that is responsive to antioxidants and xenobiotics (Jaiswal, 1994a,b). Mutational analysis of the ARE identified GTGAC***GC as the core of the ARE sequence (Xie and Jaiswal, 1996). Additional *cis*-element and nucleotide sequences flanking the core sequence have been shown to contribute to the ARE-mediated expression and induction (Wasserman and Fahl, 1997).

5.3. Antioxidant response element-binding proteins

Nuclear transcription factors c-Jun, Jun-B, Jun-D, c-Fos, Fra1, Nrf1, Nrf2, YABP, ARE-BP1, Ah (aromatic hydrocarbon) receptor and the estrogen receptor have been reported to bind to the AREs from various genes (Friling et al., 1992; Wasserman and Fahl, 1997; Montano et al., 1998). Among these transcription factors, c-Jun, Jun-B, Jun-D, c-Fos, Fra1, Nrf1 and Nrf2 bind to the human NQO1 gene ARE. Nrf1 and Nrf2 have been shown to positively regulate the ARE-mediated expression and induction of NQO1 gene in response to antioxidants and xenobiotics (Radjendirane and Jaiswal, 1999). Nrf1 and Nrf2 are leucine zipper proteins that do not heterodimerize with each other and require another leucine zipper protein for its activity (Moi et al., 1994).

Small amounts of superoxide and related reactive species are consistently required for keeping cellular defenses active. Since activation of detoxifying enzymes and other defensive proteins leads to significant reduction in the levels of superoxide and other free radicals, the cell may require negative regulatory factors like c-Fos to keep alert the expression of detoxifying enzymes and other defensive genes (Radjendirane et al., 1997).

5.4. Iron regulatory proteins

Iron regulatory proteins, IRP1 and IRP2 respond to alterations in iron levels and control mRNA translation or stability. The mechanisms involve binding of IRPs to iron responsive elements (IREs), hairpin structures in the untranslated regions of several mRNAs, primarily encoding proteins involved in cellular iron and energy metabolism (Pantopoulos and Hentze, 1995). Both IRP1 and IRP2 are homologous cytoplasmic polypeptides and belong to the family of iron-sulfur cluster isomerases that also includes mitochondrial aconitase, an enzyme of the citric acid cycle. Despite their extensive homology, IRP1 and IRP2 are regulated by distinct mechanisms following iron perturbations. Thus, activation of IRP1 involves an unusual iron-sulfur cluster switch, while IRP2 is regulated at the level of protein stability (Pantopoulos et al., 1997). Iron regulatory proteins are also modified in response to other signals, seemingly unrelated to the control of iron homeostasis. While both IRP1 and IRP2 are activated by nitric oxide, IRP1 is also induced when cells are exposed to hydrogen peroxide. These findings establish new regulatory connections between iron metabolism and oxidative stress (Pantopoulos and Hentze, 1998).

IRP1 regulates the synthesis of proteins involved in iron homeostasis by binding to iron-responsive elements of messenger RNA. IRP1 is a cytoplasmic aconitase when it contains a [4Fe–4S] cluster and an RNA-binding protein after complete removal of the metal center by an unknown mechanism. Human IRP1, obtained as the pure recombinant [4Fe–4S] form, is an enzyme as efficient toward *cis*-aconitate as the homologous mitochondrial aconitase. The aconitase activity of IRP1 is rapidly lost by reaction with hydrogen peroxide as the [4Fe–4S] cluster is quantitatively converted into the [3Fe–4S] form with release of a single ferrous ion per molecule. The IRE binding capacity of IRP1 is not elicited with H_2O_2. Ferrous sulfate (but not other more tightly coordinated ferrous ions, such as the complex with ethylenediamine tetraacetic acid) counteracts the inhibitory action of hydrogen peroxide on cytoplasmic aconitase, probably by replenishing iron at the active site. These results cast doubt on the ability of ROS to directly increase IRP1 binding to IRE and support a signaling role for hydrogen peroxide in the posttranscriptional control of proteins involved in iron homeostasis in vivo (Gehring et al., 1999).

IRP-1 controls the expression of several mRNAs by binding to IREs in their untranslated regions. In iron-replete cells, a 4Fe–4S cluster converts IRP-1 to cytoplasmic aconitase. IRE binding activity is restored by cluster loss in response to iron starvation, NO, or extracellular H_2O_2. The effects of intracellular quinone-induced oxidative stress on IRP-1 have been studied. It leads to post-translational inactivation of both genetic and enzymatic functions of IRP-1 by a mechanism that lies beyond the classical Fe–S cluster switch and exerts multiple effects on cellular iron metabolism (Brazzolotto et al., 1999).

6. ROS, signaling and apoptosis

6.1. Altered signaling: In vitro cellular model for diabetic neuropathy

Diabetes mellitus is an endocrine disease characterized by the inability of the pancreas to secrete enough insulin to maintain physiological levels of blood glucose. The mechanisms underlying these pathological changes are as yet obscure, but hyperglycemia-induced neuronal damage may result from the induction of programmed cell death, or apoptosis (Phelan et al., 1997). High among the possible damaging mechanisms ranks the hyperglycemia-induced non-enzymatic modification of sugar moieties on proteins and lipids, which leads the formation of advanced glycosylation end-products (AGEs). AGEs are involved in the possible disturbances of carbohydrate-, protein-, and lipid-metabolism. Importantly, AGEs generate ROS, suggesting that hyperglycemia causes oxidative damage to the cells through NFκB dependent pathways (Donnini et al., 1996; Mohamed et al., 1999). AGEs and ROS-induced cellular dysfunctions can interfere with gene expression of peptides and cytokines involved in the regulation of cell proliferation (Chappey et al., 1997). Extensive production of ROS may mediate a signal for apoptotic cell death (Fujii et al., 1996). Apoptosis is a complex, highly controlled process which results in programmed cell death (McConkey and Orrenius, 1994; Mesner et al., 1995). Along the way, there are several characteristic stepping stones/indicators, which will distinguish apoptosis from necrosis (chaotic cell death due to overt injury). One of the early markers for apoptosis is the activation of specific caspases, such as caspase-3, which are members of the Ced/ICE family of cystein proteases (Haviv and Stein, 1999). Progressive DNA fragmentation is a marker for the later stages of apoptosis. There is increasing evidence for the enhanced induction of apoptosis of numerous cell types in diabetes including neuronal cells (O'Brien et al., 1997; Zhang et al., 1997; Barber et al., 1998). Recent studies established an in vivo link between diabetes and apoptosis in the nervous system (Phelan et al., 1997; Russell et al., 1999). Some of these studies suggest that hyperglycemia-induced apoptosis in neurons may be driven by the formation of ROS (Chappey et al., 1997). MAP-Kinases represent a central 'switchboard' of intracellular signal transduction pathways. In addition to the classical MAPK-pathway involving the growth-factor stimulated activation (phosphorylation) of the extracellular signal regulated kinase (ERK), several other MAP kinase subclasses, such as c-Jun-N-terminal kinase/stress activated protein kinase (JNK/SAPK) and p38, have recently been identified (Fig. 1). These kinases are activated by stress and may lead, by as yet unknown mechanisms, to apoptosis (Kyriakis and Avruch, 1996). Nerve growth factor (NGF)-signaling involves activation of these three different MAP-Kinase pathways which can either result in differentiation and cell survival, mediated through the ERK pathway, or lead to apoptosis, through the p38 and the JNK/SAPK pathways (Kaplan and Miller, 1997). It has been suggested that hyperglycemia causes diabetic polyneuropathies by a mechanism which involves generation of ROS and alterations in the

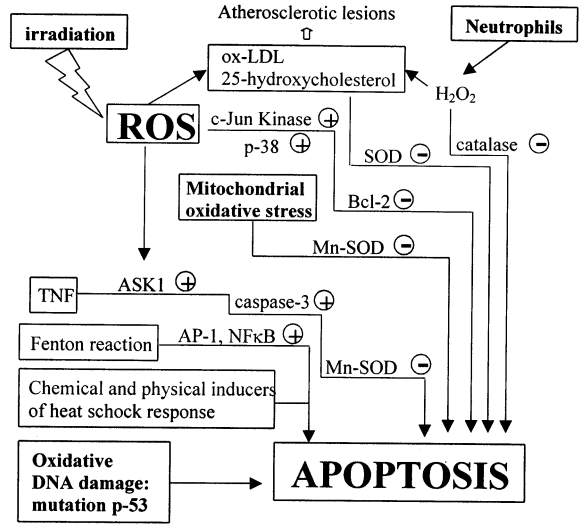

Fig. 1. Main critical steps in the signal transduction cascade leading to apoptosis that are sensitive to oxidants and antioxidants. AP-1, activated protein-1; ASK1, apoptosis signal-regulating kinase 1; NFκB, nuclear transcription factor kappa B; ox-LDL, oxidized low-density lipoproteins; TNF, tumor necrosis factor.

MAPK-dependent cellular signal transduction pathways leading to apoptosis.

6.2. ROS involvement in apoptosis

In the apoptotic process initial stress-induced damage does not kill cells directly, rather it triggers an apoptotic signalling programme that leads to cell death (Gabai et al., 1998).

Apoptotic cell death is characterized by controlled autodigestion of the cell. This differs from necrosis by distinct morphological and biochemical features, such as chromatin condensation, membrane surface blebbing, oligonucleosomal DNA fragmentation and finally, the breakdown of the cell into a series of smaller units (membrane-bound fragments). These are called apoptotic bodies and in most tissues are phagocytosed by adjacent cells (Thompson, 1995). Such events are associated with activation of specific proteases (caspases) and loss of membrane phospholipid asymmetry resulting in phosphatidylserine externalization (Fabisiak et al., 1998). Apoptosis can be initiated by a variety of stimuli, including hyperthermia, growth-factor or hormone withdrawal, glucocorticoids, oxidants, ionizing radiation and multiple classes of chemotherapeutic agents (Hockenbery et al., 1993; Bojes et al., 1997). Cell viability depends on the type of stress exerted on them. Following an apoptotic signal, cells sustain progressive lipid peroxidation. Thus, ROS and oxidative damage have been implicated in the induction of apoptosis (Amstad et al., 1994; Czene et al., 1997; Dimmeler et al., 1998; Tamarit et al., 1998). The Bcl-2 proto-oncogene is unique among cellular genes for its ability in many contexts to block apoptotic deaths. Moreover, a mechanism has been proposed in which Bcl-2 regulates antioxidant pathways at sites of free radical generation (Hockenbery et al., 1993). The protein Bcl-2 protects against apoptosis by blocking cytochrome *c* release (preventing superoxide production when it is overexpressed) hence this protein may have an antioxidant function (Cai and Jones, 1998).

ROS may contribute a novel redox system of regulatory control superimposed upon established growth signal pathways. Levels of GSH may also be involved in these processes as catalase or SOD treatment of fibroblast increase cellular levels of GSH. In addition, α-tocopherol stimulates growth. Thus, whilst hydrogen peroxide may have a role in promoting the growth of transformed and immortalized cells oxidant protection is important (Burdon et al., 1990). On the other hand, Murrell (1992) found how free radicals stimulated fibroblast proliferation and Burdon et al. (1996) show that higher oxidant concentrations not only depress proliferation rates but actually lead to an increase in the appearance of apoptotic-like cells. Inhibitors of GPX, SOD and catalase have a similar effect. Therefore intracellular conditions that are considered more prooxidant than normal, appear to favour apoptosis over proliferation in fibroblasts (Matés and Sánchez-Jiménez, 2000).

7. Summary and future prospects

7.1. State of the art

ROS can be toxic at molecular level and they are important effectors in aging and lifespan determination. The specific cell types, however, in which oxidative damage acts as toxic and to limit lifespan of the whole organism have not been explicitly identified (Parkes et al., 1998). It is fully demostrated, however, that reactive oxygen metabolites are implicated in a wide range of degenerative processes including ischemic heart disease (Melov et al., 1998) as well as in the initiation and promotion of cancer (Urano et al., 1995; Janssen et al., 1998). Free radicals may also play an important role in several pathological conditions of the central nervous system where they directly injure tissue and where their formation may also be a consequence of tissue injury (Table 1). ROS produce tissue damage through multiple mechanisms, including excito-toxicity, metabolic dysfunction, and disturbance of intracellular homeostasis of calcium (Facchinetti et al., 1998).

Many groups are studying about possibilities for antioxidant therapy: they are using adenovirus containing manganese superoxide dismutase cDNA (AdMn-SOD) in the treatment of various

cancers both in vitro and in vivo. This AdMn-SOD has an antitumor effect by itself, but this effect is more pronounced in the presence of the commonly used anticancer drug 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU) (Yang et al., 1999). Additionally, preliminary experiments on bladder protection with intravesicle injection of Mn–SOD plasmid liposomes into the rat have shown reversal of acute physiologic alterations associated with bladder damage. Radioprotective gene therapy with organ-specific targeting of Mn–SOD plasmid liposome provides a valuable technique by which to minimize radiationtoxicity and allow dose escalation to target volumes to which the transit of sensitive normal organs is required to achieve local tumor control. It has been generated two recombinant adenoviral vectors expressing the radical-scavenging enzymes Mn-SOD and Cu, Zn-SOD to test therapeutic strategies of radioprotection. It has been stated that the increase in SOD expression reduced the level of apoptosis, providing the foundation for radioprotective gene therapies in the treatment of cancer (Zwacka et al., 1998). On the other hand, several SODs (plan, bovine and recombinant) are being assayed against HIV infection (Edeas et al., 1997).

Concerning the study of mechanisms by which antioxidant induce gene expression, future research will be required to completely understand the molecular mechanism of signal transduction from antioxidants and xenobiotics. Jaiswal's group has stated the steps of signal transduction from antioxidants and xenobiotics: (1) antioxidants and xenobiotics undergo metabolism to generate superoxide and related reactive species leading to the generation of a signal to activate detoxifying/defensive genes expression; (2) the generation of superoxide and related reactive species is followed by activation of yet to be identified cytosolic factor(s), by unknown mechanism(s); (3) activated cytosolic factor(s) catalyze modification of Nrf2 and/or INrf2; (4) release of Nrf2 from INrf2 followed by nuclear localization of Nrf2 to the nucleus; (5) transcriptional activation and modification? of c-Jun; (6) heterodimerization of Nrf2 with c-Jun; (7) binding of Nrf2-c-Jun complex to the ARE from the various detoxifying enzyme genes; and (8) the coordinated increased transcription of genes encoding detoxifying/defensive proteins.

In addition to Nrf-Jun pathway, the mammalian cells also contain other pathways that activate gene expression in response to oxidative stress. These include NFκB, HIF-1 and Mac-1 mediated pathways. It is expected that collectively these pathways increase transcription of more than four dozen genes to protect cells against oxidative stress.

There is considerable interest in the therapeutic use of antioxidants. This may involve the use of naturally occurring antioxidants or completely synthetic molecules. In addition, there is evidence that some drugs already used clinically may exert part or all their effect by antioxidant mechanisms. Infusions of SOD in liposomes (usually with catalase) have been reported to protect animals against O_2 toxicity. Cu, Zn-SOD has an anti-inflammatory effect in animal models of acute inflammation, in part because it can decrease the number of neutrophils entering sites of inflammation. A wide variety of Cu, Zn-SOD conjugates are available, including polyethylene glycol (PEG)-SOD, Ficoll–SOD, lecithinized SOD, polyamine conjugated SOD, cationized SOD, genetically engineered SOD polymers, pyran-SOD and albumin-SOD complexes. All have longer circulating half-lives than the unconjugated SOD molecules (Halliwell and Gutteridge, 1999).

7.2. Metal complexes interacting with biological systems

It was supposed that metal compounds would be of particular interest because of coordination capacity of the metal center and their ability to catalyze redox processes involving (di)oxygen and active oxygen species as well as biogenic substrates. The interactions of metal complexes with biological systems, which is the field of biocoordination chemistry, is receiving increasing interest. Some authors have presented the first results of their studies concerning the biological activity of organometallic compounds, in particular several alkyne-cobalt carbonyl complexes which inhibited the growth of human melanoma and lung carcinoma cell lines (Jung et al., 1997). It is relevant

to notice that cobalt compounds have been under the increased interest as potential radiosensitizers during the last 10 years. It was observed that cobalt(III) complexes have shown specific hypoxic radiosensitization and thermosensitization as well as antitumor activity in vivo (Stratford, 1992; Teicher et al., 1990; Denny et al., 1996). Some current observations have suggested that further investigations with cobalt-containing complexes are warranted (El-Naggar et al., 1998; Perrin et al., 1999). In this connection, Dori and Gershon (1993)patent claims antitumor action of cobalt(III) complexes with tetradentate Schiff bases derived from aliphatic beta-diketones and diamines, which was observed in the case of ascite form of Erlich carcinoma. Carrying out a more thorough biomedical examination of the complexes in question, it was found a moderate conventional antitumor activity, similar to that described in the just mentioned patent, they exhibit a pronounced modifying effect sharply enhancing the action of radiation and local microwave hyperthermia (Osinsky et al., 1998). Furthemore, the complexes alone are found to display a significant antimetastatic activity exceeding that of platidiam and *cyclo*-phosphamid (Osinsky et al., 1998).

Mutations to Cu, Zn-SOD linked to familial amyotrophic lateral sclerosis (ALS) enhance an unknown toxic reaction that leads to the selective degeneration of motor neurons. However, the question of how >50 different missense mutations produce a common toxic phenotype remains perplexing. Crow et al. (1997) found that the zinc affinity of four ALS-associated SOD mutants was decreased up to 30-fold compared to wild-type SOD but that both mutants and wild-type SOD retained copper with similar affinity. Neurofilament-L (NF-L), one of the most abundant proteins in motor neurons, bound multiple zinc atoms with sufficient affinity to potentially remove zinc from both wild-type and mutant SOD while having a lower affinity for copper. The loss of zinc from wild-type SOD approximately doubled its efficiency for catalyzing peroxynitrite-mediated tyrosine nitration, suggesting that one gained function by SOD in ALS may be an indirect consequence of zinc loss (Liochev et al., 1998). Nitration of protein-bound tyrosines is a permanent modification that can adversely affect protein function. Thus, the toxicity of ALS-associated SOD mutants may be related to enhanced catalysis of protein nitration subsequent to zinc loss. By acting as a high-capacity zinc sink, NF-L could foster the formation of zinc-deficient SOD within motor neurons.

7.2.1. Small molecules mimicing antioxidant enzymes

Modern molecular biology is geared towards genes and proteins, with small molecule chemistry coming in a distant third. Small molecules are important as drugs, but largely ignored in understanding how the cell works. The revival of small molecules started with the discovery that endothelium-relaxing factor, so important for controlling blood pressure, was NO. With a string of recent discoveries, superoxide has now taken center stage. This oxygen radical has been implicated in a long list of normal and disease processes, including reperfusion injury (when a blood is reestablished following surgery, a heart attack, or stroke), neurodegenerative and autoimmune diseases, and inflammatory and mitogenic signaling. Getting rid of superoxide has become a major priority (Krishna et al., 1996).

In 1969, Fridovich and Joe McCord (University of Colorado Health Sciences Center, Denver) discovered the body's primary mode of defense against this leakage: superoxide dismutase. It is remarkable for its use of electrostatic guidance of substrates to exceed diffusion-limited catalytic rates. But as a treatment SOD was found inadequate as it was unstable, did not penetrate into cells, and provoked an immune response. Metals are good at doing redox chemistry, but indiscriminate redox chemistry by free metals is very toxic to the cell. Therefore, to use it as a redox drug you need to have it in a stable ligand. The way to do it is the way nature does it: with a macrocycle. Examples include iron porphyrins, a complex of manganese ions with the chelatins agents desferrioxamine and copper ions chelated to amino acids or to anti-inflammatory drugs (Halliwell and Gutteridge, 1999).

Now a group of companies, including MetaPhore Pharmaceuticals, Inc. (St. Louis, Missouri), is trying to mimic the SOD enzyme with cell-permeable small molecules. MetaPhore's human testing is only scheduled to start late in 2000, but already they have had good success. They started with manganese, which is far less toxic than copper or iron, the other two metals used in native SODs. In his first series of macrocycles there was just one structure (a cyclic penta-aza compound) that was both catalytically active and reasonably stable (Weiss et al., 1996).

Improving on the initial compound took an understanding of the reaction mechanism. Salvemini et al. (1999) found that the rate-limiting step of the reaction cycle was the oxidation of the Mn(II) state to the Mn(III) state. In the Mn(II) state the five nitrogen ligands were in a single plane, with two additional Mn ligands above and below this plane. But in the Mn(III) state one of these axial ligands was replaced by one of the five nitrogens. He therefore added substituents to hold the Mn(II) compound in a folded conformation, with one of the nitrogens out of the plane, ready for the conversion to the Mn(III) state. This compound behaves like an enzyme. They are truly little synthetic enzymes, termed synzymes (Fig. 2). The Mn(II) complex M40403 possesses catalytic SOD activity approaching that of the native Mn-SOD enzyme while at the same time possessing outstanding chemical and biological stability. M40403 selectively removes superoxide without interfering with other relevant biological oxidants, such as nitric oxide, peroxynitrite, or hydrogen peroxide. The end result is a compound with a catalytic rate constant that exceeds 109 M/s, which is ten times faster than the original Mn-based protein. Now it is ready for clinical trials.

Soon after its discovery as a byproduct of oxidative metabolism, superoxide began turning up in many biological systems: as a product of NADPH oxidase in phagocytes, which use a burst of superoxide to help kill bacteria; in signaling cascades involving NFκB in immune cells and ras in cancer cells; and after reperfusion or brain excitotoxicity when metabolism is suddenly resumed or increased. Thus the list of diseases that may be treatable with SOD mimics now includes heart attacks, stroke, autoimmune diseases (such as osteoarthritis), neurodegenerative diseases (including Alzheimer's and Parkinson's) and aging (Table 1). It is the first time that a molecule is a totally selective SOD catalyst. And by eliminating superoxide, the production of pro-inflammatory cytokines can be also eliminated.

Stroke is a severe and prevalent syndrome for which there is a great need for treatment, including agents to block the cascade of brain injury that occurs in the hours after the onset of ischemia. ROS have been implicated in this destructive process, but antioxidant enzymes such as SOD have been unsatisfactory in experimental stroke models. Baker et al. (1998) carried out an evaluation of the effectiveness of salen-manganese complexes, a class of synthetic SOD/catalase mimetics, in a rat focal ischemia model involving middle cerebral artery occlusion. They focus on EUK-134, a newly reported salen-manganese

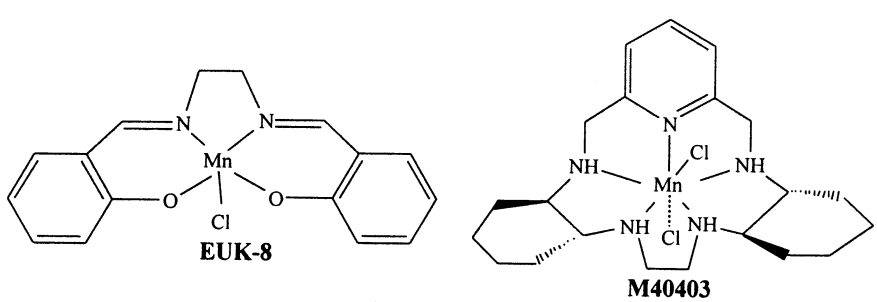

Fig. 2. Structures of some antioxidants synthesized by Metaphore Pharmaceuticals. EUK-8 was the prototype having SOD activity. M40403, a manganese (II) complex with a 20bis(*cyclo*-hexylpyridine)-substituted macrociclic ligand, has SOD activity but does not react with NO, H_2O_2 or $OONO^-$.

complex demonstrated here to have greater catalase and cytoprotective activities and equivalent SOD activity compared with the previously described prototype EUK-8 (Fig. 2). The administration of EUK-134 at 3 h after middle cerebral artery occlusion significantly reduced brain infarct size, with the highest dose apparently preventing further infarct growth. These findings support a key role for ROS in the cascade of brain injury after stroke, even well after the onset of ischemia. The enhanced activity of EUK-134 suggests that, in particular, hydrogen peroxide contributes significantly to this injury. Overall, this study suggests that synthetic SOD/catalase mimetics might serve as novel, multifunctional therapeutic agents for stroke (Baker et al., 1998).

MetaPhore is in the process of choosing its disease target. The company's current leads are not orally bioavailable, so it may decide on an accessible target like radiation-treatment-induced injury in cancer, which is an early indication for the compounds of Eukarion (Bedford, Massachusetts). Eukarion is also interested in neurodegenerative diseases, as their compounds reach the central nervous system. In these longer-term applications there will be more concern about immune suppression, although Eukarion vice president of research S. Doctrow states her company has not seen signs of immune problems in animal models.

Eukarion started with Mn compounds made by Eric Jacobsen (Harvard University, Cambridge, Massachusetts) as catalysts for asymmetric epoxidation. The compounds already had SOD activity, but the company modified them to increase catalase activity: the ability to break down hydrogen peroxide.

John Grow at Princeton University (Princeton, New Jersey) has yet another target: peroxynitrite. $OONO^-$ is formed extremely rapidly when superoxide combines with NO. They are trying to destroy superoxide before it combines with NO. Peroxynitrite scavengers are being developed by Inotek Corporation of Beverly, Massachusetts. As the companies fine-tune their respective compounds, that sorting out will become easier to do.

Metalloporphyrins are able to inhibit lipid peroxidation as prototypical antioxidants, being a novel and potent class of lipid peroxidation inhibitors. This inhibition was dependent on the transition metal ligated to the porphyrin, indicating that metal centered redox chemistry was important to the mechanism of their antioxidant activities. Manganese porphyrins with the highest SOD activities, are the most potent inhibitors of lipid peroxidation. The potencies of the manganese porphyrins were related not only to their redox potentials and SOD activities, but also to other factors that may contribute to their ability to act as electron acceptors. The broad array of antioxidant activities possessed by metalloporphyrins make them attractive therapeutic agents in disease states that involve the overproduction of ROS (Day et al., 1999).

Porphyrin-like SOD mimics are also being investigated by Aeolus Pharmaceuticals (Research Triangle Park, North Carolina) and they think that companies making SOD mimics have chosen their target well as the catalyst is just taking the electron from one to another. If it was a more complicated organic reaction it would be hopeless. Moreover, a similar analysis should be possible for each damage reaction. If the chemistry is easy, perhaps it can also be tailored to cure the ultimate modern obsession: aging. Toxic free radical reactions are the death of cells.

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