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Review

Free radicals and antioxidants in normal physiological functions and human disease

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Abstract

Reactive oxygen species (ROS) and reactive nitrogen species (RNS, e.g. nitric oxide, $NO^{\bullet}$) are well recognised for playing a dual role as both deleterious and beneficial species. ROS and RNS are normally generated by tightly regulated enzymes, such as NO synthase (NOS) and NAD(P)H oxidase isoforms, respectively. Overproduction of ROS (arising either from mitochondrial electron-transport chain or excessive stimulation of NAD(P)H) results in oxidative stress, a deleterious process that can be an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA. In contrast, beneficial effects of ROS/RNS (e.g. superoxide radical and nitric oxide) occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defence against infectious agents, in the function of a number of cellular signalling pathways, and the induction of a mitogenic response. Ironically, various ROS-mediated actions in fact protect cells against ROS-induced oxidative stress and re-establish or maintain "redox balance" termed also "redox homeostasis". The "two-faced" character of ROS is clearly substantiated. For example, a growing body of evidence shows that ROS within cells act as secondary messengers in intracellular signalling cascades which induce and maintain the oncogenic phenotype of cancer cells, however, ROS can also induce cellular senescence and apoptosis and can therefore function as anti-tumourigenic species. This review will describe the: (i) chemistry and biochemistry of ROS/RNS and sources of free radical generation; (ii) damage to DNA, to proteins, and to lipids by free radicals; (iii) role of antioxidants (e.g. glutathione) in the maintenance of cellular "redox homeostasis"; (iv) overview of ROS-induced signaling pathways; (v) role of ROS in redox regulation of normal physiological functions, as well as (vi) role of ROS in pathophysiological implications of altered redox regulation (human diseases and ageing). Attention is focussed on the ROS/RNS-linked pathogenesis of cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease), rheumatoid arthritis, and ageing. Topics of current debate are also reviewed such as the question whether excessive formation of free radicals is a primary cause or a downstream consequence of tissue injury.

Keywords: Oxidative stress; Reactive oxygen species; Nitric oxide; Antioxidants; Human disease; Redox regulation

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1. Introduction

The causes of the poisonous properties of oxygen were obscure prior to the publication of Gershman's free radical theory of oxygen toxicity in 1954, which states that the toxicity of oxygen is due to partially reduced forms of oxygen (Gerschman, Gilbert, Nye, Dwyer, & Fenn, 1954). In the same year, observations of a weak electron paramagnetic resonance (EPR) signal attributable to the presence of free radicals in a variety of lyophilised biological materials were reported by Commoner, Townsend, and Pake (1954). The world of free radicals in biological systems was soon thereafter in 1956 explored by Denham Harman who proposed the concept of free radicals playing a role in the ageing process (Harman, 1956). This work gradually triggered intense research into the field of free radicals in biological systems. A second epoch of the research of free radicals in biological systems was explored in 1969 when McCord and Fridovich discovered the enzyme superoxide dismutase (SOD) and thus provided convincing evidence about the importance of free radicals

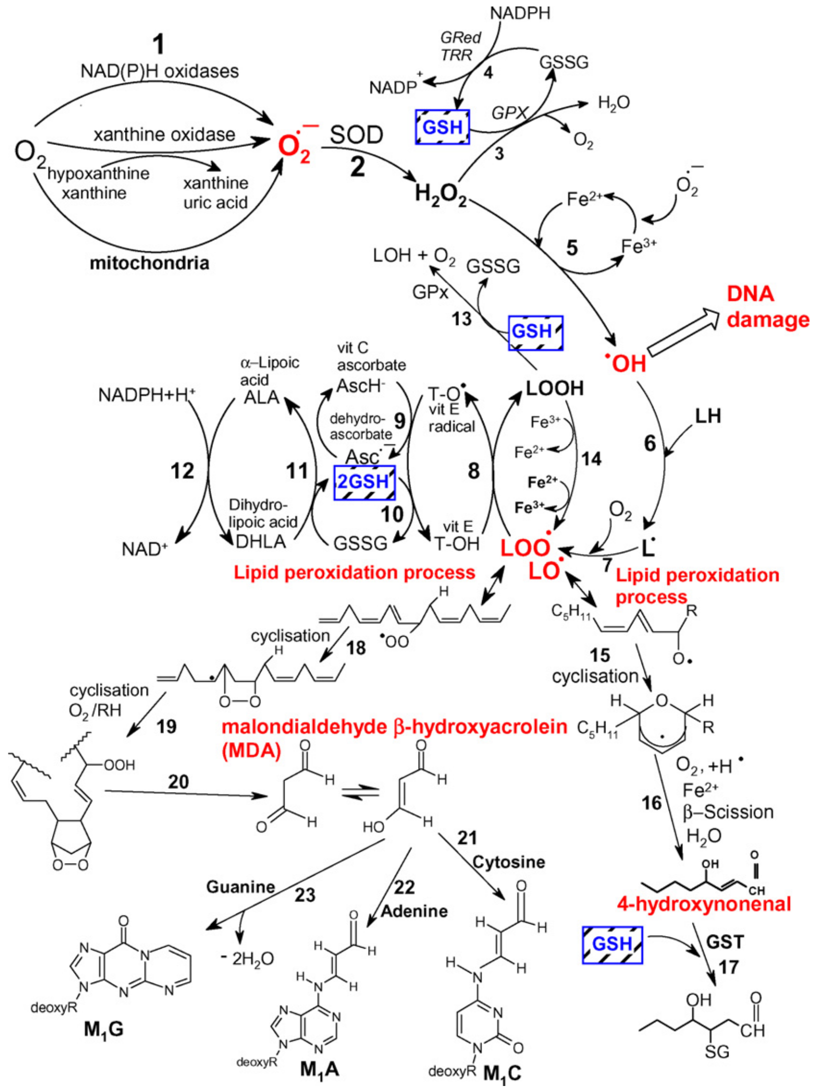

Fig. 1. Pathways of ROS formation, the lipid peroxidation process and the role of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, lipoic acid) in the management of oxidative stress (equations are not balanced). Reaction 1: The superoxide anion radical is formed by the process of reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. Reaction 2: Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. Reaction 3: Hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor. Reaction 4: The oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) which uses NADPH as the electron donor. Reaction 5: Some transition metals (e.g. Fe^{2+}, Cu^{+} and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction). Reaction 6: The hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical ($L^{\bullet}$). Reaction 7: The lipid radical ($L^{\bullet}$) can further interact with molecular oxygen to give a lipid peroxyl radical ($LOO^{\bullet}$). If the resulting lipid peroxyl radical $LOO^{\bullet}$ is not reduced by antioxidants, the lipid peroxidation process occurs (reactions 18–23 and 15–17). Reaction 8: The lipid peroxyl radical ($LOO^{\bullet}$) is reduced within the membrane by the reduced form of Vitamin E (T-OH) resulting in the formation of a lipid hydroperoxide and a radical of Vitamin E ($T\text{-}O^{\bullet}$). Reaction 9: The regeneration of Vitamin E by Vitamin C: the Vitamin E radical ($T\text{-}O^{\bullet}$) is reduced back to Vitamin E (T-OH) by ascorbic acid (the physiological form of ascorbate is ascorbate monoanion, $AscH^{-}$) leaving behind the ascorbyl radical ($Asc^{\bullet-}$). Reaction 10: The regeneration of Vitamin E by GSH: the oxidised Vitamin E radical ($T\text{-}O^{\bullet}$) is reduced by GSH. Reaction 11: The oxidised glutathione (GSSG) and the ascorbyl radical ($Asc^{\bullet-}$) are reduced back to GSH and ascorbate monoanion, $AscH^{-}$, respectively, by the dihydrolipoic acid (DHLA) which is itself converted to α-lipoic acid (ALA). Reaction 12: The regeneration of DHLA from ALA using NADPH. Reaction 13: Lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor. *Lipid peroxidation process*: Reaction 14: Lipid hydroperoxides can react fast with Fe^{2+} to form lipid alkoxyl radicals ($LO^{\bullet}$), or much slower with Fe^{3+} to form lipid peroxyl radicals ($LOO^{\bullet}$). Reaction 15: Lipid alkoxyl radical ($LO^{\bullet}$) derived for example from arachidonic acid undergoes cyclisation reaction to form a six-membered ring hydroperoxide. Reaction 16: Six-membered ring hydroperoxide udergoes further reactions (involving β-scission) to from

in living systems (McCord & Fridovich, 1969). A third era of free radicals in biological systems dates from 1977 when Mittal and Murad provided evidence that the hydroxyl radical, $^{\bullet}OH$, stimulates activation of guanylate cyclase and formation of the "second messenger" cyclic guanosine monophosphate (cGMP) (Mittal & Murad, 1977). Since then, a large body of evidence has been accumulated that living systems have not only adapted to a coexistence with free radicals but have developed various mechanisms for the advantageous use of free radicals in various physiological functions.

Oxygen free radicals or, more generally, reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS are well recognised for playing a dual role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). Beneficial effects of ROS occur at low/moderate concentrations and involve physiological roles in cellular responses to noxia, as for example in defence against infectious agents and in the function of a number of cellular signalling systems. One further beneficial example of ROS at low/moderate concentrations is the induction of a mitogenic response.

The harmful effect of free radicals causing potential biological damage is termed oxidative stress and nitrosative stress (Kovacic & Jacintho, 2001; Ridnour et al., 2005; Valko, Morris, Mazur, Rapta, & Bilton, 2001). This occurs in biological systems when there is an overproduction of ROS/RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidants on the other. In other words, oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant/antioxidant reactions in living organisms. The excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal function. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the ageing process. The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms and is achieved by mechanisms called "redox regulation". The process of "redox regulation" protects living organisms from various oxidative stresses and maintains "redox homeostasis" by controlling the redox status in vivo (Dröge, 2002).

This review examines the available evidence for the involvement of cellular oxidants in the maintenance of "redox homeostasis" in the redox regulation of normal physiological functions as well as pathogenesis of various diseases, including cancer, diabetes mellitus, ischemia/reperfusion injury, inflammatory diseases, neurodegenerative disorders and ageing. A discussion is also devoted to the various protective pathways that may be provided by the antioxidant network against the deleterious action of free radicals.

2. Reactive oxygen species (ROS)

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell & Gutteridge, 1999). This unpaired electron(s) usually gives a considerable degree of reactivity to the free radical. Radicals derived from oxygen represent the most important class of radical species generated in living systems (Miller, Buettner, & Aust, 1990). Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2^{\bullet-}$) (Miller et al., 1990) (see Fig. 1). Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes (Valko, Morris, & Cronin, 2005). Various pathways of ROS formation are outlined in Fig. 1.

The production of superoxide occurs mostly within the mitochondria of a cell (Cadenas & Sies, 1998). The mitochondrial electron transport chain is the main source of ATP in the mammalian cell and thus is essential for life. During energy transduction, a small number of electrons "leak" to oxygen prematurely, forming the oxygen free radical superoxide, which has been implicated in the pathophysiology of a variety of diseases (Kovacic, Pozos, Somanathan, Shangari, & O'Brien, 2005; Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). Measurements on submitochondrial particles suggest an upper

4-hydroxy-nonenal. Reaction 17: 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST, glutathione *S*-transferase). Reaction 18: A peroxyl radical located in the internal position of the fatty acid can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical. Reaction 19: This radical can then either be reduced to form a hydroperoxide (reaction not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide. Reaction 20: Formed compound is an intermediate product for the production of malondialdehyde. Reactions 21, 22, 23: Malondialdehyde can react with DNA bases Cytosine, Adenine, and Guanine to form adducts M_1C, M_1A and M_1G, respectively.

limit of 1–3% of all electrons in the transport chain "leaking" to generate superoxide instead of contributing to the reduction of oxygen to water. Superoxide is produced from both Complexes I and III of the electron transport chain, and once in its anionic form it is too strongly charged to readily cross the inner mitochondrial membrane. Recently, it has been demonstrated that Complex I-dependent superoxide is exclusively released into the matrix and that no detectable levels escape from intact mitochondria (Muller, Liu, & Van Remmen, 2004). This finding fits well with the proposed site of electron leak at Complex I, namely the iron–sulphur clusters of the (matrix-protruding) hydrophilic arm. In addition, experiments on Complex III show direct extramitochondrial release of superoxide, but measurements of hydrogen peroxide production revealed that this could only account for <50% of the total electron leak even in mitochondria lacking Cu, Zn-SOD. It has been proposed that the remaining 50% of the electron leak must be due to superoxide released to the matrix.

The hydroxyl radical, $^{\bullet}OH$, is the neutral form of the hydroxide ion. The hydroxyl radical has a high reactivity, making it a very dangerous radical with a very short *in vivo* half-life of approx. 10^{-9} s (Pastor, Weinstein, Jamison, & Brenowitz, 2000). Thus when produced *in vivo* $^{\bullet}OH$ reacts close to its site of formation. The redox state of the cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits. It has been suggested that iron regulation ensures that there is no free intracellular iron; however, *in vivo*, under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules. The release of iron by superoxide has been demonstrated for [4Fe–4S] cluster-containing enzymes of the dehydratase-lyase family (Liochev & Fridovich, 1994). The released Fe^{2+} can participate in the Fenton reaction, generating highly reactive hydroxyl radical ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$). Thus under stress conditions, $O_2^{\bullet -}$ acts as an oxidant of [4Fe–4S] cluster-containing enzymes and facilitates $^{\bullet}OH$ production from H_2O_2 by making Fe^{2+} available for the Fenton reaction (Valko et al., 2005; Leonard, Harris, & Shi, 2004). The superoxide radical participates in the Haber–Weiss reaction ($O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^-$) which combines a Fenton reaction and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen ($Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$) (Liochev & Fridovich, 2002).

The Fe–S cluster contains also iron responsive elements (IRE)-binding protein (IRE-BP). This Fe–S cluster has been implicated as the region of the protein that senses intracellular iron levels and accordingly modifies the ability of the IRE-BP to interact with iron-responsive elements (IREs). IRE-BP produced in iron-replete cells has aconitase activity (Han et al., 2005). In mammalian cells, oxidants are able to convert cytosolic aconitase into active IRE-BP, which increases the "free iron" concentration intracellularly both by decreasing the biosynthesis of ferritin and increasing biosynthesis of transferrin receptors.

The most realistic *in vivo* production of hydroxyl radical according to the Fenton reaction occurs when M^{n+} is iron, copper, chromium, or cobalt. However, Rae and co-workers recently reported that the upper limit of so-called "free pools" of copper was far less than a single atom per cell (Rae, Schmidt, Pufahl, & O'Halloran, 1999). This finding casts serious doubt on the *in vivo* role of copper in Fenton-like generation of hydroxyl radical. Although Fenton chemistry is known to occur *in vitro*, its significance under physiological conditions is not clear, noting particularly the negligible availability of "free catalytic iron" due to its effective sequestration by the various metal-binding proteins (Kakhlon & Cabantchik, 2002). However, organisms overloaded by iron (as in the conditions of hemochromatosis, b-thalassemia, hemodialysis) contain higher amounts of "free available iron" and this can have deleterious effects. "Free-iron" is transported into an intermediate, labile iron pool (LIP), which represents a steady state exchangeable, and readily chelatable iron compartment (Kakhlon & Cabantchik, 2002).

Additional reactive radicals derived from oxygen that can be formed in living systems are peroxyl radicals ($ROO^{\bullet}$) (see Fig. 1). The simplest peroxyl radical is $HOO^{\bullet}$, which is the protonated form (conjugate acid; $pK_a \sim 4.8$) of superoxide ($O_2^{\bullet -}$) and is usually termed either hydroperoxyl radical or perhydroxyl radical. Given this pK_a value, only ∼0.3% of any superoxide present in the cytosol of a typical cell is in the protonated form (De Grey, 2002). It has been demonstrated that hydroperoxyl radical initiates fatty acid peroxidation by two parallel pathways: fatty acid hydroperoxide (LOOH)-independent and LOOH-dependent (Aikens & Dix, 1991). The LOOH-dependent pathway of $HO_2^{\bullet}$-initiated fatty acid peroxidation may be relevant to mechanisms of lipid peroxidation initiation *in vivo*.Xanthine oxidase (XO, EC 1.1.3.22) and xanthine dehydrogenase (XD, EC 1.1.1.204) are interconvertible forms of the same enzyme, known as xanthine oxidoreductase (XOR) (Borges, Fernandes, & Roleira, 2002; Vorbach, Harrison, & Capecchi, 2003). In purine catabolism, XOR catalyzes the oxidative hydroxylation of hypoxanthine to xanthine and subsequently of xanthine to uric acid. Uric acid acts as a potent antioxidant and free radical scavenger. XOR has, therefore, important functions as a cellular defense enzyme against oxidative stress. With both XO and XD forms, but particularly with the XO form, numerous ROS and RNS are synthesized (Vorbach et al., 2003). Thus, the synthesis of both an antioxidant (uric acid) and numerous free radicals (ROS and RNS) makes XOR an important protective regulator of the cellular redox potential.

Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet-}$, under physiologic conditions (Valko et al., 2004). Peroxisomes are major sites of oxygen consumption in the cell and participate in several metabolic functions that use oxygen. Oxygen consumption in the peroxisome leads to H_2O_2 production, which is then used to oxidize a variety of molecules. The organelle also contains catalase, which decomposes hydrogen peroxide and presumably prevents accumulation of this toxic compound. Thus, the peroxisome maintains a delicate balance with respect to the relative concentrations or activities of these enzymes to ensure no net production of ROS. How the organelle maintains this equilibrium is unclear. When peroxisomes are damaged and their H_2O_2 consuming enzymes downregulated, H_2O_2 releases into the cytosol which is significantly contributing to oxidative stress.

If a phagocytic cell such as the neutrophil is exposed to a stimulus, it has the ability of recognising the foreign particle and undergoing a series of reactions called the respiratory burst (DeCoursey & Ligeti, 2005). Nicotine adenine dinucleotide phosphate (NAD(P)H) oxidase is best characterised in neutrophils, where its production of $O_2^{\bullet-}$ generates the respiratory burst necessary for bacterial destruction. The enzyme complex consists of two membrane-bound components, $gp91^{phox}$ and $p22^{phox}$, which comprise cytochrome b558, the enzymatic centre of the complex. After activation, cytosolic components, involving $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and the small G coupled proteins, Rac and Rap1A, translocate to the membrane to form the active enzyme complex. The non-phagocytic NAD(P)H oxidases produce superoxide at a fraction (1–10%) of the levels produced in neutrophils and are thought to function in intracellular signalling pathways (see also below).

3. Reactive nitrogen species (RNS)

$NO^{\bullet}$ is a small molecule that contains one unpaired electron on the antibonding $2\pi_y^*$ orbital and is, therefore, a radical. $NO^{\bullet}$ is generated in biological tissues by specific nitric oxide synthases (NOSs), which metabolise arginine to citrulline with the formation of $NO^{\bullet}$ *via* a five electron oxidative reaction (Ghafourifar & Cadenas, 2005). Nitric oxide ($NO^{\bullet}$) is an abundant reactive radical that acts as an important oxidative biological signalling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Bergendi, Benes, Durackova, & Ferencik, 1999). Due to its extraordinary properties, in 1992 was $NO^{\bullet}$ acclaimed as the "molecule of the year" in *Science Magazine* (Koshland, 1992).

$NO^{\bullet}$ has a half-life of only a few seconds in an aqueous environment. $NO^{\bullet}$ has greater stability in an environment with a lower oxygen concentration (half-life >15 s). However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes (Chiueh, 1999). $NO^{\bullet}$ has effects on neuronal transmission as well as on synaptic plasticity in the central nervous system. In the extracellular milieu, $NO^{\bullet}$ reacts with oxygen and water to form nitrate and nitrite anions.

Overproduction of reactive nitrogen species is called nitrosative stress (Klatt & Lamas, 2000; Ridnour et al., 2004). This may occur when the generation of reactive nitrogen species in a system exceeds the system's ability to neutralise and eliminate them. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function.

Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$), which is a potent oxidising agent that can cause DNA fragmentation and lipid oxidation (Carr, McCall, & Frei, 2000):

$$NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^- \quad (1)$$

Reaction (1) has one of the highest rate constants known for reactions of $NO^{\bullet}$, $7.0 \times 10^9\ M^{-1}\ s^{-1}$. Thus $NO^{\bullet}$ toxicity is predominantly linked to its ability to combine with superoxide anions.

Nitric oxide readily binds certain transition metal ions; in fact many physiological effects of $NO^{\bullet}$ are exerted as a result of its initial binding to Fe^{2+}-Haem groups in the enzyme soluble guanylate cyclase (sGC) (Archer, 1993)

$$Fe^{2+}\{sGC\} + NO^{\bullet} \rightarrow Fe^{2+}\{sGC\}\text{–}NO \quad (2)$$

The product is represented here as $\{Fe^{2+}\text{–}NO^{\bullet}\}$, however, $\{Fe^{3+}\text{–}NO^-\}$ is also commonly seen. The convention $\{FeNO\}^7$, where the superscript is the sum of the metal d electron count (here 6 or 5) and the occupancy of the relevant NO π^* orbital (here 1 or 2), is often employed to avoid specific assignment of oxidation states.

4. Oxidative damage to DNA, lipids and proteins

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids, lipids and proteins (Valko et al., 2006). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell & Gutteridge, 1999). The most extensively studied DNA lesion is the formation of 8-OH-G. Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis, and ageing.

It is known that metal-induced generation of ROS results in an attack not only on DNA, but also on other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Siems, Grune, & Esterbauer, 1995). Once formed, peroxyl radicals ($ROO^{\bullet}$) can be rearranged *via* a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) (Fedtke, Boucheron, Walker, & Swenberg, 1990; Fink, Reddy, & Marnett, 1997; Mao, Schnetz-Boutaud, Weisenseel, Marnett, & Stone, 1999; Marnett, 1999; Wang et al., 1996) (Fig. 1). The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Hydroxynonenal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation.

Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionising radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed (Stadtman, 2004). The side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS/RNS (Stadtman, 2004). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (*S*-glutathiolation). The concentration of carbonyl groups, generated by many different mechanisms is a good measure of ROS-mediated protein oxidation. A number of highly sensitive methods have been developed for the assay of protein carbonyl groups (Dalle-Donne, Giustarini, Colombo, Rossi, & Milzani, 2003; Dalle-Donne et al., 2005).

Advanced glycation end products (AGEs) is a class of complex products. They are the results of a reaction between carbohydrates and free amino group of proteins. The intermediate products are known, variously, as Amadori, Schiff Base and Maillard products, named after the researchers who first described them (Dalle-Donne et al., 2005). Most of the AGEs are very unstable, reactive compounds and the end products are difficult to be completely analysed. The brown colour of the AGEs is probably related to the name of melanoidins initially proposed by Maillard, and well known from food chemistry. The best chemically characterised AGEs compounds found in human are pentosidine and carboxyl methyl lysine (CML).

5. Antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). Defence mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanisms, (iii) physical defences, and (iv) antioxidant defences. Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT). Non-enzymatic antioxidants are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, and other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health. Various pathways for the management of oxidative stress by GSH and other antioxidants are shown in Fig. 1.

Here we briefly desribe the role of major thiol antioxidant and redox buffer of the cell, the tripeptide, glutathione (GSH) (Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005). The oxidised form of glutathione is GSSG, glutathione disulphide. Glutathione is highly abundant in the cytosol (1–11 mM), nuclei (3–15 mM), and mitochondria (5–11 mM) and is the major soluble antioxidant in these cell compartments. Because GSH is synthesized in the cytosol by the sequential action of glutamate–cysteine ligase and glutathione synthetase, its mitochondrial presence requires inner membrane transport. Two mitochondrial electroneutral antiport carrier proteins have been shown to have the capacity to transport GSH, the dicarboxylate carrier protein and the 2-oxoglutarate carrier protein. Recently, it has been shown that externally added GSH is readily taken up by mitochondria, despite the ∼8 mM GSH present in the mitochondrial matrix (Shen, Dalton, Nebert, & Shertzer, 2005). It therefore appears that GSH is taken up against a concentration gradient.

GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. Oxidised glutathione is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Nogueira, Zeni, & Rocha, 2004; Jones et al., 2000). Too high a concentration of GSSG may damage many enzymes oxidatively.

The main protective roles of glutathione against oxidative stress are (Masella et al., 2005): (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathionetransferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathionperoxidase; (iv) glutathione is able to regenerate the most important antioxidants, Vitamins C and E, back to their active forms; glutathione can reduce the tocopherol radical of Vitamin E directly, or indirectly, *via* reduction of semidehydroascorbate to ascorbate (Fig. 1). The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide-glutathione couple (GSSG/2GSH) (Pastore, Federici, Bertini, & Piemonte, 2003).

The various roles of enzymatic antioxidants (SOD, Catalase, glutathione peroxidase) and non-enzymatic antioxidants (Vitamin C, Vitamin E, carotenoids, lipoic acid and others) in the protection against oxidative stress can be found in a numerous reviews and original papers (see Fig. 1) (Burton & Ingold, 1984; Cameron & Pauling, 1976; Carr & Frei, 1999; Catani et al., 2001; El-Agamey et al., 2004; Hirota et al., 1999; Kojo, 2004; Landis & Tower, 2005; Makropoulos, Bruning, & SchulzeOsthoff, 1996; Mates, Perez-Gomez, & De Castro, 1999; Miller et al., 2005; Nakamura, Nakamura, & Yodoi, 1997; Packer & Suzuki, 1993; Pryor, 2000; Schrauzer, 2006; Sharoni, Danilenko, Dubi, Ben-Dor, & Levy, 2004; Smith, Shenvi, Widlansky, Suh, & Hagen, 2004; White, Shannon, & Patterson, 1997).

6. ROS and mechanisms of maintenance of "redox homeostasis"

Free radicals and reactive diamagnetic species derived from radicals operate at low, but measurable concentrations in the cells. Their "steady state" concentrations are determined by the balance between their rates of production and their rates of removal by various antioxidants. Thus each cell is characterised by a particular concentration of electrons (redox state) stored in many cellular constituents and the redox state of a cell and its oscillation determines cellular functioning (Schafer & Buettner, 2001). In recent years the term "redox state" has not only been used to describe the state of a redox pair, e.g. GSSG/2GSH, $Asc^{\bullet-}/AcsH^{-}$ and others, but also to describe more generally the redox environment of a cell (Butler, 2000; Schafer & Buettner, 2001). The redox state of a cell is kept within a narrow range under normal conditions—similar to the manner in which a biological system regulates its pH. Under pathological conditions, the redox state can be altered to lower or higher values. A 30 mV change in the redox state means a 10-fold change in the ratio between reductant and oxidant species (Schafer & Buettner, 2001).

The intracellular "redox homeostasis" or "redox buffering" capacity is substantiated primarily by GSH and thioredoxin (TRX). The glutathione (2GSH/GSSG couple) represents the major cellular redox buffer and therefore is a representative indicator for the redox environment of the cell (Dröge, 2002; Schafer & Buettner, 2001). Under enhanced oxidative stress conditions, GSSG content increases, this in turn increases the content of protein mixed disulphides. A significant number of proteins involved in signalling that have critical thiols, such as receptors, protein kinases and some transcription factors can be altered in their function by formation of mixed disulphides. In this regard, GSSG appears to act as a non-specific signalling molecule.

The high ratios of reduced to oxidised GSH and TRX are maintained by the activity of GSH reductase and TRX reductase, respectively. Both of these "redox buffering" thiol systems counteract intracellular oxidative stress; in addition to antioxidant functioning in the cell, GSH and TRX are involved in cell signalling process (Dröge, 2002; Thannickal & Fanburg, 2000).

In addition to GSH and TRX, there are other relatively low molecular weight antioxidants, that when present at high concentration, can significantly contribute to overall ROS scavenging activity (McEligot, Yang, & Meyskens, 2005; Sies, 1993). These include various free amino acids, peptides, and proteins. Oxidised proteins are substrates for proteolytic digestion and contribute to maintenance of redox homeostasis in the cell (Dröge, 2002). Oxidative modifications of proteins increase their susceptibility to proteolytic attack; proteolytic degradation is executed mainly by proteasomes. Proteolysis was estimated to increase more than 10-times after exposure to superoxide radical or hydrogen peroxide. It should be noted that proteins significantly vary in their susceptibility to oxidative damage. For example intact proteins are less sensitive to oxidation than misfolded proteins.

The term redox signalling is used to describe a regulatory process in which the signal is delivered through redox reactions. Redox signalling requires that the steady state of "redox balance" is disturbed either by an increase in ROS formation or a decrease in the activity of antioxidant system(s). The regulated increase in free radicals (ROS/RNS) leads to a temporary imbalance that represents the physiological basis for redox regulation. Thus physiological demonstration of redox regulation involves a temporary shift of the intracellular redox state toward more oxidising conditions. Signalling mechanisms that respond to changes in the thiol/disulphide redox state involve: (i) transcription factors AP-1 and NF-κB; (ii) bacterial OxyR; (iii) protein tyrosine phosphatases; (iv) Src family kinases; (v) JNK and p38 MAPK signalling pathways; (vi) insulin receptor kinase activity, and others (Dröge, 2002; Galter, Mihm, & Dröge, 1994; Hehner et al., 2000; Kuge & Jones, 1994; Aslund, Zheng, Beckwith, & Storz, 1999). Under pathological conditions, however, abnormally large concentrations of ROS/RNS may lead to permanent changes in signal transduction and gene expression, typical for disease states.

The process of redox signalling is adopted by various organisms including bacteria to induce protective responses against oxidative stress and to restore the original state of "redox homeostasis" after temporary exposure to ROS/RNS. For example, the production of $NO^{\bullet}$ is the subject of direct feedback inhibition of NOS by $NO^{\bullet}$.

Prokaryotes have several different signalling pathways for responding to ROS or to alterations in the intracellular redox state. Studies on *Escherichia coli* explored that low levels of ROS activate expression of several gene products involved in antioxidant defence including Mn-SOD, catalase, glutathione reductase, and others. Several proteins that are synthesised in *E. coli* after exposure to hydrogen peroxide are under the control of the OxyR locus. The OxyR protein controls protective responses against lethal doses of hydrogen peroxide or against killing by heat (Aslund et al., 1999). Hydrogen peroxide or an oxidative shift in the intracellular thiol/disulphide redox state converts the reduced form of OxyR (containing –SH groups) into its oxidised and regulatory active form containing –S–S– groups. The formation of disulphide bonds can be reversed by glutaredoxin and by thioredoxin.

One of the best studied models of redox regulation in mammalian cells is the redox control of heme oxygenase-1 (HO-1) (Keyse & Tyrrell, 1989). HO-1 induction in skin fibroblasts may serve as an inducible defence pathway to remove heme liberated by oxidants. The HO-1 protein and mRNA are strongly induced by ROS, UVA irradiation and various stressors; thus the inducibility of HO-1 mRNA in many tissues and various mammalian species has rendered HO-1 mRNA a useful marker for cellular oxidative stress at the mRNA level.

As menioned above, the cell cycle is characterised by fluctuations in the redox environment of a cell, mediated, in particular by intracellular changes in concentration of glutathione (Arrigo, 1999; Kern & Kehrer, 2005; Schafer & Buettner, 2001). GSH has been shown to play a role in the rescue of cells from apoptosis; depletion of GSH, which renders the cellular environment more oxidising, was concomitant with the onset of apoptosis. Generally, a more reducing environment (maintained by elevated levels of glutathione and thioredoxin) of the cell stimulates proliferation and a slight shift towards a mildly oxidising environment initiates cell differentiation. A further shift towards a more oxidising environment in the cell leads to apoptosis and necrosis. While apoptosis is induced by moderate oxidising stimuli, necrosis is induced by an intense oxidising effect (Cai & Jones, 1998; Evens, 2004; Voehringer et al., 2000).

From the above discussion is clear that the redox environment is the critical determinant for the trigger of apoptosis. Recent studies indicate that a knowledge of the mechanisms by which TRX, GSH, and Ref-1 maintain the intracellular "redox buffering" capacity can conveniently be used in the development of targeted cancer-preventive and therapeutic drugs (Evens, 2004).

7. ROS, antioxidants and signal transduction—an overview

Cells communicate with each other and respond to extracellular stimuli through biological mechanisms called cell signalling or signal transduction (Poli, Leonarduzzi, Biasi, & Chiarpotto, 2004). Signal transduction is a process enabling information to be transmitted from the outside of a cell to various functional elements inside the cell. Signal transduction is triggered by extracellular signals such as hormones, growth factors, cytokines and neurotransmitters (Thannickal & Fanburg, 2000). Signals sent to the transcription machinery responsible for expression of certain genes are normally transmitted to the cell nucleus by a class of proteins called transcription factors. By binding to specific DNA sequences, these factors regulate the activity of RNA polymerase II. These signal transduction processes can induce various biological activities, such as muscle contraction, gene expression, cell growth, and nerve transmission (Thannickal & Fanburg, 2000).

While ROS are predominantly implicated in causing cell damage, they also play a major physiological role in several aspects of intracellular signalling and regulation (Dröge, 2002). It is a well-known feature that cells are capable of generating endogenously and constitutively ROS which are utilized in the induction and maintenance of signal transduction pathways involved in cell growth and differentiation.

Most cell types have been shown to elicit a small oxidative burst generating low concentrations of ROS when they are stimulated by cytokines, growth factors and hormones, e.g. interleukin-1β (IL-1β), interleukin 6 (IL-6), interleukin 3 (IL-3), tumor necrosis factor-α (TNF-α), angiotensin II (ANGII), platelet derived growth factor (PDGF), nerve growth factor (NGF), transforming growth factor-β1 (TGF-β1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and fibroblast growth factor (FGF-2) (Thannickal & Fanburg, 2000). This led to the assumption that the initiation and/or proper functioning of several signal transduction pathways rely on the action of ROS as signalling molecules which may act on different levels in the signal transduction cascade. ROS can thus play a very important physiological role as secondary messengers (Lowenstein, Dinerman, & Snyder, 1994; Storz, 2005). Probably the most significant effect of metals and ROS on signalling pathways has been observed in the mitogen-activated protein kinase (MAPK) pathways (Sun & Oberley, 1996). Fig. 2 summarises activation of MAPK signalling pathways.

7.1. Cytokines and growth factor signalling

A variety of cytokines and growth factors that bind to receptors of different classes have been reported to generate ROS in nonphagocytic cells. Growth factor receptors are tyrosine kinases (RTKs) that play a key role in the transmission of information from outside the cell into the cytoplasm and the nucleus (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999). The information is transmitted *via* the activation of mitogen-activated protein kinases (MAPKs) signalling pathways (Mulder, 2000). ROS production as a result of activated growth factor receptor signalling includes epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, vascular endothelial growth factor (VEGF) (Neufeld et al., 1999). Further examples involve cytokine receptors (TNF-α and IFN-γ) or interleukin receptors (IL-1β) (Sundaresan et al., 1996). Cytokines receptors fall into a large and heterogenous group of

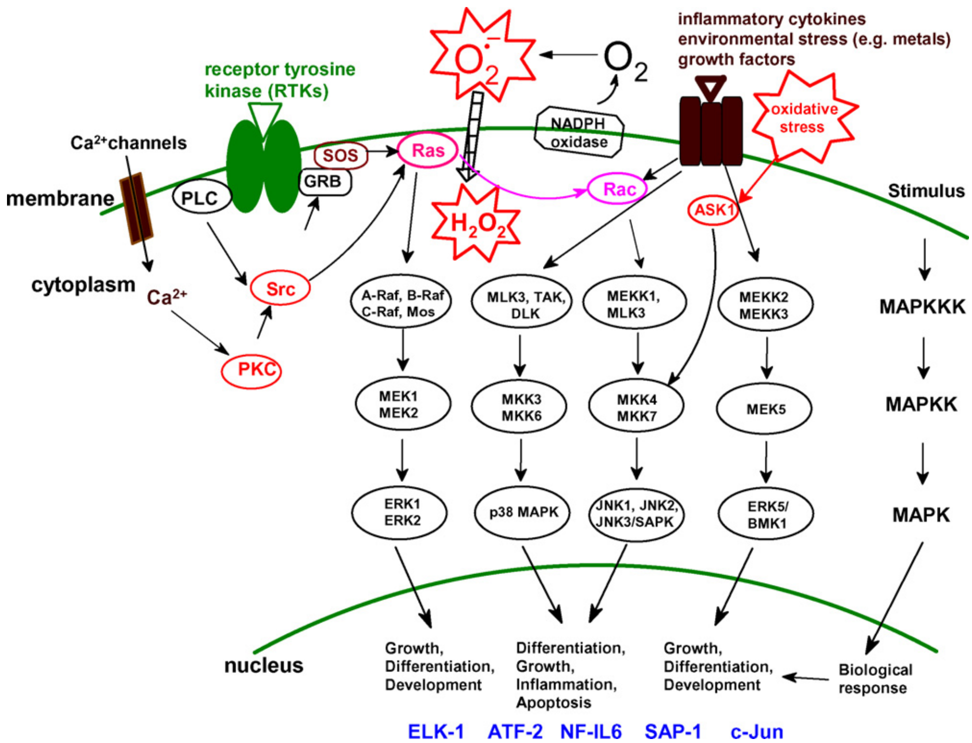

Fig. 2. ROS-induced MAPK signalling pathways.

receptors that lack intrinsic kinase activity and are most directly linked to ion channels or G proteins. Cytokines such as TNF-α, IL-1 and interferon (IFN-γ) were among those first reported to generate ROS in nonphagocytic cells (Chapple, 1997). It is generally accepted that ROS generated by these ligand/receptor-initiated pathways can function as true second messengers and mediate important cellular functions such as proliferation and programmed cell death.

7.2. Non-receptor tyrosine kinases

In addition to receptor tyrosine kinases, several non-receptor protein kinases (PTKs) belonging to the Src family (Src kinases) and Janus kinase (JAK) are also activated by ROS (Abe & Berk, 1999). For example hydrogen peroxide and superoxide radical induce tyrosine phosphorylation of several PTKs in different cell types, including fibroblasts, T and B lymphocytes, macrophages and myeloid cells. Activated Src binds to cell membranes by myristilation and initiates MAPK, NF-κB, and PI3K signalling pathways (Fig. 2).

7.3. Protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPs) are probably the best characterised direct targets of ROS. Reversible inactivation of PTPs by ROS plays an important role in the redox control and cell signalling. It has been shown that inhibition of PTPs by ROS may directly trigger PTKs. The effects of ROS occur through targeting the cysteine-containing residues of the active sites of tyrosine phosphatases (Salmeen & Barford, 2005). Cystein residues are most susceptible to oxidative damage by hydrogen peroxide and other oxidants, producing sulfenic acid intermediates which can further react with thiols to form catalytically inactive PTP disulfides. Superoxide radical was also shown to regulate the activity of PTPs very efficiently, in particular PTP-1B *via* cysteine residues.

7.4. Serine/threonine kinases

All receptor serine/threonine kinases described in mammalian cells are members of TGF-β superfamily. The TGF-β1 has been shown to stimulate ROS production in a variety of cells and typically inhibits the growth of most target cells (Shaw, Cohen, & Alessi, 1998). TGF-β1 has also been shown to suppress the expression of antioxidant enzymes in some cells. TGF-β1 inhibited the expression of Mn-SOD, Cu, Zn-SOD and catalase in rat hepatocytes.

Akt is a serine/threonine kinase, recruited to the cell membrane by PI3k and activated by phosphorylation. The end result of Akt activation is stimulation of growth pathways and inhibition of apoptotic pathways. Conversely, inhibition of Akt may result in apoptosis. VEGF activation by ROS in mouse muscle cells occurs *via* the PI3K/Akt pathway.

Calcium has been well recognised as a signalling factor involved in the regulation of a wide range of cellular processes involving cell proliferation, cell differentiation and apoptosis (Parekh & Penner, 1997). Experiments revealed that ROS induce release of calcium from intracellular stores, resulting in the activation of kinases, such as protein kinases C (PKCs) a member of serine/threonine kinases.

Among serine/threonine kinases, PKC is subjected to a rather complicated cellular redox regulation. PKC contains several cysteine rich regions both in the zinc finger of the regulatory domain and in the catalytic site which can be modified by various oxidants (Gopalakrishna & Jaken, 2000). One of the possible mechanisms of the PKC activation is tyrosine phosphorylation and conversion to the Ca^{2+}/phospholipid-independent form. It appears certain that oxidant-induced PKC activation plays a critical role in cancer proliferation and clearly this has important functional consequences on downstream signalling pathways; i.e. activation of MAPKs, defined transcription factors and proto-oncogenes (Dempsey et al., 2000).

The group of proteins termed mitogen-activated protein kinases relay signals generated by exogenous and endogenous stimuli to intracellular space *via* phosphorylation of proteins. During this process of intracellular communication, MAPKs interact with upstream mediators, involving growth factor receptors, G-proteins, tyrosine kinases and downstream mediators, such as nuclear transcription factors (Lopez-Ilasaca, Crespo, Pellici, Gutkind, & Wetzker, 1997).

A number of studies reported that the serine/threonine kinases of the MAPK family can be regulated by oxidants. There are four known MAPK families: extracellular-regulated (ERKs), c-jun-NH_2-terminal kinase (JNKs), p38 MAPK and the big MAPK-1 (BMAPK-1), of which serine/thereonine kinases are important in the process of carcinogenesis including cell proliferation, differentiation and apoptosis (Kyriakis & Avruch, 2001). Products of NOX1 activity, superoxide, hydrogen peroxide can activate the MAPK cascade at the level of MEK and ERK1/2. The experimental studies on the up-regulation of MAPKs by H_2O_2 treatment have shown that the activation of each signalling pathway is type- and stimulus-specific. For example, it has been reported that endogenous H_2O_2 production by the respiratory burst induces ERK but not p38 kinase activity (Iles & Forman, 2002). Conversely, exogenous H_2O_2 activates p38 kinase, but not ERK in rat alvedor macrophages. The ERK pathway has most commonly been associated with the regulation of cell proliferation. The balance between ERK and JNK activation is a key factor for cell survival since both a decrease in ERK and an increase in JNK are required for the induction of apoptosis.

7.5. Nuclear transcription factors

As already mentioned above, probably the most significant effect of ROS on signalling pathways has been observed in the MAPK pathways (Sun & Oberley, 1996). This involves activation of nuclear transcription factors. These factors control the expression of protective genes that repair damaged DNA, power the immune system, arrest the proliferation of damaged cells, and induce apoptosis. The nuclear transcription factor NF-κB, is involved in inflammatory responses and AP-1 is important for cell growth and differentiation. p53 is a gene whose disruption is associated with more than half of all human cancers (Sun & Oberley, 1996). The p53 protein guards a cell-cycle checkpoint, as inactivation of p53 allows uncontrolled cell division. The nuclear factor of activated T cells (NFAT) regulates cytokine formation, muscle growth and differentiation, angiogenesis and adipogenesis. HIF-1 regulates the expression of many cancer-related genes including VEGF, enolase, heme oxygenase 1 and lactate dehydrogenase A.

7.5.1. AP-1

AP-1 is a collection of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (FosB, Fra-1, Fra-2), Maf, and ATF subfamilies, all of which can bind the tumour-promoting agent (TPA) or cAMP response elements. c-Jun, a potent transcriptional regulator, often forms stable heterodimers with Jun proteins, which aid the binding of Jun to DNA (Rao, Luo, & Hogan, 1997). AP-1 activity is induced in response to certain metals in the presence of H_2O_2 as well as by several cytokines and other physical and chemical stresses.

7.5.2. NF-κB

A number of reports published during recent years indicate that some ROS/metals are able to affect the activation or activity of NF-κB transcription factors (Pande & Ramos, 2005). NF-κB is an inducible and ubiquitously expressed transcription factor for genes involved in cell survival, differentiation, inflammation, and growth.

NF-κB is a DNA binding protein that interacts with the enhancing domain of target genes in the configuration of a dimer of two members of the NF-κB /Rel/Dorsal (NRD) family of proteins (Pande & Ramos, 2005). Although there are five known NRD members, RelA (also called p65), cRel, RelB, p50 (also called NF-κB1) and p52 (also called NF-κB2), the classical dimer is composed of p50 and RelA. Only RelA contains a transactivation domain that activates transcription by an interaction with the basal transcription apparatus. In unstimulated cells, NF-κB is sequestered in the cytoplasm because of an interaction with a member of the inhibitory (IκB) family. Activation of NF-κB occurs in response to a wide variety of extracellular stimuli that promote the dissociation of IκB, which unmasks the nuclear localization sequence and thereby allows entry of NF-κB into the nucleus and binds κB regulatory elements. NF-κB regulates several genes involved in cell transformation, proliferation, and angiogenesis (Amiri & Richmond, 2005). Expression of NF-κB has been shown to promote cell proliferation, whereas on the other inhibition of NF-κB activation blocks cell proliferation. Reactive oxygen species have been implicated as second messengers involved in the activation of NF-κB *via* tumour necrosis factor (TNF) and interleukin-1 (Baud & Karin, 2001).

7.5.3. p53

The nuclear factor p53 plays a key role in protecting a cell from tumourigenesis (Hofseth, Hussain, & Harris, 2004). Due to its ability to halt the cell cycle or initiate apoptosis if cell is damaged, it is often called a "tumour suppressor". Mutations in p53 leading to its inactivation has been found in more than half of human cancers (Hofseth et al., 2004). P53 is activated by UV radiation, hypoxia, gamma-radiation, nucleotide deprivation and others. Several cysteine residues in the central domain of the protein are critical for p53 binding to the specific DNA sequence.

The p53 family commonly upregulate at least two proteins that participate in ROS mediated apoptosis: ferrodoxin reductase (FDXR) and REDD1/HIF-1. In addition to the generation of ROS, p53 induces the expression of p85, which may function as a signalling molecule during ROS-mediated p53-dependent apoptosis. p85 is a known regulator of phosphatidyl inositol-3 kinase (PI3K); however, its function during ROS-induced apoptosis is independent of PI3K.

Recent results by Sablina and coworkers (Sablina et al., 2005) indicate that under normal/low cellular stress, low concentrations of p53 induce the expression of antioxidant genes, whereas in severe cellular stress, high concentrations of p53 promote the expression of genes that contribute to ROS formation and p53-mediated apoptosis. Thus under normal/low stress conditions, p53 appears to have an antioxidant role that protects cells from oxidative DNA damage and although this effect might depend on the concentration of p53, other cellular factors likely participate in a cell's final fate. The relative pro-apoptotic and anti-apoptotic functions of p53 would appear to depend at least partly on the cellular p53 concentration as well as on other factors, such as p53 subcellular localization, phosphorylation status and others (Tomko, Bansal, & Lazo, 2006).

7.5.4. NFAT

The nuclear factor of activated T cells (NFAT) family of nuclear transcription factors regulates muscle growth and differentiation, cytokine formation, angiogenesis and other processes. Four of five NFAT proteins are calcium dependent (Rao et al., 1997). NFAT is activated by phosphatase calcineurin, which is in turn activated by high intracellular calcium levels. Various ROS/metals are known to increase intracellular calcium and this may represent a probable mechanism by which metals activate NFAT.

7.5.5. HIF-1

HIF-1 (hypoxia-inducible factor) is a heterodimer and is composed of two bHLH proteins, HIF-1α and HIF-1β. HIF-1α is expressed and HIF-1β accumulated only in hypoxic cells (Semenza, 2000). HIF-1 regulates the expression of many cancer-related genes including VEGF, aldolase, enolase, lactate dehydrogenase A and others. HIF-1 is induced by the expression of oncogenes such as Src and Ras and is overexpressed in many cancers. VEGF as one of the HIF-1 regulated proteins plays an important role in tumour progression and angiogenesis.

8. ROS and redox regulation of physiological functions

A great number of physiological functions are controlled by redox-responsive signalling pathways (Dröge, 2002). These, for example involve: (i) redox regulated production of NO; (ii) ROS production by phagocytic NAD(P)H oxidase (oxidative burst); (iii) ROS production by NAD(P)H oxidases in nonphagocytic cells; (iv) regulation of vascular tone and other regulatory functions of $NO^{\bullet}$; (v) ROS production as a sensor for changes of oxygen concentration; (vi) redox regulation of cell adhesion; (vii) redox regulation of immune responses; (viii) ROS-induced apoptosis and other mechanisms. Here we very briefly discuss the basic principles of the above-mentioned redox-regulated physiological functions.

(i) $NO^{\bullet}$ is generated in biological tissues by specific nitric oxide synthases (NOSs) which exist in three isoforms, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Bredt et al., 1991; Lamas, Marsden, Li, Tempst, & Michel, 1992; Xie et al., 1992). Many tissues express one or more of these isoforms. While nNOS and eNOS are constitutively expressed and their activity is regulated by the intracellular calcium concentration, the isoform iNOS is inducibly expressed in macrophages following stimulation by lipopolysaccharides, cytokines and other agents. Expression of iNOS is regulated at the transcriptional and posttranscriptional level by signalling pathways involving redox-dependent transcription factor NF-κB or mitogen activated protein kinases (MAPKs).

(ii) Oxidative burst is characterised by massive production of ROS in an inflammatory environment and plays a key role in defence against environmental pathogens. In an inflammatory environment, activated neutrophils and macrophages produce large quantities of superoxide radical and other ROS *via* the phagocytic isoform of NAD(P)H oxidase (Keisari, Braun, & Flescher, 1983). For example, the concentration of hydrogen peroxide under such conditions may reach a level of 10–100 μM. Thus the physiological role of NAD(P)H is to act as a defence agent. Activation of NAD(P)H oxidase is controlled by the rac isoform rac2 in neutrophils and rac1 in macrophages (Tauber, Borregaard, Simons, & Wright, 1983). Stimulated neutrophils and macrophages are known also to generate singlet oxygen by reactions involving NAD(P)H oxidase or myeloperoxidase.

(iii) Various types of nonphagocytic cells involving fibroblasts, vascular smooth muscle cells, cardiac myocytes, and endothelial cells are known to produce ROS by NAD(P)H oxidase to regulate intracellular signalling cascades (Jones et al., 1996; Thannickal & Fanburg, 1995). Most often rac1 is involved in the NAD(P)H induction (Jones et al., 1996). Nonphagocytic cells produce only one-third of that produced by neutrophils. Of interest is that, in contrast to neutrophils, endothelial cells and fibroblasts, vascular smooth muscle cells produce superoxide radical mainly intracellularly. Upon stimulation by growth factors and cytokines, NAD(P)H oxidases of vascular cells produce superoxide and other ROS, which in turn activate multiple intracellular signalling pathways. Thus ROS play an important role in the regulation of cardiac and vascular cell functioning (Griendling, Sorescu, Lasse‘gue, & Ushio-Fukai, 2000). Angiotensin II is known to enhance NAD(P)H-mediated superoxide formation in vascular smooth cells and fibroblasts; thrombin, PDGF (platelet-derived growth factor) and TNF-α (tumour necrosis factor-α) stimulate NAD(P)H-mediated superoxide formation in vascular smooth muscle cells; TNF-α, Interleukin-1 (IL-1) and platelet-activating factor increase NADPH-mediated formation of superoxide in fibroblasts.

(iv) The regulation of vascular tone by cGMP is a special case. The enzyme soluble guanylate cyclase (sGC) is known to be activated by both hydrogen peroxide and $NO^{\bullet}$ radical (Ignarro & Kadowitz, 1985; Wolin, Burke-Wolin, & Mohazzab-H, 1999). Guanylate cyclase belongs to the family of heterodimeric heme proteins and catalyses the formation of cGMP, which is used as an intracellular amplifier and second messenger in a variety of physiological responses. $NO^{\bullet}$ binds to Fe^{2+}-Haem groups in sGC (see reaction (3) above) resulting in a conformational change at Fe^{2+} that activates the enzyme. Its product, cGMP modulates the function of protein kinases, ion channels, and other physiologically important targets, the most important ones being regulation of smooth muscle tone and the inhibition of platelet adhesion.

(v) Oxygen homeostasis is preserved in higher organisms by a tight regulation of the red blood cell mass and respiratory ventilation (Acker & Xue, 1995). It has been proposed that changes in oxygen concentration are sensed independently by several different ROS-producing proteins involving b-type cytochrome. Some other studies suggested that change in the rate of mitochondrial ROS may play a role in oxygen sensing by the carotid bodies which are sensory organs that detect changes in arterial blood oxygen. Other responses to changes in oxygen pressure include the regulated production of certain hormones such as erythropoietin, VEGF and IGF-II (insulin-like growth factor) all of which are controlled by the transcription hypoxia inducible factor-1 (HIF-1) (Wang, Jiang, Rue, & Semenza, 1995).

(vi) Cell adhesion plays an important role in embryogenesis, cell growth, differentiation, wound repair, and other processes and therefore the changes in the adhesive properties of cells and tissues are tightly redox regulated (Albelda, Smith, & Ward, 1994; Frenette & Wagner, 1996). The expression of cell adhesion molecules is stimulated by bacterial lipopolysaccharides and by various cytokines such as TNF, interleukin-1 and interleukin-1β (Albelda et al., 1994). The adhesion of leukocytes to endothelial cells is induced by ROS. ROS-treated endothelial cells induce the phosphorylation of the focal adhesion kinase $pp125^{FAK}$, a cytosolic tyrosine kinase that has been implicated in the oxidant-mediated adhesion process (Schaller et al., 1992).

(vii) Even small amounts of environmental pathogens activate the immune response involving the lymphocyte receptor for antigen, receptors for co-stimulatory signals and various types of cytokines (Linsley & Ledbetter, 1993). The immune response is redox regulated process; the activation of T lymphocytes is significantly enhanced by ROS or by a shift in intracellular glutathione redox state. T-cell functions such as interleukin-2 production can be induced by physiologically relevant concentrations of superoxide radical and hydrogen peroxide (Los, Dröge, Stricker, Baeuerle, & Schulze-Osthoff, 1995). There exists evidence that the intracellular redox state also modulates the immunological functions of macrophages (Hamuro, Murata, Suzuki, Takatsuki, & Suga, 1999).

(viii) Programmed cell death (apoptosis) is needed both for proper development and to destroy cells that represent a threat to the integrity of the organism. The decision of a cell to commit suicide is based on the balance between the withdrawal of positive signals (those needed for continued survival, e.g. growth factors for neurons, interleukin-2, etc.) and the receipt of negative signals (e.g. increased levels of oxidants within the cell, damage to DNA by oxidants, or other harmful effects such as high-energy irradiation, chemotherapeutics, etc.) (Hengartner, 2000). Generally, there are three different mechanisms by which a cell commits suicide by apoptosis: one triggered by internal signals: the intrinsic or mitochondrial pathway; another triggered by external signals: the extrinsic or death receptor pathway; and a third triggered by apoptosis inducing factor (AIF) (Hale et al., 1996). The mechanism triggered by internal signals is represented by intracellular damage to the cell (e.g. from ROS, irradiation, etc.) which causes Bcl-2 (a protein located in the outer membranes of mitochondria) to activate a related protein, Bax, which "makes holes" in the outer mitochondrial membrane, causing cytochrome *c* to release from mitochondria. Using the energy provided by ATP, the released cytochrome *c* binds to the protein—apoptotic protease activating factor-1 (Apaf-1), followed by aggregation of these complexes to form apoptosomes which bind to and activate one of the proteases, caspase-9 (Philchenkov, Zavelevich, Kroczak, & Los, 2004). Proteases are known to cleave proteins predominantly at aspartate residues. Cleaved caspase-9 activates other "executive" caspases (3 and 7) leading finally to digestion of structural proteins in the cytoplasm, degradation of DNA and phagocytosis of the cell. NO-dependent apoptosis is associated with a decrease in the concentration of cardiolipin, decreased activity of the mitochondrial electron transport chain and release of mitochondrial cytochrom *c* into cytosol (Brune et al., 1997). However, endothelial cells are resistant to the induction of apoptosis by $NO^{\bullet}$. Resistance against apoptosis in such cells has been related to high intracellular levels of glutathione (Albina & Reichner, 1998). More detailed accounts on apoptosis can be found in recent excellent reviews (Adams, 2003; Danial & Korsmeyer, 2004; Orrenius, Zhivotovsky, & Nicotera, 2003; Newmeyer & Ferguson-Miller, 2003).

9. ROS, human disease and ageing: pathophysiological implications of altered redox regulation

Oxidative stress has been implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing (Dalle-Donne et al., 2006; Dhalla, Temsah, & Netticadan, 2000; Jenner, 2003; Sayre, Smith, & Perry, 2001). These diseases fall into two groups: (i) the first group involves diseases characterised by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance—the so-called "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus); (ii) the second group involves disease characterised by "inflammatory oxidative conditions" and enhanced activity of either NAD(P)H oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS (implicated in ischemia and reperfusion injury). The process of ageing is to a large extent due to the damaging consequence of free radical action (lipid peroxidation, DNA damage, protein oxidation) (Harman, 1956).

Table 1
Biomarkers of oxidative damage associated with some human diseases

Disease/biomarker	
Cancer	Parkinson's disease
MDA	HNE
GSH/GSSG ratio	GSH/GSSG ratio
NO_2-Tyr	Carbonylated proteins
8-OH-dG	Iron level
Cardiovascular disease	Ischemia/reperfusion
HNE	F_2-isoprostanes
GSH/GSSG ratio	GSH/GSSG ratio
Acrolein	Atherosclerosis
NO_2-Tyr	MDA
F_2-isoprostanes	HNE
Acrolein	Acrolein
Rheumatoid arthritis	F_2-isoprostanes
F_2-isoprostanes	NO_2-Tyr
GSH/GSSG ratio	Diabetes mellitus
Alzheimer's disease	MDA
MDA	GSH/GSSG ratio
HNE	*S*-glutathionylated proteins
GSH/GSSG ratio	F_2-isoprostanes
F_2-isoprostanes	NO_2-Tyr
NO_2-Tyr	AGE
AGE	

Abbreviations: MDA, malondialdehyde; HNE, 4-hydroxy-2-nonenal; AGE, advanced glycation end products; 8-OH-dG, 8-hydroxy-20-deoxyguanosine; GSH, reduced glutathione; GSSG, oxidised glutathione; NO_2-Tyr, 3-nitro-tyrosine.

Convincing evidence for the association of oxidative/nitrosative stress and acute and chronic diseases lies on validated biomarkers of oxidative stress. Such biomarkers have to be objectively measured and evaluated on healthy and ill subjects for long periods. Table 1 summarises most representative biomarkers of oxidative damage associated with human diseases discussed below. An excellent review on biomarkers of oxidative stress in human diseases has very recently been published by Dalle-Donne and coworkers (Dalle-Donne et al., 2006).

9.1. Cancer

Oxidative stress induces a cellular redox imbalance which has been found to be present in various cancer cells compared with normal cells; the redox imbalance thus may be related to oncogenic stimulation. Permanent

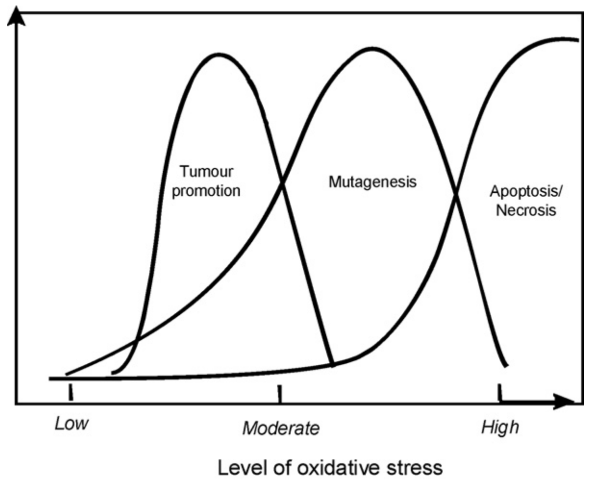

Fig. 3. The dose-dependent effect of relationship between level of oxidative stress and the tumour promotion process, process of mutagenesis and the process of apoptosis/necrosis.

modification of genetic material resulting from "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis, and ageing. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions have been noted in various tumours, strongly implicating such damage in the etiology of cancer. To date, more than 100 oxidised DNA products have been identified. ROS-induced DNA damage involves single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result in either arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which are associated with carcinogenesis (Marnett, 2000; Valko et al., 2006). The most extensively studied DNA lesion is the formation of 8-OH-G. This lesion is important because it is relatively easily formed and is mutagenic and therefore is a potential biomarker of carcinogenesis. DNA damage, mutations, and altered gene expression are thus all key players in the process of carcinogenesis. The involvement of oxidants appears to be the common denominator to all these events (Valko et al., 2006, 2001, 2004). The role of oxidative stress at various stages of carcinogenic process and the process of apoptosis are outlined in the Fig. 3.

In addition to ROS, various redox metals, due to their ability to generate free radicals, or non-redox metals, due to their ability to bind to critical thiols, have been implicated in the mechanisms of carcinogenesis and ageing (Leonard et al., 2004; Pourahmad & O'Brien, 2001; Roy & Saha, 2002; Santos et al., 2005; Stayner, Dankovic, & Lemen, 1996; Valko et al., 2005; Waalkes, Liu, Ward, & Diwan, 2004).

Iron-induced oxidative stress is considered to be a principal determinant of human colorectal cancer (Valko et al., 2001). Occupational exposure to asbestos containing about 30% (weight) of iron is related to increased risk of asbestosis—the second most important cause of lung cancer (Stayner et al., 1996).

Occupational exposure to cadmium has been associated with occurence of increased oxidative stress and cancer (Santos et al., 2005). Cadmium itself is unable to generate free radials directly, however, *via* indirect mechanisms, it can cause free radical-induced damage to the gene expression. It has ben reported that cadmium can cause activation of cellular protein kinases (protein kinase C), which result in enhanced phosphorylation of transcription factors and consequently lead to the transcriptional activation of target gene expression (Valko et al., 2005). It has been suggested that cadmium might also be implicated in the pathogenesis of human pancreatic cancer and renal carcinoma.

Hexavalent chromium is considered a potential lung carcinogen; Cr(VI)-induced cytotoxicity is associated with mitochondrial/lysosomal toxicity substantiated by the enhanced formation of free radicals (Pourahmad & O'Brien, 2001).

Arsenic compounds are well-established human carcinogens, capable of binding to –SH groups and thus inhibiting various enzymes, including glutathione reductase (Roy & Saha, 2002). Studies support the hypothesis that arsenic may act as a co-carcinogen—not by causing cancer directly, but by allowing other factors, such as cigarette smoke or UV radiation, to cause DNA mutations more effectively (Waalkes et al., 2004). It has been shown that exposure of JB6 cells to arsenic induced phosphorylation and activation of ERKs and JNKs (Valko et al., 2005). The effect of arsenic on p53 is not fully understood. The experimental results suggest both p53-dependent and p53-independent induction of apoptosis, and also both an increased and decreased expression of the protein (Valko et al., 2005).

Tobacco smoke, a well known carcinogenic source of ROS, increased the oxidative DNA damage rate by 35–50%, as estimated from the urinary excretion of 8-OH-G, or by 20–50%, estimated from the level of 8-OH-G in leukocytes (Loft & Poulsen, 1996). The main endogenous source of ROS, oxygen consumption, showed a close correlation with the 8-OH-G excretion rate although moderate exercise appeared to have no immediate effect. Cross-sectional studies of diet composition and intervention studies, including energy restriction and antioxidant supplements, have generally failed to show an influence on the oxidative DNA modification (Dreher & Junod, 1996).

In addition to ROS, reactive nitrogen species (RNS), such as peroxynitrites and nitrogen oxides, have also been implicated in DNA damage (Hehner et al., 2000). Upon reaction with guanine, peroxynitrite has been shown to form 8-nitroguanine. Due to its structure, this adduct has the potential to induce G:C → T:A transversions. While the stability of this lesion in DNA is low, in RNA, however, this nitrogen adduct is stable. The potential connection between 8-nitroguanine and the process of carcinogenesis is unknown.

In addition to the extensive studies devoted to the role of oxidative nuclear DNA damage in neoplasia, there exists evidence about the involvement of mitochondrial oxidative DNA damage in the carcinogenesis process (Valko et al., 2006). Mutations and altered expression in mitochondrial genes encoding for complexes I, III, IV and V, and in the hypervariable regions of mitochondrial DNA, have been identified in various human cancers. Hydrogen peroxide and other reactive oxygen species have been implicated in the activation of nuclear genes that are involved in mitochondrial biogenesis, transcription, and replication of the mitochondrial genome. Although the region of tumour cells that possess mutated mitochondrial DNA and the extent to which mitochondrial DNA alterations participate in the cancer process have not been satisfactorily established, a significant amount of information supporting the involvement of the mitochondria in carcinogenesis exists (Valko et al., 2006). This connection supports the fact that fragments of mitochondrial DNA have been found to be inserted into nuclear DNA, suggesting a possible mechanism for activation of oncogenes.

Apart from DNA damage, the lipid peroxidation process has been implicated in the mechanism of carcinogenesis. Once formed, lipoperoxyl radicals ($ROO^{\bullet}$) can be rearranged *via* a cyclisation reaction to endoperoxides with the final product of the peroxidation process being malondialdehyde (MDA) (Fig. 1) (Marnett, 1999). The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxynonenal (HNE) (Fig. 1). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. HNE is weakly mutagenic but appears to be the major toxic product of lipid peroxidation. In addition, HNE has powerful effects on signal transduction pathways which in turn have a major effect on the phenotypic characteristics of cells. MDA can react with DNA bases G, A, and C to form adducts M_1G, M_1A and M_1C, respectively (Fig. 1, reactions 21–23) (Marnett, 1999). M_1G adducts were detected in tissue at levels as high as 1.2 adducts per 10^6 nucleosides (which corresponds to approximately 6000 adducts per cell). M_1G has also been detected in human breast tissue by ^{32}P-post-labelling as well as in rodent tissues (Wang et al., 1996). M_1G adducts were found to range in tissue at levels ranging from below the limit of detection to as high as 1.2 adducts per 10^6 nucleosides (which corresponds approximately 6000 adducts per cell). Site-specific experiments confirmed that M_1G is mutagenic in *E. coli*, inducing transversions to T and transitions to A (Fink et al., 1997; Mao et al., 1999).

There are also other exocyclic DNA adducts that arise from lipid peroxidation. For example etheno-dA, etheno-dC and etheno-dG have been detected by both ^{32}P-post-labelling and GC–MS (Fedtke et al., 1990). It has been demonstrated that etheno-dA and etheno-dC are strongly genotoxic but weakly mutagenic when introduced on single-stranded vectors in *E. coli*. In addition, it has been demonstrated that hydroxypropanodeoxyguanosines (HO-PdGs) are present in human DNA (Marnett, 2000). These adducts are most probably derived from the reaction of DNA with acrolein and crotonaldehyde generated by a lipid peroxidation process. Acrolein and crotonaldehyde are mutagenic in bacteria and mammalian cells.

9.1.1. ROS, signal transduction and cancer

As mentioned above cell signalling refers to the process by which extracellular substances produce an intracellular response. Aberrant signalling mechanisms are related to various disease states (Brown & Borutaite, 2001). Since one of the most fundamental processes regulated through signal transduction mechanisms is cell growth, alterations in the normal regulatory processes of cells may lead to cancer. The abnormal behaviour of neoplastic cells can often be traced to an alteration in cell signalling mechanisms, such as receptor or cytoplasmic tyrosine kinases, altered levels of specific growth factors, intracellular processes for conveying membrane signals to the nucleus, portions of the transcription apparatus, and genes involved in the cell cycle and the regulation of DNA replication. It has been clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways and are thus instrumental in the process of carcinogenesis (Poli et al., 2004; Valko et al., 2006). The mechanism of cell growth regulation is very complex and therefore the role of ROS in this process depends on the type and concentration of the particular radical involved. The activation of transcription factors including MAP-kinase/AP-1 and NF-κB pathways has a direct effect on cell proliferation and apoptosis (Valko et al., 2006).

Abnormalities in growth factor receptor functioning are closely associated with the development of many cancers (Drevs, Medinger, Schmidt-Gersbach, Weber, & Unger, 2003). Several growth factor receptors (EGF, PDGF, VEGF) are affected by ROS and carcinogenic metals such as nickel, arsenic, cobalt and beryllium (Drevs et al., 2003). Activation of both EGF and VEGF results in increases in cellular Ca(II). Increased expression of the EGF receptors and overexpression of the EGF receptor has been observed in lung and urinary cancers (Drevs et al., 2003). The PDGF is found in endothelial cells, fibroblasts and mesenchymal cells; the overexpression of PDGF has been found in lung and prostate cancers.

The role of cellular oxidants and AP-1 activation in the cancer process is now well documented by a number of experiments (Valko et al., 2006). One effect of AP-1 activation is to increase cell proliferation. It has been demonstrated that c-fos and c-Jun are positive regulators of cell proliferation. Expression of c-fos and c-jun can be induced by a variety of compounds, involving reactive radicals and nongenotoxic and tumour promoting compounds (various metals, carbon tetrachloride, phenobarbital, TPA, TCDD, alcohol, ionising radiation, asbestos) (Valko et al., 2006). In addition to affecting cell proliferation, AP-1 proteins also function as either positive or negative regulators of apoptosis. Whether AP-1 induces or inhibits apoptosis is dependent upon the balance between the pro- and anti-apoptotic target genes, the stimulus used to activate AP-1 and also on the duration of the stimulus. AP-1 proteins have also been found to participate in oncogenic transformation through interaction with activated oncogenes such as Ha-ras (Storz, 2005).

NF-κB regulates several genes involved in cell transformation, proliferation, and angiogenesis (Thannickal & Fanburg, 2000). NF-κB activation has been linked to the carcinogenesis process because of its role in differentiation, inflammation, and cell growth. Carcinogens and tumour promoters involving toxic metals, UV radiation, phorbol esters, asbestos, alcohol, and benzopyrene are among the external stimuli that activate NF-κB (Leonard et al., 2004). On the one hand, expression of NF-κB has been shown to promote cell proliferation, whereas on the other inhibition of NF-κB activation blocks cell proliferation. Several studies documented that tumour cells from blood neoplasms, and also colon, breast, and pancreas cell lines have all been reported to express activated NF-κB (Storz, 2005; Valko et al., 2006). Reactive oxygen species have been implicated as second messengers involved in the activation of NF-κB *via* tumour necrosis factor (TNF) and interleukin-1 (Poli et al., 2004; Valko et al., 2006).

The tumour suppressor protein p53 plays a key role in protecting a cell from tumourigenesis (Hollstein, Sidransky, Vogelstein, & Harris, 1991). p53 exerts its activity by preventing DNA-damaged cells from dividing until either the chromosomal repair is effected or the cell undergoes apoptosis. ROS are enhanced through the action of p53-mediated transcription of apoptosis-promoting genes; however, p53 also can promote the expression of many antioxidant genes that prevent apoptosis (see below). Mutations in p53 leading to its inactivation has been found in more than half of human cancers (Hollstein et al., 1991). p53 is activated by UV radiation, hypoxia, gamma-radiation, nucleotide deprivation and others. Many studies have been devoted to mutations in p53 caused by direct action of ROS or by carcinogenic metals (Hollstein et al., 1991).

As discussed above, the cell cycle is characterised by fluctuations in the redox environment of a cell, mediated, in particular by intracellular changes in concentration of glutathione (Schafer & Buettner, 2001). Generally, a more reducing environment of the cell stimulates proliferation and a slight shift towards a mildly oxidising environment initiates cell differentiation. A further shift towards a more oxidising environment in the cell leads to apoptosis and necrosis. Thus the redox environment is the critical determinant for the trigger of apoptosis. Apoptosis is closely tied to the Bcl-2 gene family. The Bcl-2 gene family exert either pro-apoptotic (e.g. Bax) or anti-apoptopic effect (e.g. Bcl-2) (Kluck, BossyWetzel, Green, & Newmeyer, 1997). It has been discovered that the over-expression of Bcl-2 acts to inhibit cytochrome *c* release, thereby blocking caspase activation and the apoptotic process (Kluck et al., 1997). Since the cytochrome *c* release from mitochondria is linked with glutathione depletion, the Bcl-2 driven blockage of cytochrome *c* release in turn inhibits a decrease in glutathione concentration, shifting thus the redox environment of the cell away from apoptosis (towards more reducing environment). Cancer cells are characterised by overexpressed Bcl-2 which may enhance resistance against oxidative stress (ROS)-induced apoptosis. In view of these findings, cancer is characterised by a more reducing environment of the cell and can be considered as a disturbed balance between cell proliferation and cell death shifted more greatly towards cell proliferation (Schafer & Buettner, 2001). It should be noted, that the depletion of intracellular glutathione is just one of the factors involved in the commitment to undergo apoptosis.

9.1.2. ROS, antioxidant status and cancer

Many of the biological effects of antioxidants appear to be related to their ability not only to scavenge deleterious free radicals but also modulate cell-signalling pathways (Mates et al., 1999). Thus the modulation of cell signalling pathways by antioxidants could help prevent cancer by (i) preserving normal cell cycle regulation; (ii) inhibiting proliferation and inducing apoptosis; (iii) inhibiting tumour invasion and angiogenesis; (iv) suppressing inflammation; (v) stimulating phase II detoxification enzyme activity and other effects.

It has been demonstrated that activation of NF-κB by nearly all stimuli can be blocked by antioxidants, including L-cysteine, *N*-acetyl cysteine (NAC), thiols, green tea polyphenols, and Vitamin E.

As described above, while the role of Mn-SOD in catalyzing the conversion of superoxide to hydrogen peroxide in mitochondria has been well characterised, the potential role of Mn-SOD in cancer development is not yet clear (Behrend, Henderson, & Zwacka, 2003). Because Mn-SOD level seems to be lowered in certain cancer cells and stimulated expression of Mn-SOD appears to suppress malignant phenotypes in certain experimental models, this enzyme has been considered to be a tumour suppressor protein; however, the general statement of Mn-SOD as a tumour-suppressor protein is far from clear. Enhanced Mn-SOD expression, detected in various primary human cancer tissues and in blood samples from patients with leukemia has been shown to be incosistent with its proposed tumour suppression function (Behrend et al., 2003). Thus more rational is to assume that over-expression of Mn-SOD may be related to a cellular response to intrinsic oxidative stress in cancer cells. The increased SOD activity decreases superoxide content in the cells and thus reduces the ROS-mediated stimulation of cell growth. It may be hypothesised that Mn-SOD would decrease cancer cell proliferation indirectly through reduction of ROS, unlike conventional tumour suppressors, which regulate cell growth and decrease expression of cancer cells.

A large number of studies have established an association between cancer incidence and various disorders of GSH-related enzyme functions, alterations of glutathione *S*-transferases (GSTs) being most frequently reported (Pastore et al., 2003). GSTs are a family of enzymes that utilize glutathione in reactions contributing to the transformation of a wide range of compounds, including carcinogens, therapeutic drugs, and products of oxidative stress. GSTs are separated into five classes (α, μ, π, σ and θ) of which μ class is comprised of five different isoenzymes termed GST-M1–GST-M5. Most frequently reported links between cancer and mutations in GSTs concern predominantly GST-M1. The GSH/GSSG ratio measured in the blood of patients with colon and breast cancer has been found to be significantly decreased compared to the control (Pastore et al., 2003). This has been explained by an increased level of oxidised glutathione GSSG, especially in advanced stages of cancer progression. These findings may be explained by increased generation of peroxide, which causes an increased release of GSSG from various tissues within the red blood cells.

There exists significant experimental and clinical evidence connecting thioredoxin to cancer (Baker, Payne, Briehl, & Powis, 1997): (i) elevated levels of TRX have been reported in a wide range of human cancers including cervical carcinoma, hepatoma, gastric tumours, lung, and colorectal carcinomas; (ii) many cancer cells have been shown to secrete TRX; (iii) TRX is able to stimulate the growth of a wide variety of human leukemia and solid tumour cell lines; (iv) overexpression of TRX protected cells from oxidative-stress induced apoptosis and provided a survival as well as a growth advantage to tumours; (v) the elevated levels of thioredoxin in human tumours may cause resistance to chemotherapy (e.g. doxorubicin, *cis*-platin and others).

As it is well known, low-molecular weight antioxidants are involved directly in the conversion of ROS to less reactive species. However, antioxidant protection therapy in cancer patients should be used only with caution since its effects depend on the stage at which it is introduced (Dreher & Junod, 1996; Valko et al., 2004). Since apoptosis is caused by elevated levels of free radicals, decreased concentrations of free radicals due to the excessive administration of antioxidants might actually stimulate survival of damaged cells and proliferation into neoplastic state and thus rather promote process of carcinogenesis than interrupt it. In addition, antioxidant therapy during the progression stage of cancer might actually stimulate growth of tumours through the enhanced survival of tumour cells. Another important issue which should be taken into consideration is a prooxidant character of some antioxidants which may occur depending on the concentration and environment (oxygen pressure) in which they act (Mortensen, Skibsted, & Truscott, 2001; Valko et al., 2004).

9.1.3. Matrix metalloproteinases, angiogenesis, and cancer

An important step in the growth of any tumour beyond a few millimeters is the generation of new blood supplies that feed the malignant cells (Folkman, 1995). Angiogenesis is a multi-step process, involving degradation of the endothelial cell basement membrane, endothelial cell migration to the perivascular stroma and capillary sprouting. Previously, the tumour suppressor p53 was understood to regulate the process of angiogenesis through the activation of genes that inhibit neovascularization and the repression of genes that promote vessel growth. With the identification of p63 and p73, p53 family regulation of angiogenesis has broadened and become more complex (Carmeliet & Jain, 2000).

Angiogenesis and the development of metastasis are intrinsically connected. The cancer cell invasion is a critical point for cancer metastasis. It is generally accepted that remodeling of the extracellular matrix (ECM) is a required process for cancer cell invasion (Westermarck & Kahari, 1999). The process of ECM remodeling occurs in both normal physiological conditions as well as pathological sates such as cancer invasion. Cancer invasion is a disordered and uncontrolled behaviour that usually involves the interaction of tumour cells and their surrounding stromal cells, leading to the loss of matrix function and a compromised matrix boundary. Although several classes of proteases have been suggested in ECM remodelling, it has been clearly demonstrated that the activation of zinc-dependent matrix metalloproteinases (MMPs) was the primary response for the degradation of components of the ECM (Nagase & Brew, 2003). Currently more than 20 members of the MMP family are known, and they can be subgrouped based on their structures. Common properties of the MMPs include the requirement of zinc ion in their catalytic site for activity and their synthesis as inactive zymogens that generally need to be proteolytically cleaved to be active. Normally the MMPs are expressed only when and where needed for tissue remodelling that accompanies various processes such as during embryonic development, wound healing, uterine and mammary involution, cartilage-to-bone transition during ossification, and trophoblast invasion into the endometrial stoma during placenta development. However, aberrant expression of various MMPs has been correlated with pathological conditions, such as rheumatoid arthritis, tumour cell invasion and metastasis (Galis & Khatri, 2002).

Angiogenesis involves multiple interactions between endothelial cells, surrounding pericytes, and smooth muscle cells, ECM, and angiogenic cytokines/growth factors. MMPs contribute to angiogenesis not only by degrading basement membrane and other ECM components, allowing endothelial cells to detach and migrate into new tissue, but also by releasing ECM-bound proangiogenic factors: Basic fibroblast growth factor (bFGF), VEGF and transforming growth factor beta (TGFß) (Konig et al., 1997). These factors bind to their respective cell-surface receptors on endothelial cells, leading to their activation, which includes the induction of cell proliferation, increased expression of cell adhesion molecules (for example integrins $\alpha1\beta1$, $\alpha2\beta1$, $\alpha5\beta1$, and $\alpha v\beta3$), secretion of MMPs, and increased migration and invasion. By directly binding to $\alpha v\beta3$, MMP-2 may itself initiate integrin signalling and thereby contribute to endothelial cell survival and proliferation. Interestingly, MMPs also are able to generate endogenous angiogenesis inhibitors from larger precursors: cleavage of plasminogen by MMPs releases angiostatin. Thus, the MMPs are required for angiogenesis and have both pro- and anti-angiogenic functions. Matrix metaloproteinase inhibitors (MMPIs) have been shown to inhibit angiogenesis in various models (see for example Skiles, Gonnella, & Jeng, 2004).

9.2. Cardiovascular disease

The ROS-induced oxidative stress in cardiac and vascular myocytes has been linked with cardiovascular tissue injury (Dhalla et al., 2000). Regardless of the direct evidence for a link between oxidative stress and cardiovascular disease, ROS-induced oxidative stress plays a role in various cardiovascular diseases such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure (Kukreja & Hess, 1992). The major sources of oxidative stress in cardiovascular system involve: (i) the enzymes xanthine oxidoreductase (XOR), (ii) NAD(P)H oxidase (multisubunit membrane complexes) and (iii) NOS as well as (iv) the mitochondrial cytochromes and (v) hemoglobin (Berry & Hare, 2004; Hare & Stamler, 2005). NOSs and hemoglobin are also principal sources of RNS, including $NO^\bullet$ and SNOs (NO-modified cysteine thiols in amino acids, peptides, and proteins), which convey $NO^\bullet$ bioactivity. These pathways are shown in Fig. 4.

Oxidative stress is associated with increased formation of ROS that modifies phospholipids and proteins leading to peroxidation and oxidation of thiol groups (Molavi & Mehta, 2004). The assaults by ROS lead to changes in membrane permeability, membrane lipid bilayer disruption and functional modification of various cellular proteins. In addition to cellular protein and lipid damage, abnormalities in myocyte function due to increased oxidative stress are considered to be associated with the effects of ROS on subcellular organelles. For example, incubation of sarcolemma with hydrogen peroxide and Fe^{2+} inhibited the ecto-ATPase activity and a similar effect was observed on the sarcolemmal ATP-independent Ca^{2+}-binding activity (Kaneko, Elimban, & Dhalla, 1989). Sarcolemma incubated for 1 min with superoxide resulted in 15% drop in the sarcolemmal ATP-dependent Ca^{2+} accumulation and Ca^{2+}-stimulated ATPase activities. These effects correlate well with an

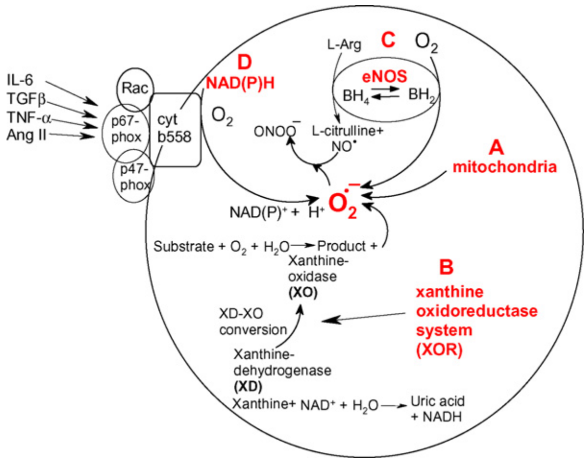

Fig. 4. Major pathways of ROS generation in cardiovascular system. (A) Uncoupling of mitochondrial oxidative phosphorylation. (B) The xanthine-oxidoreductase (XOR) system. XOR exists in two enzymatic forms, as a XD (xanthine dehydrogenase) and as an XO (xanthine oxidase). XD is the form that predominates in purine catabolism resulting in the synthesis of the antioxidant uric acid. The XO form is associated with the synthesis of large amount of ROS and RNS, which at low levels are important second messengers but at high levels have microbicidal action. (C) Uncoupling of $NO^•$ synthesis. The endothelial nitric oxide synthase (eNOS) with the deficiency of cofactors L-arginine and BH_4 ((6*R*)-5,6,7,8-tetrahydrobiopterin) switches from a coupled state (generating nitric oxide, $NO^•$) to an uncoupled oxide (generating superoxide, $O_2^{•-}$). Superoxide can further react with pre-formed $NO^•$ and generate oxidising agent peroxynitrite $ONOO^-$. (D) Activation of NAD(P)H oxidase system by various mediators. The enzyme complex is activated in response to a variety of vasoactive (Angiotensin II, Ang II), inflammatory (IL-6, TNF-α), and growth (TGF-β) factors.

increase of MDA in sarcolemma. Superoxide radical, hydroxyl radical, and nitric oxide have been reported to promote sarcoplasmic reticular Ca^{2+} release by the interaction with sulphydryl groups of the cardiac and skeletal ryanodine receptor (Stoyanovsky, Murphy, Anno, Kim, & Salama, 1997).

Mitochondrial creatine kinase activity of rat heart was reported to decrease upon exposure to xanthine plus xanthine oxidase or hydrogen peroxide (Hayashi, Iimuro, Matsumoto, & Kaneko, 1998). Cardiac mitochondria treated with ROS exhibited decreased Ca^{2+} membrane transport; cardiac mitochondria exposed to 4-hydroxy-2-nonenal cause a rapid decrease in NAD(P)H state 3 and uncoupled respiration. In view of these results, it may be concluded that oxidative stress may alter the activities of different subcellular structures, proteins, and lipids and thus changing myocyte function.

The critical role of intracellular Ca^{2+} overload in the genesis of myocyte dysfunction has been well established (Perez, Gao, & Marban, 1998). In general, Ca^{2+}-overload can be induced by direct effect of ROS on Ca^{2+}-handling proteins or indirectly, by inducing membrane lipid peroxidation. In addition, other mechanisms involving an increase in the concentration of Na^+ and accumulation of long chain fatty acids in cardiac membranes should be considered. Deficiency in ATP in the ischemic heart may also impair Ca^{2+}-handling mechanisms in the sarcolemmal and sarcoplasmic reticular membranes and thus induce Ca^{2+}-overload. Reperfusion of the ischemic heart may also increase the uptake of extracellular Ca^{2+} into the myocardium and thus be another factor for Ca^{2+}-overload. Intracellular Ca^{2+} overload seems to be a common denominator for stimulation of neointimal hyperplasia and thus the occurrence of atherosclerosis, vasoconstriction for the development of hypertension, myocardial cell damage observed in ischemia-reperfusion, and cardiac hypertrophy in heart failure. Evidence for the participation of oxidative stress in these types of cardiovascular disease is described below.

Animal experiments revealed significant amounts of iron pool in atherosclerotic lesions which indicate that the iron-catalysed formation of free radicals (e.g. Fenton chemistry) may take place in the process development of atherosclerosis (Yuan & Li, 2003). Human endothelial cells show increased levels of intracellular level of Ca^{2+} suggesting that Ca^{2+}-overload induced oxidative stress is another factor participating in atherosclerosis. In addition to high levels of cholesterol, uptake of oxidised low-density cholesterol (LDLox) seems to be a key step in the development of atherosclerosis (Podrez, Abu-Soud, & Hazen, 2000). Oxidised lipoprotein and LDLox have been reported to mediate enhanced superoxide formation, which in turn leads to apoptosis of cells in the umbilical vascular wall. LDLox-mediated formation of ROS also causes plaque formation. These effects can be prevented by treatment with SOD and catalase. Besides the direct effect of $O_2^{•-}$, oxidation of $NO^•$ by $O_2^{•-}$ results in the formation of peroxynitrite which is known to initiate lipid peroxidation or lipoproteins oxidation, both important events in the incidence of atherosclerosis.

Since increased amounts of superoxide radical and hydrogen peroxide have been reported in hypertensive patients, the etiology of ROS-induced oxidative stress in the pathogenesis of hypertension is well established (Romero & Reckelhoff, 1999). Superoxide promotes cell proliferation whereas hydrogen peroxide induces apoptosis and activates protein kinase C, suggesting a role for protein kinase C in ROS-mediated vascular disease. ROS-induced oxidative stress in hypertensive patients is accompanied by decreased levels of antioxidants such as Vitamin E, GSH, and SOD, all good scavengers of free radicals. The interaction of $O_2^{•-}$ and $NO^•$ (leading to peroxynitrite formation) seems to be also involved in the process of hypertension (Li & Forstermann, 2000). In a rat model of renal hypertension, it was observed that elevated superoxide levels are linked with suppressed formation of $NO^{\bullet}$ from aortic rings. Since vascular endothelial cells are known to generate $NO^{\bullet}$, suppressed formation of $NO^{\bullet}$ accompanied by endothelial dysfunction is an important factor in the development of hypertension.

Angiotensin II (AngII) is considered a multifunctional hormone influencing many cellular processes involving the regulation of vascular function, including cell growth, apoptosis, migration, inflammation, and fibrosis (Romero & Reckelhoff, 1999; Sowers, 2002). AngII plays a key role in regulating blood pressure and fluid homeostasis. A growing body of evidence indicates that production of ROS is tightly linked with AngII-induced action. It is known that all vascular cell types, including endothelial cells, smooth muscle cells, fibroblasts, and macrophages generate superoxide radicals and hydrogen peroxide and therefore are of particular interest as inter- and intra-cellular signalling species. Links between oxidative stress and hypertension have been established with the demonstration that AngII increases formation of ROS by vascular smooth muscle cells (VSCM). It was also demonstrated that AngII-induced hypertension was associated with increased vascular superoxide production. Liposomal SOD reduced blood pressure by 50 mm Hg in AngII-infused rats (Laursen et al., 1997). Since overproduction of AngII is a critical step in hypertension, inhibitors of angiotensin converting enzymes (ACE), inhibiting the conversion of AngI to AngII, play a role in treatment of hypertension.

The heart is rich in cardiolipin, a phospholipid acylated in four sites, predominately with linoleic acid. Cytochrome *c* is normally bound to the inner mitochondrial membrane by association with cardiolipin (Robinson, 1993). Increasing evidence suggests that ROS play a key role in promoting cytochrome *c* release from mitochondria. Peroxidation of cardiolipin leads to dissociation of cytochrome *c* and its release through the outer mitochondrial membrane into the cytosol. The mechanism by which cytochrome *c* is released through the outer membrane is not clear. One mechanism may involve mitochondrial permeability transition (MPT), with swelling of the mitochondrial matrix and rupture of the outer membrane. ROS may promote MPT by causing oxidation of thiol groups on the adenine nucleotide translocator, which is believed to form part of the MPT pore (Petrosillo, Ruggiero, & Paradies, 2003). Cytochrome *c* release may also occur *via* MPT-independent mechanisms and may involve an oligomeric form of Bax.

The cells' growth known as cardiomyocyte hypertrophy, can lead to congestive heart failure and other forms of cardiovascular disease (Molkentin & Dorn, 2001). Heart muscle cells become enlarged when an intricate intracellular signalling pathway regulated by a messenger molecule, called muscle-specific A-kinase anchoring proteins (mAKAP), is perturbed. Cyclic adenosine 3′, 5′-monophosphate (cAMP) is a ubiquitous mediator of intracellular signalling events. It acts principally through stimulation of cAMP-dependent protein kinases (PKAs). Metabolism of cAMP is catalysed by phosphodiesterases (PDEs). Very recently, a cAMP-responsive signalling complex maintained by the muscle-specific A-kinase anchoring protein (mAKAP) that includes PKA, PDE4D3 and Epac1 has been identified (Dodge-Kafka et al., 2005). The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. It has been shown that mAKAPs tether the enzyme, called protein kinase A (PKA), to particular locations in the cell. It is known that the PKA signalling pathway is perturbed in certain cases of heart disease. According to this study, the mAKAP signalling system has been linked to excessive heart cell enlargement, which increases the potential for heart disease.

9.2.1. Cardiac $NO^{\bullet}$ signalling

There is growing evidence that the altered production, spatiotemporal distribution of ROS/RNS induces oxidative/nitrosative stresses in the failing heart and vascular tree, which contribute to the abnormal cardiac and vascular phenotypes (Ignarro et al., 2002). ROS are known to contribute to cardiac injury both by oxidizing cellular constituents (mainly proteins critical for excitation–contraction (E–C) coupling) and by diminishing $NO^{\bullet}$ bioactivity (Khan et al., 2004).

Protein *S*-nitrosylation represents the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine and has emerged as an important mechanism for dynamic, post-translational regulation of most or all main classes of proteins. Protein *S*-nitrosylation has been established as a route through which $NO^{\bullet}$ can modulate diverse cellular processes, including cardiac E–C coupling, endothelial/vascular function, and tissue oxygen delivery (Foster & Stamler, 2004).

ROS may reduce the effect of $NO^{\bullet}$ by directly inactivating it; however, the mechanism is unclear. In addition, ROS are able to affect $NO^{\bullet}$ responses by oxidizing sites in proteins with which $NO^{\bullet}$ reacts. It appears that this mode of ROS action may contribute to cardiac pathophysiology. The wide range of $NO^{\bullet}$ effects in the heart is closely associated with the subcellular location of the NOS isoforms. For example NOS3 is found within membrane caveolae in proximity to the L-type channel, and NOS1 localizes to the SR in a complex with RyR (Xu, Huso, Dawson, Bredt, & Becker, 1999). Generally, NO$^{\bullet}$ specificity is carried out through spatial localization of NOSs to signalling modules and direct interactions between NOSs and their targets (Matsumoto, Comatas, Liu, & Stamler, 2003). The NOS isoforms contribute independently to other cardiac phenotypes—mainly cardiac hypertrophy. For example, a cumulative hypertrophic phenotype emerges, at both structural and genetic levels, when both NOS isoforms are absent from myocardium. Termination of NO-based signalling is partly governed by the actions of enzymes; cGMP is metabolized by phosphodiesterase-5 (PDE5), which is spatially localized in proximity to NOS. Generally, NO$^{\bullet}$ may be a global modulator of ROS production in congestive heart failure (Cote, Yu, Zulueta, Vosatka, & Hassoun, 1996). Oxidant-producing enzymes are upregulated in congestive heart failure, and NO-producing enzymes – NOSs and XO – are altered in either their abundance or spatial localization (Damy et al., 2004). The abundance of vascular NADPH oxidases is increased in the failing circulation, at least in part due to increased levels of angiotensin II, which suggests a link between neurohormonal activation and NO/redox disequilibrium (Hilenski, Clempus, Quinn, Lambeth, & Griendling, 2004). Elevated superoxide may inactivate NO$^{\bullet}$, reducing its control over the vascular oxidase (Clancy, Leszczynskapiziak, & Abramson, 1992). In heart failure, increased XO activity is directly reflected in dysregulation of NO$^{\bullet}$ signalling. At low physiologic concentration, NO$^{\bullet}$ may act as an antioxidant, abating Fenton reactions, terminating radical chain reactions, and inhibiting peroxidases and oxidases (Valko et al., 2005). The relative concentration of NO$^{\bullet}$ and superoxide and location of both NOSs and oxidases in the heart directly determines the chemical fate of their interactions: In the normal physiological state, the concentration of NO$^{\bullet}$ is higher than the concentration of superoxide and favors protein *S*-nitrosylation. NO/superoxide disequilibrium (characteristic of heart failure) favors oxidation reactions. In addition, NO reactions with $O_2^{\bullet-}$ produce nitrosating reagents that react preferential with thiols (Foster & Stamler, 2004). Thus, controlled production of RNS and ROS not only preserves an antioxidant environment, but may also serve as a mechanism of channelling NO$^{\bullet}$ to cysteine substrates (Wink et al., 1997). Conversely, when NO$^{\bullet}$ and/or $O_2^{\bullet-}$ are elevated, both the nature of target modification and the specificity of targeting are impaired. In other words, superoxide/ROS production may facilitate protein *S*-nitrosylation at basal conditions but disrupts this signalling mechanism at higher concentrations (Foster & Stamler, 2004). Insights into the mechanism of NO$^{\bullet}$/redox-mediated signalling may help in the development of novel therapeutic approaches for heart failure. Angiotensin-converting enzyme inhibitors stimulate NO$^{\bullet}$ production by increasing bradykinin formation and reduce ROS formation by suppressing angiotensin II—stimulation of NADPH oxidase. Many currently used drugs influence NO$^{\bullet}$/redox balance, including inhibitors of the renin-angiotensin aldosterone pathway, the sympathetic nervous system, and the HMG-CoA reductase pathways.

9.2.2. *Ischemic preconditioning*

Preconditioning ischemia triggers endogenous protective mechanisms in heart muscle, and is the most effective means for myocardial protection. The protection induced by short preconditioning ischemia periods disappears within 1–2 h, but reappears after 24–72 h (the so called "second window protection"). The mechanism of ischemic preconditioning is very complex and involves both triggers and mediators and involves multiple second messenger pathways. The process involves such components as a paradoxical protective role of oxygen free radicals, adenosine, adenosine receptors, heat shock proteins (HSP), nitric oxide, the epsilon isoform of protein kinase C (PKC), mitogen-activated protein kinases, the mitochondrial ATP-dependent potassium (K^+(ATP)) channels (Kalikiri & Sachan, 2004; Skyschally, Schulz, Gres, Korth, & Heusch, 2003; Zhao, Hines, & Kukreja, 2001). It has been proposed that ischemia-induced release of endogenous agents such as adenosine and nitric oxide, activation of adenosine receptors, protein kinase C (PKC), mitogen-activated protein kinases (MAPK) and opening of ATP-sensitive mitochondrial potassium (K^+(ATP)) channels in sarcolemmal or mitochondrial membranes are the potential mechanisms of this preconditioning phenomenon.

ROS are known to trigger preconditioning by activating the mitochondrial K^+(ATP) channel, followed by generation of ROS and NO$^{\bullet}$, which are essential for preconditioning protection (Kalikiri & Sachan, 2004). The opening of K^+(ATP) channels ultimately confers cytoprotection by decreasing cytosolic and mitochondrial Ca^{2+} overload. Nitric oxide acts as a trigger in the first window of protection *via* activation of a constitutive nitric oxide synthase (NOS) isoform and cGMP pathway (Das, Maulik, Sato, & Ray, 1999). Nitric oxide is also involved in the second window of protection (SWOP), however, *via* a different mechanism, through the activation of a protein kinase C (PKC), which in turn activates ATP sensitive potassium (K^+(ATP)) channels. In the second window of protection, the origin of nitric oxide is attributed to the activity of an endothelial nitric oxide synthase (eNOS) (Laude, Favre, Thuillez, & Richard, 2003). Thus an increase in the release of nitric oxide as well as adenosine may be responsible for both windows of protection. Adenosine-induced preconditioning involves p38 MAP kinase, and mitochondrial K^+(ATP) channels (Zhao et al., 2001). Recently, it has been suggested that the K^+(ATP) channels involved in the protection are mitochondrial rather than sarcolemmal (McCully & Levitsky, 2003).

9.3. Ischemic/reperfusion injury

Ischemia-reperfusion injury is a clinically relevant problem occuring as damage to the myocardium following blood restoration after a critical period of coronary occlusion. Despite the low oxygen tension during ischemia, moderate ROS generation is substantiated to occur most probably from a mitochondria source (Becker, 2004; Kasparova et al., 2005; Lombardi et al., 1998). The massive burst of ROS seen during reperfusion may originate from a different cellular source than during ischemia and is not yet convincingly identified. Massive production of ROS during ischemia/reperfusion in turn lead to tissue injury causing thus serious complications in organ transplantation, stroke, and myocardial infarction (Kasparova et al., 2005). The consequences of oxidative stress and the cardioprotective role of antioxidants in ischemia/reperfusion injury are shown in Fig. 5.

Neutrophils are the principal effector cells of reperfusion injury. Under the conditions of ischemia/reperfusion, xanthine dehydrogenase is converted into xanthine oxidase which uses oxygen as a substrate. During ischemia, oversized ATP consumption leads to accumulation of the purine catabolites hypoxanthine and xanthine, which upon subsequent reperfusion and influx of oxygen are metabolized by xanthine oxidase to produce enormous amounts of superoxide radical and hydrogen peroxide (Granger et al., 2001). The harmful effect of ischemia/reperfusion injury was shown to be suppressed by synthetic SOD mimetic compounds in a rat model. Ischemia/reperfusion induced in a model of the rat heart was shown to activate the redox-sensitive transcription factors NF-kB and AP-1 and the MAPKs JNK and p38 in the presence of minimal activation of ERK (Lazou et al., 1994); activation may be due to inflammatory responses and apoptotic cell death in the affected tissue (Schreck, Rieber, & Baeuerle, 1991).

Numerous studies have investigated the deleterious effects of ischemia-reperfusion-induced oxidant production using various pharmacological interventions (Chen et al., 1998). The role of cellular antioxidant enzymes in the pathogenesis of myocardial injury in vivo in gene-targeted mice revealed that neither deficiency nor overexpression of Cu–ZnSOD altered the extent of myocardial necrosis (Jones, Hoffmeyer, Sharp, Ho, & Lefer, 2003). Overexpression of glutathione peroxidase did not affect the degree of myocardial injury. Conversely, overexpression of MnSOD significantly attenuated myocardial necrosis after myocardial injury/reperfusion. These findings indicate an important role for MnSOD but not Cu/ZnSOD or glutathione peroxidase in mice after in vivo MI/R.

9.4. Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disease that causes chronic inflammation of the joints and tissue around the joints with infiltration of macrophages and activated T cells (Bauerova & Bezek, 1999). The pathogenesis of this disease is linked predominantly with the formation of free radicals at the site of inflammation. Oxidative injury and inflammatory status in various rheumatic diseases was confirmed by increased levels of isoprostanes and prostaglandins in serum and synovial fluid compare to controls. Oxidative conditions in synovial tissue are also associated with a higher incidence of p53 mutations (Firestein, Echeverri, Yeo,

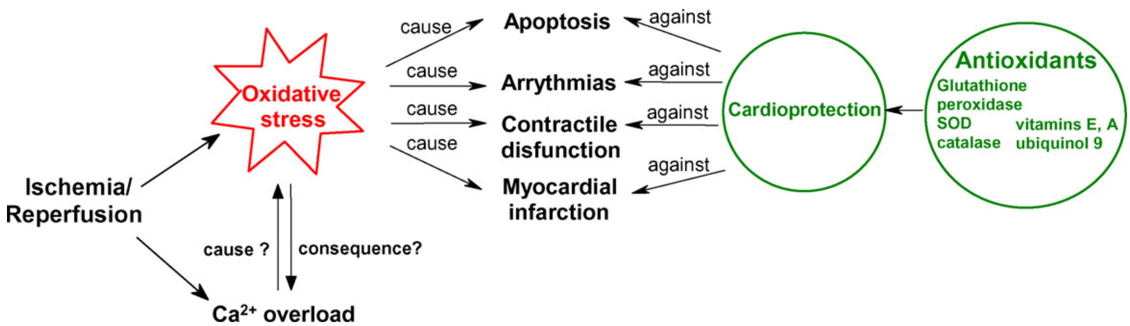

Fig. 5. Effect of oxidative stress and antioxidants in pathophysiology of ischemia-reperfusion injury in the heart.

Zvaifler, & Green, 1997). T cells isolated from the synovial fluid of patients with rheumatoid arthritis show signs of decreased intracellular GSH level, impaired phosphorylation of the adaptor protein linker for T-cell activation (LAT) and the "primed" CD45RO phenotype (Maurice et al., 1997). Altered subcellular localisation of LAT has been shown to be caused by the changes in intracellular GSH level. The migration of monocytes and lymphocytes into the rheumatoid arthritis synovium is mediated by the abnormal expression of several adhesion molecules (ELAM-1, VCAM-1, ICAM-1, ICAM-2) (Cunnane, FitzGerald, Beeton, Cawston, & Bresnihan, 2001); this can be explained by the abnormal induction of redox-sensitive signalling pathways.

9.5. Diabetes

A relatively small amount (10%) of patients suffering from diabetes mellitus has type 1, or insulin dependent diabetes (Brownlee & Cerami, 1981; Niedowicz & Daleke, 2005). However, the majority of diabetes patients are non-insulin-dependent and capable at least initially of producing insulin, but are deficient in their cellular response. This type of diabetes is the type 2 diabetes mellitus and is the most common form of diabetes. Decreased uptake of glucose into muscle and adipose tissue leads to chronic extracellular hyperglycemia resulting in tissue damage and pathophysiological complications, involving heart disease, atherosclerosis, cataract formation, peripheral nerve damage, retinopathy and others (Brownlee & Cerami, 1981). Increased oxidative stress has been proposed to be one of the major causes of the hyperglycemia-induced trigger of diabetic complications. Hyperglycemia in an organism stimulates ROS formation from a variety of sources. These sources include oxidative phosphorylation, glucose autooxidation, NAD(P)H oxidase, lipooxygenase, cytochrome P450 monooxygenases, and nitric oxide synthase (NOS).

9.5.1. A brief overview of insulin signalling

Insulin rapidly interacts with its receptor at target tissues. The insulin receptor (IR, composed of two extracellular α-subunits and two transmembrane β-subunits linked by –S–S– bonds) possess an intrinsic tyrosine kinase activity. Tyrosine autophosphorylation of the IR β-subunit is induced following binding of insulin to the α-subunit (White, 1998). The activated IR phosphorylates the insulin receptor substrate (IRS) proteins and other substrates. The process of phosphorylation leads to activation of different signalling pathways. While the ERK pathway is mainly involved in growth, the activation of phosphatidylinositol 3-kinase (PI 3-kinase), mainly through insulin receptor substrates 1 and 2 (IRS1, IRS2), is involved in the metabolic actions of insulin. IRS1 belongs to the IRS family and plays a key role in insulin signalling. While the phosphorylation of IRS1 on tyrosine residue is critical for insulin-stimulated responses, the phosphorylation of IRS1 on serine residues has a dual role: either to enhance or to terminate the insulin effects. The imbalance between the positive IRS1 tyrosine phosphorylation and the negative IRS1 serine phosphorylation is strongly stimulated by "diabetogenic" factors including free fatty acids, TNFα and oxidative stress. Insuline-activated protein kinase B (PKB) propagates insulin signalling and promotes the phosphorylation of IRS1 on serine residue which in turn generates a positive-feedback loop for insulin action (Lawlor & Alessi, 2001).

Various insulin resistance-inducing agents such as angiotensin II, cytokines, free fatty acids, endothelin-1, cellular oxidative stress and hyperinsulinemia lead to both activation of several serine/threonine kinases and phosphorylation of IRS1 (Vicent et al., 2003). These agents are known to negatively regulate the IRS1 functions by phosphorylation, however also *via* other molecular mechanisms such as suppressor of cytokine signalling proteins (SOCS) expression, IRS degradation, *O*-linked glycosylation. Understanding the mechanisms of IRS1 inhibition and identification of kinases involved in these processess may reveal novel targets for development of strategies to prevent insulin resistance. Future work to understand the molecular mechanisms implicated in insulin resistance might reveal "key-regulatory" kinases and/or direct or indirect inhibitors of these kinases which are responsible for the inhibition of the functions of IRS1 but also of IRS2.

9.5.2. Sources of ROS in diabetes

Under normal conditions, the key sites of superoxide formation in the mitochondrial membrane are complex I and the ubiquinone–complex III interface, where the presence of long lived intermediates allows reaction of electrons with molecular dioxygen (Kwong & Sohal, 1998). However, diabetes alters the primary sites of superoxide generation so that complex II becomes the primary source of electrons that contribute to superoxide formation under diabetic conditions (Nishikawa et al., 2000). This conclusion comes from the study of a complex II inhibitor, 2-thenoyltrifluoroacetone and an uncoupler of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenyldihydrazone leading to a decrease in ROS formation in various cells exposed to high concentration of glucose (Yamagishi, Edelstein, Du, & Brownlee, 2001).

Another source of ROS in diabetes is NAD(P)H. The enzyme is a complex of two membrane-bound components, gp91^{phox} and p22^{phox}, which comprise cytochrome b558, the enzymatic center of the complex (Babior, 1999). Several lines of evidence support that NAD(P)H oxidases are a major source of glucose-induced ROS production in the vasculature and kidney cells, confirming thus NAD(P)H as a mediator of diabetic complications (Li & Shah, 2003). Involvement of other cells has not been satisfactorily confirmed. Since hypertension is a common complication of diabetes, it is possible that expression of NAD(P)H oxidase is regulated similarly in both these disease states. This arises from increased angiotensin II labelling in cardiac myocytes and endothelial cells from human diabetic patients (Li & Shah, 2003). High glucose-induced formation of ROS and p47^{phox} (cytosolic component of activated NAD(P)H) can be blocked with AngII type 1 receptor antagonists, confirming thus a link between the two pathways of NAD(P)H oxidase activation. The NAD(P)H oxidase-mediated production of ROS in diabetes can be suppressed by a variety of PKC inhibitors, implicating this family of kinases in the regulation of hyperglycemia-induced NAD(P)H oxidase activity.

Since hyperglycemia-induced oxidative stress occurs in nonnucleated cells lacking mitochondria and the NAD(P)H oxidase (erythrocytes), another mechanism of ROS formation in such cells must exist. A possible explanation for such behaviour is glucose auto-oxidation (Robertson, Harmon, Tran, Tanaka, & Takahashi, 2003). Glucose itself, as well as its metabolites, is known to react with hydrogen peroxide in the presence of iron and copper ions to form hydroxyl radical. Evidence for this comes from *in vitro* experiments and therefore *in vivo* studies should be carried out.

In addition to ROS, RNS have been implicated as one of the sources of nitrosative stress in diabetes. NO$^{\bullet}$ can react with superoxide forming peroxynitrite, a highly reactive oxidant linked with many disease states including diabetes (Zou, Shi, & Cohen, 2002). Peroxynitrite reacts with the zinc-cluster of NOS leading to its uncoupling, suggesting that peroxynitrite not only depletes exisiting NO$^{\bullet}$ but also reduces a tissue's ability to produce more NO$^{\bullet}$. Hyperglycemia is linked with the regulation of NOS expression and the production of peroxynitrite. Glucose induced aortic expression of eNOS (endothelial NOS) can be suppressed by the addition of PKC inhibitor, suggesting that PKC activation is a key event in hyperglycemia-induced NOS upregulation (perhaps mediated by NF-kappa B) (Hink et al., 2001).

Xanthine oxidase (XO) has been proposed to be a major source of ROS in diabetes mellitus (Butler, Morris, Belch, Hill, & Struthers, 2000). Treatment of non-insulin dependent diabetes patients with the XO inhibitor allopurinol reduces the level of oxidised lipids in plasma and improves blood flow. Experimental data suggests that the role for XO is tissue-dependent.

Lipooxygenases catalyse conversion of arachidonic acid into a broad class of signalling molecules, such as leukotrienes, lipoxins, and hydroxyeicosatetraenoic acid. Diabetes is associated with increased lipoxygenase expression, resulting in eicosanoid formation (Brash, 1999).

Production of ROS and RNS depletes both enzymatic and non-enzymatic antioxidants leading to additional ROS/RNS accumulation causing cellular damage. Vitamin E is depleted in diabetes and has a protective effect mainly through suppressed lipid peroxidation. Vitamin C levels in plasma have also been found to be reduced in diabetes patients; however, the relation between reduced levels of Vitamin C and diabetic complications is unclear (VanderJagt, Harrison, Ratliff, Hunsaker, & Vander Jagt, 2001).

The effect of diabetes on glutathione (GSH) peroxidase activity is highly variable with respect to the model of diabetes and type of tissue used. The data suggest hyperglycemia and diabetes complications affect the regulation of GSH peroxidase expression; however the effect to which GSH peroxidase inhibition affects cell health is unclear (VanderJagt et al., 2001). Hyperglycemic treatment impaired no change in activity of GSH reductase, thus it does not appear that GSH reductase plays a role in the onset of diabetic complications.

Several of the most significant biomarkers of oxidative stress linked with diabetes mellitus are listed in Table 1. Various consequences of oxidative stress in diabetic subjects involve accumulation of MDA. However, the role for 4-hydroxy-nonenal in diabetes is not yet clear; a few studies have reported the accumulation of 4-HNE and the activation of signalling pathways (Traverso et al., 2002). Isoprostanes, non-enzymatic products of arachidonic acid oxidation, have been found to be elevated in diabetic rats and in plasma and urine of non-insulin dependent patients (Table 1). The data are suggestive, but do not definitively support an active role for isoprostanes in the onset of diabetic complications. Isoprostanes have become popular markers of lipid peroxidation in diabetes and other disease, in part because of their specificity and sensitivity of detection.

Glucose can react directly with free amine groups on protein and lipids, finally yielding a diverse group of modifications referred as advanced glycation end products (AGE) (Ling et al., 2001). AGE are observed primarily in long-lived structural proteins, such as collagen.

AGE are found in almost all tissues examined from diabetic rats and in human non-insulin dependent patients. Some tissues, such as liver, kidneys, and erythrocytes are more susceptible to AGE formation than others. Thus AGE formation is probably a significant contributor to the onset of diabetic complications, mainly atherosclerosis.

9.5.3. Molecular basis of type 2 diabetes mellitus

Type 2 diabetes mellitus is the most common form of diabetes. As discussed above, type 2 diabetes (formerly called non insulin-dependent diabetes) causes abnormal carbohydrate, lipid and protein metabolism associated with insulin resistance and impaired insulin secretion. Insulin resistance is a major contributor to progression of the disease and to complications of diabetes (Weyer et al., 2001). Currently, a cascade of following events is recognized to be one of the most important among polygenic causes of type-2 diabetes: certain oxidative stress-related defect(s) in oxidative phosphorylation machinery and mitochondrial β-oxidation lead to excess accumulation of intracellular triglyceride in muscle and liver and subsequent insulin resistance (Rosca et al., 2005). The β-oxidation of long-chain fatty acids is central to the provision of energy for the organism and is of particular importance for cardiac and skeletal muscle.

When pancreatic β-cells are no longer able to compensate for insulin resistance by adequately increasing insulin production, impaired glucose tolerance appears, characterized by excessive postprandial hyperglycemia (Gerich, 2003). Insulin resistance in skeletal muscle and abnormal pancreatic β-cell function are earliest detectable defects preceding hyperglycemia even 10 years before diabetes is diagnosed and those dysfunctions are due to the oxidative stress. Impaired glucose tolerance may evolve into overt diabetes. These three conditions, insulin resistance, impaired glucose tolerance, and overt diabetes, are associated with an increased risk of cardiovascular disease.

Many studies have suggested that β-cell dysfunction results from prolonged exposure to high glucose, elevated free fatty acid levels, or a combination of both (Evans, Goldfine, Maddux, & Grodsky, 2003). β-Cells are particularly sensitive to ROS because they are low in free-radical quenching (antioxidant) enzymes such as catalase, glutathione peroxidase, and superoxide dismutase. Therefore, the ability of oxidative stress to damage mitochondria and markedly blunt insulin secretion is not surprising. For example, it has been demonstrated that oxidative stress generated by short exposure of β-cell to H_2O_2 increases production of cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/CIP1/Sdi1}$ and decreases insulin mRNA, cytosolic ATP, and calcium flux in cytosol and mitochondria (Kaneto et al., 1999).

One of the hypotheses for induction of β cell dysfunction, focuses on changes in the expression and function of a mitochondrial inner membrane protein called uncoupling protein-2 (UCP2) (Krauss et al., 2003). It has been proposed that UCPs activity and expression contribute to an increase in superoxide formation under diabetic conditions.

The mechanism of β-cells toxicity involves pancreas duodenum homeobox-1 (PDX-1) and insulin gene expression (Robertson et al., 2003). It has been observed that the chronic exposure of HIT-T15 cells to supraphysiological concentrations of glucose over several months caused gradual loss of insulin gene expression (Robertson, Zhang, Pyzdrowski, & Walseth, 1992). It was explored that the mechanism involves the loss of mRNA and protein levels of pancreas duodenum homeobox-1 (PDX-1), a critical regulator of insulin promoter activity. It appears that these adverse effects of glucose toxicity involve JNK pathway. Demonstration of the actual mechanism by which JNK might interfere with PDX-1 gene expression and insulin mRNA level requires further work.

More than two decades ago, it was demonstrated that pancreatic islets contain relatively small amounts of the antioxidant enzymes CuZn-SOD, Mn-SOD, catalase, and glutathione peroxidase (GPx) (Grankvist, Marklund, & Taljedal, 1981). Further work demonstrated that β-cells in rats were sensitive to peroxide and that the activity of GPx was low (Malaisse, Malaisse-Lagae, Sener, & Pipeleers, 1982). These and many other observations have reinforced the notion that the intrinsically low levels of antioxidant activity of islets render them particularly at risk for ROS-induced damage. Due to the low level of antioxidant enzyme expression and activity, the β-cells are at greater risk of oxidative damage than tissues with higher levels of antioxidant protection. For protection from the highly toxic hydroxyl radical and other ROS, the β-cells must metabolize hydrogen peroxide *via* catalase and GPx. However, a potentially major problem for the β-cells is their unusually low complement of SOD, catalase, and GPx. This unusual situation sets up the β-cell as an easy target for ROS, whether generated by interactions with cytokines or elevated levels of glucose. Consideration of antioxidants in clinical treatment as adjunct therapy in type 2 diabetes is warranted because of the many reports of elevated markers of oxidative stress in patients with this disease, which is characterized by imperfect management of glycemia, consequent chronic hyperglycemia, and relentless deterioration of β-cell function (Ceriello & Motz, 2004).

In this connection, of interest is a very recent epidemiological trial on the preservation of pancreatic beta-cell function and prevention of type 2 diabetes by pharmacological treatment of insulin resistance in high-risk Hispanic women. Treatment with troglitazone delayed or prevented the onset of type 2 diabetes in these patients. The protective effect was associated with the preservation of pancreatic β-cell function and appeared to be mediated by a reduction in the secretory demands placed on β-cells by chronic insulin resistance (Buchanan et al., 2002).

Generally, antioxidant treatment can exert beneficial effects in diabetes, with preservation of *in vivo* β-cell function. Antioxidant treatment suppresses apoptosis in β-cells without changing the rate of β-cell proliferation, supporting the hypothesis that in chronic hyperglycemia, apoptosis induced by oxidative stress causes reduction of β-cell mass. The antioxidant treatment also preserved the amounts of insulin content and insulin mRNA, making the extent of insulin degranulation less evident. Furthermore, expression of pancreatic and duodenal homeobox factor-1, a β-cell-specific transcription factor, was more clearly visible in the nuclei of islet cells after the antioxidant treatment (Kaneto et al., 1999). Thus, in concluding, recent results suggests a potential usefulness of antioxidants for treating diabetes and provide further support for the implication of oxidative stress in β-cell dysfunction in diabetes.

An increasing recognition that diabetic patients suffer from additional cardiac insult is termed 'diabetic cardiomyopathy' (Sharma & McNeill, 2006). Diabetic cardiomyopathy refers to a disease process which affects the myocardium in diabetic patients causing a wide range of structural abnormalities eventually leading to LVH (left ventricular hypertrophy) and diastolic and systolic dysfunction or a combination of these. The concept of diabetic cardiomyopathy is based upon the idea that diabetes is the factor which leads to changes at the cellular level, leading to structural abnormalities.

The major molecular abnormalities and their consequences in the pathogenesis of diabetic cardiomyopathy involve (Wakasaki et al., 1997): (i) Hyperglycemia; the mechanisms involve excess of AGE and ROS formation with deactivation of NO, myocardial collagen deposition and fibrosis; (ii) the increase in and dependence of diabetic myocardium on fatty acid supply results in several major cellular metabolic perturbations; the mechanism involve impaired glycolysis, pyruvate oxidation, lactate uptake results in apoptosis, and perturbation of myocardial bioenergetics and contraction/relaxation coupling; (iii) increased activation of the DAG (diacylglycerol)-activated PKC signal transduction pathway; activation of DAG/PKC signal transduction pathway leads to reduction in tissue blood flow, increased vascular permeability, alterations in neovascularization and enhanced extracellular matrix deposition; (iv) the activation of stretch receptors in the heart activates RAS and the SNS (sympathetic nervous system) leading to changes in myocardial structure and remodelling, which impairs cardiac performance; (v) an inadequate angiogenic response to ischemia in the myocardium of diabetic patients could result in an increased propensity to infarction. The occurence of hypoxia in ischemia/infarction is mediated mainly through HIF-1, a transcriptional regulator complex which operates through a specific promoter motif [HRE (hypoxia response element)] present in many gene promoters, including VEGF. VEGF and its receptors, VEGF-R1 and VEGF-R2, are decreased significantly, leading to impaired angiogenesis; (vi) endothelial dysfunction is a precursor to and an effect of atherosclerosis; the mechanisms involve impaired endothelial NO production and increased vasoconstrictor prostaglandins, glycated proteins, endothelium adhesion molecules and platelet and vascular growth factors enhance vasomotor tone and vascular permeability and limit growth and remodelling; (vii) sarcolemmal membrane abnormalities in Na^+/K^+ ATPase, Na^+/Ca^{2+} exchange and Ca^{2+} pump activity have been proposed to lead to an overload of intracellular Ca^{2+} during the development of diabetic cardiomyopathy. A significant depression of the Na^+/K^+ ATPase a1-subunit mRNA and an increase in Na^+/Ca^{2+} exchanger mRNA has been observed in the ventricular myocardium of alloxan-induced diabetic rats. A key electrophysiological abnormality in diabetic cardiomyopathy is enhanced arrhythmogenicity, which may be associated with a decrease of repolarizing K^+ currents (Hayat, Patel, Khattar, & Malik, 2004).

9.6. Neurological disorders

The brain is particularly vulnerable to oxidative damage because of its high oxygen utilisation, its high content of oxidisable polyunsaturated fatty acids, and the presence of redox-active metals (Cu, Fe). Oxidative stress increases with age and therefore it can be considered as an important causative factor in several neurodegenerative diseases, typical for older individuals.

9.6.1. Alzheimer's disease

The brains of patients with Alzheimer's disease (AD) show a significant extent of oxidative damage associated with a marked accumulation of amyloid-β peptide (Aβ), the main constituent of senile plaques in brain, as well as deposition of neurofibrillary tangles and neurophil threads (Butterfield, Castegna, Lauderback, & Drake, 2002). Aβ is the main constituent of senile plaques and cerebrovascular amyloid deposits in AD patients. Aβ is a 39–43 amino acid peptide derived from the larger amyloid beta (A4) precursor protein (APP) by proteolytic cleavage. Aβ1-40 is the major form of Aβ, however, the minor species, Aβ1-42, has a higher propensity to aggregate and is greatly enriched in amyloid deposits.

The original version of the amyloid-β hypotheses as the main cause of Alzheimer disease claimed the fibrilized form of Aβ (fAβ) as the main component of senile plaques (Hardy & Higgins, 1992). However, since many aspects of AD could not be explained by fibrilized form of Aβ, the amyloid cascade hypothesis was modified to claim that oligomeric Aβ, rather than fAβ plays the key role in the pathogenesis of AD. In this connection, of great interest is a very recent work of Tamagno and coworkers reporting, that differential effects of Aβ are dependent on aggregation state (Tamagno, Bardini, Guglielmotto, Danni, & Tabaton, 2006). Specifically, it has been demonstrated that oligomeric Aβ species, while increasing 4-hydroxynonenal and hydrogen peroxide more than fAβ and being more toxic than fAβ, have no effect on BACE-1 expression and activity. BACE-1 (β-site AβPP Cleaving Enzyme), identified as the β-secretase that cleaves AβPP within the ectodomain is an integral type 1 transmembrane protein detected in the *trans*-Golgi network and endosomes. Conversely, fAβ is less toxic but increases BACE-1 expression and activity. Based on these findings, it has been proposed that Aβ acts *via* a biphasic neurotoxic mechanism that is conformation dependent such that oligomeric Aβ exerts toxic effects by inducing oxidative stress and leads to fAβ formation from oligomeric Aβ. Although fAβ is less toxic than oligomeric Aβ, fAβ increases the accumulation of Aβ by inducing BACE-1 expression and activity and this process may contribute further to the toxicity of fAβ.

Pathological mutations close to, or within, the Aβ domain of APP give rise to familial forms of AD (FAD). FAD mutations are also found in genes associated with Aβ processing, including presenilin 1 and 2, while risk factors for late onset AD include apolipoprotein E4 (Apo-E4) and alpha-2 macroglobulin (α2m). Although genetic lesions associated with FAD result in elevated Aβ1-42 levels, this alone does not explain the aetio-pathology of AD onset. The neurochemical factors responsible for this age-related pathological process are still poorly characterised, however, growing evidence supports an important role for biometals such as copper (Cu), iron (Fe) and zinc (Zn) in Aβ accumulation and neuronal degeneration.

Aβ has high affinity binding sites for both Cu and Zn and APP also binds these metals *via* N-terminal metal-binding domains. Cu ions bind to Aβ monomer *via* three histidine residues and a tyrosine or *via* a bridging histidine molecule in aggregated Aβ. Cu has been shown to induce significant Aβ aggregation at mildly acidic conditions (pH 6.6) which reflects the likely micro-environmental conditions in AD neuropil.

Both Aβ and APP have strong Cu-reductase activity, generating Cu^{+} from Cu^{2+}. This reaction produces hydrogen peroxide as a by-product. Interestingly, the redox potentials for different species of Aβ are Aβ42 > Aβ40 ≫ rodent Aβ which accurately reflects the role of the respective peptides in amyloid pathology (rodents do not form amyloid plaques in the brain). Cu^{+} is a potent mediator of the highly reactive hydroxyl radical ($OH^{\bullet}$) and APP or Aβ-associated Cu^{+} may contribute to the elevated oxidative stress characteristic of AD brain. The direct evidence supporting increased oxidative stress in AD brain include (i) increased Cu, Fe, Al, and Hg content; (ii) increased lipid peroxidation and decreased polyunsaturated fatty acid content, and an increase in 4-hydroxynonenal, an aldehyde product of lipid peroxidation in AD ventricular fluid; (iii) increased protein and DNA oxidation; (iv) diminished energy metabolism and decreased cytochrome *c* oxidase content; (v) advanced glycation end products (AGE), malondialdehyde, carbonyls, peroxynitrite, heme oxygenase-1, and SOD-1 in neurofibrillary tangles (Butterfield et al., 2002); (vi) the presence in activated microglia surrounding most senile plaques of nitrotyrosine, formed from peroxynitrite ($ONOO^{\bullet -}$).

As discussed above, elevated production of Aβ, as a preventive antioxidant for brain lipoproteins under the action of increased oxidative stress and neurotoxicity in ageing, is postulated to represent a major event in the development of Alzheimer's disease (Butterfield et al., 2002). Individuals with genetic alterations in one of the genes that code the three transmembrane proteins – amyloid precursor protein, presenilin-1, and presenilin-2 – deposit large amounts of the amyloid fragment Aβ(1–42). Aβ peptide toxicity depends on its conformational state and peptide length. As already noted, it is known that Aβ aggregates into two different conformational states: (i) the non-β-sheet, an amorphous, nonfibrillar, state and (ii) the β-sheet, a highly ordered, fibrillar, state. The aggregated state and structure of Aβ peptide are influenced by the concentration of peptide, pH, and ionic concentration of zinc, copper and iron. The neurotoxicity of Aβ depends also on peptide length, with

Aβ(1-42) being more toxic than Aβ(1-40). Aβ(1-42) is the most likely candidate to generate hydrogen peroxide and other ROS (Butterfield et al., 2002).

As already mentioned above, copper binds to Aβ with a high affinity *via* histidine (His13, His14, His6) and tyrosine (Tyr10) residues and copper in abnormally high concentrations has been found in amyloid plaques. In addition to Cu^{2+}, Aβ also binds Zn^{2+} and Fe^{3+} *in vitro* and the amounts of these metals are also markedly elevated in the neocortex and especially enriched in amyloid plaque deposits in individuals with Alzheimer's disease (Butterfield et al., 2002). Zn^{2+} precipitates Aβ *in vitro* and Cu^{2+} promotes the neurotoxicity of Aβ, which correlates with metal reduction [$Cu^{2+} \rightarrow Cu^{+}$] and the generation of hydrogen peroxide (Cuajungco et al., 2000). The effect of copper is greater for Aβ (1-42) than for Aβ (1-40), corresponding to the capacity to reduce Cu^{2+} to Cu^{+}, respectively and form hydrogen peroxide. The copper complex of Aβ (1-42) has a highly positive reduction potential, characteristic of strongly reducing cupro-proteins (Huang et al., 1999).

Dikalov et al., 2004 reported that neurotoxic forms of amyloid-β, Aβ (1-42), Aβ (1-40), and also Aβ (25-35), stimulated copper-mediated oxidation of ascorbate, whereas nontoxic Aβ (40-1) did not. It was concluded that toxic Aβ peptides stimulate copper-mediated oxidation of ascorbate ($AscH^{-}$) and the generation of hydroxyl radicals. Therefore, Aβ-Cu^{2+} stimulated free radical generation may be involved in the pathogenesis of Alzheimer's disease. This can be described by the following set of equations

$$A\beta\text{-}Cu^{2+} + AscH^{-} \leftrightarrow A\beta\text{-}Cu^{+} + Asc^{\bullet-} + H^{+} \quad (3)$$

$$A\beta\text{-}Cu^{2+} + Asc^{\bullet-} \leftrightarrow A\beta\text{-}Cu^{+} + Asc \quad (4)$$

$$A\beta\text{-}Cu^{+} + H_2O_2 \leftrightarrow A\beta\text{-}Cu^{2+} + {}^{\bullet}OH + HO^{-} \text{ (Fenton)} \quad (5)$$

$$A\beta\text{-}Cu^{+} + O_2 \leftrightarrow A\beta\text{-}Cu^{2+} + O_2^{\bullet-} \quad (6)$$

In the presence of oxygen or H_2O_2, Cu^{+} may catalyse free radical oxidation of the peptide *via* the Fenton reaction (reaction (5)).

The *N*-terminally complexed Cu^{2+} can be reduced by electrons originating from the C-terminal methionine (Met-35) residues according to reaction Huang et al. (1999).

$$\text{Met-S} + A\beta\text{-}Cu^{2+} \leftrightarrow \text{Met-S}^{\bullet+} + A\beta\text{-}Cu^{+} \quad (7)$$

forming the sulphide radical of Met-35 (Met-$S^{\bullet+}$) and reducing Cu^{2+}. While thermodynamic calculations based on the reduction potentials of the Cu^{2+}/Cu^{+} and Met/ Met-$S^{\bullet+}$ couples show that reaction (7) is rather unfavourable, electron transfer between Met-S and Aβ-Cu^{2+} may be accelerated by the subsequent reaction of deprotonation of Met-$S^{\bullet+}$, leaving behind the 4-methylbenzyl radical, thus making the reaction (7) viable *in vivo* (Pogocki, 2003). The sulphide radical Met-$S^{\bullet+}$ may also undergo very fast reactions with, e.g. superoxide radical anion, originating from reaction (7). This reaction leads to the formation of methionine sulphoxide (Met-SO)

$$\text{Met-S}^{\bullet+} + O_2^{\bullet-} \xrightarrow{\text{Met}} 2\text{Met-SO} \quad (8)$$

which has been isolated from AD senile plaques. Methionine-35 is strongly related to the pathogenesis of AD, since it represents the residue in Aβ most susceptible to oxidation *in vivo*. It has been proposed that Met-35 oxidation to Met-sulphoxide reduced toxic and pro-apoptotic effects of the amyloid beta protein fragment on isolated mitochondria (Pogocki, 2003). Thus these results point to the critical role of Met-35 in Alzheimer's amyloid-beta-peptide (1-42)-induced oxidative stress and neurotoxicity.

Recently Apolipoprotein E (apoE), a lipid transport molecule that has been linked to the pathogenesis of AD, has been found to be subject to free radical attack and a direct correlation exists between Apo E peroxidation and Alzheimer's disease (Butterfield et al., 2002).

While the role of redox-metals in the etiology of AD seems to be a clear-cut, the role of zinc appears to be very questionable. A growing number of reports indicate that zinc in micromolar concentration inhibits Aβ-induced toxicity. The exact mechanisms of the protective effect of zinc against Aβ toxicity is unclear, however a reason might be cytoprotection through blockage of the membrane calcium channel pore formed by Aβ(1-40) (Bush, 2003).

Zinc and copper have a clear relationship in the context of AD (Cuajungco et al., 2000). The overall Zn level in the brain has been estimated as approximately 150 μM. Although the normal intracellular concentration is probably sub-nanomolar, the extracellular level may be in order of 500 nM [66]. However, extraordinarily high levels of Zn occur in the synaptic cleft with concentrations estimated at greater than 1 mM. This puts Zn at one to two orders of magnitude higher than synaptic Cu and highlights the fact that Zn is not a trace element but a major ionic regulator of synaptic transmission and other neuronal processes. The highest concentrations of Zn have also been associated with brain regions most affected in AD pathology, including the hippocampus, neocortex and amygdala. Aβ1-40 binds Zn at high and low affinities. Binding of Zn is mediated *via* histidine residues and is thus abolished at acid pH. Zn levels as low as 300 nM can rapidly precipitate synthetic Aβ1-40. Although Zn appears to contribute to amyloid aggregation and deposition *in vivo*, there is evidence that Zn may also act to inhibit the toxic action of Aβ. The argument advocating a protective role of zinc is its competition with copper (or iron) to bind Aβ. Zinc binding to Aβ changes the protein conformation to the extent that copper ions cannot reach its metal-binding sites. Prevention of copper from interacting with Aβ may preclude the Cu^{2+}-Aβ induced formation of hydrogen peroxide and free radicals.

On the other hand, a trigger caused by endogenous (genetic) and exogenous (e.g. environmental) factors results in oxidative and nitrosative stress which in turn leads to abnormal metabolism of Aβ accompanied by uncontrolled flooding of the vesicular zinc pool (Cuajungco & Lees, 1998). Thus, while low levels of zinc protect against Aβ toxicity, the excess of zinc released by oxidants could trigger neuronal death that is independent or even synergistic with the toxic effect of Aβ. This conclusion is in agreement with other studies documenting that at higher concentrations of zinc, binding to Aβ forces Aβ to precipitate over a wide range of pH (6–8) (Cuajungco & Faget, 2003). Zinc binding has been found to preserve the α-helical conformation of Aβ(1-40) and the highly ordered conformational state of Aβ(1-40) upon binding of zinc has been interpreted as producing toxic, fibrillar, Aβ aggregates. Consequently, the immunological/inflammatory response to nonsoluble Aβ plagues is disruption of zinc homeostasis followed by uncontrolled cerebral zinc release, typical for oxidative stress. It can be hypothesized that under normal physiological conditions a sensitive balance exists between zinc, copper, and Aβ metabolism. However, oxidative and nitrosative stress may perturb this balance which leads to uncontrolled zinc elevation and amyloid deposition. Uncontrolled accumulation of zinc or Aβ may lead to zinc-induced and Aβ-mediated oxidative stress and cytotoxicity.

Of fundamental importance in future research is determining whether loss of biometal homeostasis drives aberrant amyloid metabolism, aggregation, deposition and toxicity, or if other causes of amyloidogenesis result in perturbations to metal homeostasis. In reality, it is likely that both mechanisms will have important roles to play in progression of AD pathology.

A fundamental question in understanding AD is where inside a neuron most Aβ is made. It is clear that Aβ gets deposited in the terminal fields of neurons. Rodent experiments in which the perforant pathway was cut showed subsequent loss of Aβ deposition in the terminal field. These studies provide evidence for APP's axonal transport, processing at the nerve terminal, and presynaptic release of Aβ.

While all these studies are in progress, animal trials will no doubt continue with well-known and novel metal ligands. Recent reports have described the exciting development of potential therapeutic agents based on modulation of metal bioavailability. The metal-chelating ligands, clioquinol (CQ) and 1,2-bis(2-aminophenyloxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (DP-109) have shown promising results in animal models and in small clinical trials. A new generation of metal-ligand based therapeutics for AD is under development.

9.6.2. *Parkinson's disease*

Parkinson's disease (PD) involves a selective loss of neurons in an area of the midbrain called the substantia nigra (Sayre et al., 2001). The cells of the substantia nigra use dopamine (a neurotransmitter-chemical messenger between brain and nerve cells) to communicate with the cells in another region of the brain called the stratium. Thus, a reduction in nigral dopamine levels results in a decrease in stratial dopamine that is believed to cause PD symptoms (Jenner, 2003). Neuronal loss and Lewy bodies, the pathological hallmarks of PD, have been fond in cerebral cortex, anterior thalamus, hypothalamus, amygdala and basal forebrain. Lewy Bodies are tiny spherical protein deposits found in nerve cells. Their presence in the brain disrupts the brain's normal functioning, interrupting the action of the important chemical messenger's, including acetylcholine and dopamine; they are most likely formed as the cells try to protect themselves from attack (Sayre et al., 2001). The major component of intracytoplasmic Lewy bodies are filaments consisting of α-synuclein. Two recently identified point mutations in α-synuclein are the genetic causes of PD (Jin & Yang, 2006).

A majority of studies explored the effect of oxidative stress that contributes to the cascade of events leading to dopamine cell degeneration in PD (Tretter, Sipos, & Adam-Vizi, 2004). The occurrence of oxidative stress in PD is supported by both postmortem studies and by studies demonstrating the capacity of oxidative stress to induce nigral cell degeneration. There is evidence that there are high levels of basal oxidative stress in the substantia nigra pars compacta (SNc) in the normal brain, but that this increases in PD patients. However, other factors involving inflammation, excitotoxic mechanisms, toxic action of nitric oxide, and mitochondrial dysfunction play roles in the etiology of PD (Andersen, 2004).

Since it is known that the c-Jun N-terminal kinase (JNK) pathway plays an important role in regulating many of the cellular processes which are affected in Parkinson's disease, the possible importance of JNK pathway in pathogenesis of PD is being increasingly recognised (Peng & Andersen, 2003).

One of the earliest detectable changes in the PD brain is a dramatic depletion in substantia nigra levels of the glutathione. It has been demonstrated that glutathione depletion in dopaminergic cells in culture results in a selective decrease in mitochondrial complex I activity (a major hallmark of PD) and a marked reduction in mitochondrial function. Current evidence suggests that mitochondrial complex I inhibition may be the central cause of sporadic PD and that derangements in complex I cause α-synuclein aggregation, which contributes to the demise of dopamine neurons. The complex I inhibition appears to be due to production of nitric oxide ($NO^{\bullet}$), which can interact with the proteins within complex I and thereby inhibit its activity. Treatment of glutathione-depleted, cultured dopaminergic cells with inhibitors of nitric oxide synthetase (NOS), the enzyme that makes $NO^{\bullet}$, prevents mitochondrial complex I inhibition. In addition, increased iron levels have been reported in the Parkinsonian midbrain. Interestingly, genetically or pharmacologically chelated iron (e.g. Fe–clioquinol complex, see also above) in a form which cannot participate in oxidative events prevents degeneration of dopaminergic midbrain neurons (Kaur et al., 2003). This suggests that increased level of iron is actively involved in subsequent neurodegeneration and that iron chelation may prevent or delay PD progression.

The above mentioned biochemical abnormalities, such as mitochondrial complex I deficiency, depletion of intracellular thiols, and increased nigral iron result in aberrant oxidation of dopamine to 6-hydroxydopamine or dopamine-quinone, both neurotoxic either directly or in conjugation with cystein (Sayre et al., 2001). The entry and release of iron from iron-storage protein, ferritin, occurs *via* the "free iron (ferrous) labile pool", active in Fenton chemistry. Besides superoxide, ferritin iron can be released by 6-hydroxydopamine a neurotoxin implicated in PD.

It has been recently shown that the loss of inherited PD gene DJ-1 leads to striking sensitivity to the herbicide paraquat and the insecticide rotenone (Meulener et al., 2005), which suggests that DJ-1, may have a role in protection from oxidative stress from environmental toxins. Thus exposure to various environmental toxins acting through oxidative stress seems to be associated with PD.

Levodopa (L-dopa) (often combined with carbidopa) is a dopamine precursor and the most commonly used medicine to treat Parkinson's disease. It is possible that the use of L-dopa for prolonged periods causes oxidation and toxicity to brain cells. If this turns out to be true, it would further justify the recommendations that antioxidants be added to standard Parkinson's disease therapy.

Since oxidative stress appears to represent a portion of a cascade of biochemical changes leading to dopaminergic death, one of a major problem in understanding the pathogenesis of PD is separating out the effect and extent of oxidative stress from other components of the cascade that themselves can play a primary role in the initiation of ROS and RNS.

9.7. Ageing

The process of ageing may be defined as a progressive decline in the physiological functions of an organism after the reproductive phase of life. The free radical theory of ageing was first introduced in 1956 by Denham Harman who proposed the concept of free radicals playing a role in the ageing process (Harman, 1956); his work has gradually triggered intense research into the field of role of free radicals in biological systems.

Generally, there are two main theories describing the process of ageing: damage-accumulation theories and genetic theories (Fossel, 2003; Hayflick, 1998). Damage accumulation theories involve "free radical theory", "glycation theory", "error catastrophe theory", "membrane theory", "entropy theory" and others, among which "free radical theory" is probably the most complex approach to explain the process of ageing. The "free radical approach" is based on the fact that the random deleterious effects of free radicals produced during aerobic metabolism cause damage to DNA, lipids, and proteins and accumulate over time.

The genesis of ageing starts with oxygen, occupying the final position in the electron transport chain (Valko et al., 2004). Even under ideal conditions, some electrons "leak" from the electron transport chain. These leaking electrons interact with oxygen to produce superoxide radicals, so that under physiological conditions, about 1–3% of the oxygen molecules in the mitochondria are converted into superoxide. The primary site of radical oxygen damage from superoxide radical is mitochondrial DNA (mtDNA) (Cadenas & Davies, 2000). The cell repairs much of the damage done to nuclear DNA (nDNA), but mtDNA cannot be readily fixed. Therefore, extensive mtDNA damage accumulates over time and shuts down mitochondria, causing cells to die and the organism to age. An interesting correlation between oxygen consumption and ageing was found (Halliwell & Gutteridge, 1999): (i) lowered oxygen consumption explains why queen bees live 50 times longer than actively flying worker bees; (ii) houseflies prevented from flying by removing their wings lived much longer than normally flying insects because of decreased consumption of oxygen; (iii) larger animals consume less oxygen per unit of body mass than smaller ones and live longer; (iv) different rates of ROS generation influence the life span of animals. For example, rat and pigeon have similar metabolic rates, however different life spans (rat: 3 years; pigeon: 30 years). This fact could be explained by *in vitro* experiments that show that pigeon tissues generate ROS more slowly than rat mitochondria; (v) caloric restriction in rodents plays an important role in the process of ageing and is associated with increased DNA repair capacity, decreased production of superoxide, and decreased levels of damaged DNA, lipids, and proteins; (vi) longer-lived species have more efficient antioxidant protective mechanisms in relation to rates of oxygen uptake that short-lived species. This mostly applies to levels of SOD, carotenoids, GSH, glutathione peroxidase, and Vitamin E in animals.

In humans, the level of oxidative DNA damage, as measured by urinary biomarkers, can be modulated by caloric restriction and dietary composition. Consequently, longevity may depend not only on the basal metabolic rate but also on dietary caloric intake. The accumulation of free radical-induced damage to biomolecules is illustrated by an age-related increase in the serum 8-OH-dG level in disease-free individuals over an age range of 15–91 years. Numerous studies have reported the accumulation of 8-OH-dG and other lesions with age, both *in vivo* and *in vitro*, in nuclear and mitochondrial DNA. DNA repair capacity correlates with species-specific life span. Repair activity appears to decline with age. However, several studies on animals reported that age-related increase in 8-OH-dG in nuclear and mitochondrial DNA is due to a tissue's increased sensitivity to oxidative damage rather than decreased repair capacity with age. Interestingly, antioxidant status does not change significantly with age. Human studies have shown that comparison of SOD, GSH, catalase, and ceruloplasmin levels among the age groups of 35–39, 50–54, and 65–69 years did not alter (Barnett & King, 1995).

The concept of ageing is supported by studies in many different animals showing that ageing is frequently associated with the accumulation of oxidized forms of proteins. Generally, a role of protein modification in ageing was highlighted by the result that many different enzymes isolated from younger animals were catalytically more active and more heat stable than the same enzymes isolated from older animals (Stadtman, 2004). Because exposure of enzymes from young animals to metal-catalyzed oxidation led to changes in activity and heat-stability similar to those observed during ageing, it was proposed that ROS mediated protein damage is involved in this process.

Telomeres are unique DNA-protein structures that contain noncoding TTAGGG repeats and telomere-associated proteins (Saretzki, Petersen, Petersen, Kolble, & von Zglinicki, 2002). These specialized structures are essential for maintaining genomic integrity. Telomere dysfunction has been proposed to play a critical role in ageing as well as cancer progression. Nevertheless, ageing is a multifactorial process, and DNA and protein damage cannot be responsible for all of the pathophysiological changes seen.

10. Free radicals-induced tissue injury: Cause or consequence?

From the discussion above, it is clear that free radials act as signalling species in various normal physiological processes. It is also clear that excessive production of free radicals causes damage to biological material and is an essential event in the etiopathogenesis of various diseases (Juranek & Bezek, 2005). However, the question was recently raised whether uncontrolled formation of ROS species is a primary cause or a downstream consequence of the pathological process. While the role of free radicals as primary species causing damage to DNA in the mechanism of carcinogenesis is clear (Valko et al., 2004), the primary role of ROS in the process of post-ischemic tissue injury and some other disease states is controversial.

It is known that increased concentration of cytosolic calcium plays a role in tissue injury by activation of calcium-dependent regulatory proteins and degradative enzymes which may irreversibly alter functions of the affected bio-macromolecules. Various experiments suggest that post-ischemic tissue injury occurs as an inevitable consequence of increased cytosolic calcium which in turn leads to overproduction of free radicals causing enzymatic breakdown of essential intracellular components (see Fig. 5). Thus observed overproduction of ROS in post-ischemic injury is unlikely to be the primary cause of the pathological process, but more probably it is a consequence of increased concentration of cytosolic calcium (Juranek & Bezek, 2005). In fact, various calcium blockers were shown to inhibit lipid peroxidation and prevented ROS formation.

From the discussion on the role of ROS in the etiology of neurological disorders, in particular Parkinson's disease, it is unclear if free radical-induced oxidative stress is the primary, *initiating* event causing neurodegeneration, however, it is clear that oxidative stress is involved in the *propagation* stage of cellular injury that leads to neuropathology (Andersen, 2004). Therefore, there need not be a cascade of events initiated by oxidative stress, rather a cycle of events of which oxidative stress is a major component. Inhibition of oxidative stress might break the cycle of cell death of neurons, thus much effort is devoted to developing rational drug or genetic therapy targeted at the "oxidative stress component" of the cycle.

11. Conclusions

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism. ROS/RNS are known to act as secondary messengers controlling various normal physiological functions of the organism and therefore the production of $NO^{\bullet}$ by NOS and superoxide by NAD(P)H is tightly regulated by hormones, cytokines, and other mechanisms. In addition, ROS and RNS participate in various redox-regulatory mechanisms of cells in order to protect cells against oxidative stress and maintenance of cellular "redox homeostasis". The most prominent examples of such mechanisms involve the inhibition of NOS by $NO^{\bullet}$ and the oxidative induction of protective enzymes by the redox-sensitive bacterial OxyR protein.

Overproduction of ROS, most frequently either by excessive stimulation of NAD(P)H by cytokines, or by the mitochondrial electron transport chain and xanthine oxidase result in oxidative stress. Oxidative stress is a deleterious process that can be an important mediator of damage to cell structures and consequently various disease states and ageing. Some of the challenging areas for further research in oxidative stress-related disease are as follows:

(i) ROS appear to be key regulatory factors in molecular pathways linked to tumour development and tumour dissemination, which offer potential therapeutic invention points. For example specific understanding of the regulation of anti-proliferative pathways by MnSOD and its control of tumour invasion might aid in the design of novel therapies targeting the respective molecular pathways.

(ii) Since GSH depletion may sensitize tumour cells to some chemotherapy agents and many of the GSH-depleting agents have dose-related toxicity, development of non-toxic GSH-depleting agent has immense importance in overcoming multidrug resistance (MDR). Continued research is needed to better understand the mechanisms and specific apoptotic pathways involved in ROS-induced cell death, and to determine the most rational and effective combination of redox-active agents.

(iii) Insights into the mechanism of $NO^{\bullet}$/redox-mediated signalling may help in the development of novel therapeutic approaches for heart failure.

(iv) With respect to diabetes, several important questions remain to be answered: (a) Whether the reduction in mitochondrial function *in vivo* is due to mitochondrial loss, functional defects in the mitochondria, or both; (b) the role of uncoupling protein-2 (UCP-2) in β cell dysfunction in patients with type 2 diabetes.

(v) Future work to understand the molecular mechanisms implicated in insulin resistance might reveal "key-regulatory" kinases and/or direct or indirect inhibitors of these kinases which are responsible for the inhibition of the functions of IRS1 but also of IRS2.

(vi) A fundamental knowledge gap in Alzheimer's disease research today is where inside a neuron most Aβ is made. It is clear that Aβ gets deposited in the terminal fields of neurons. Rodent experiments in which the perforant pathway was cut showed subsequent loss of Aβ deposition in the terminal field.

(vii) Of fundamental importance in future research in Alzheimer's disease is determining whether loss of biometal homeostasis drives aberrant amyloid metabolism, aggregation, deposition and toxicity or if other causes of amyloidogenesis result in perturbations to metal homeostasis.

(viii) The powerful neuroprotective agents for the treatment of Parkinson's disease should be targeted at reducing oxidative stress, restoring complex I activity, reducing α-synuclein aggregation and enhancing protein degradation. Further, it remains an open question to what extent the mitochondrial damage seen in Parkinson's disease is of genetic origin and how much is caused by hydrogen peroxide generated during enhanced turnover of dopamine neurons.

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