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Mini-review

Free radicals, metals and antioxidants in oxidative stress-induced cancer

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Dedicated to Prof. Ladislav Valko on the occasion of his 75th birthday

Abstract

Oxygen-free radicals, more generally known as reactive oxygen species (ROS) along with reactive nitrogen species (RNS) are well recognised for playing a dual role as both deleterious and beneficial species. The "two-faced" character of ROS is substantiated by growing body of evidence 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. The cumulative production of ROS/RNS through either endogenous or exogenous insults is termed oxidative stress and is common for many types of cancer cell that are linked with altered redox regulation of cellular signalling pathways. 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. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions (8-OH-G) have been noted in various tumours, strongly implicating such damage in the etiology of cancer. It appears that the DNA damage is predominantly linked with the initiation process. This review examines the evidence for involvement of the oxidative stress in the carcinogenesis process. Attention is focused on structural, chemical and biochemical aspects of free radicals, the endogenous and exogenous sources of their generation, the metal (iron, copper, chromium, cobalt, vanadium, cadmium, arsenic, nickel)-mediated formation of free radicals (e.g. Fenton chemistry), the DNA damage (both mitochondrial and nuclear), the damage to lipids and proteins by free radicals, the phenomenon of oxidative stress, cancer and the redox environment of a cell, the mechanisms of carcinogenesis and the role of signalling cascades by ROS; in particular, ROS activation of AP-1 (activator protein) and NF-κB (nuclear factor kappa B) signal transduction pathways, which in turn lead to the transcription of genes involved in cell growth regulatory pathways. The role of enzymatic (superoxide dismutase (Cu, Zn-SOD, Mn-SOD), catalase, glutathione peroxidase) and non-enzymatic antioxidants (Vitamin C, Vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), flavonoids, selenium and others) in the process of carcinogenesis as well as the antioxidant interactions with various regulatory factors, including Ref-1, NF-κB, AP-1 are also reviewed.
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1. Introduction

In the last two decades there has been an explosive interest in the role of oxygen-free radicals, more generally known as "reactive oxygen species," (ROS) and of

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"reactive nitrogen species" (RNS) in experimental and clinical medicine [1]. ROS and RNS: (i) are generated during irradiation by UV light, by X-rays and by gamma-rays; (ii) are products of metal-catalyzed reactions; (iii) are present as pollutants in the atmosphere; (iv) are produced by neutrophils and macrophages during inflammation; (v) are by-products of mitochondria-catalyzed electron transport reactions and other mechanisms [2].

ROS/RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems [3]. Beneficial effects of ROS 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 concentrations is the induction of a mitogenic response. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acids (termed oxidative stress) [4]. The harmful effects of ROS are balanced by the antioxidant action of non-enzymatic antioxidants in addition to antioxidant enzymes [5]. Despite the presence of the cell's antioxidant defence system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle, and radical-related damage to DNA, to proteins and to lipids has been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions [1]. This paper examines the available evidence for the involvement of cellular oxidants in the incidence of cancer, in damage to cellular structures and in the role of signalling cascades by ROS. A discussion of the various protective pathways that may be provided by the antioxidant network against the deleterious action of free radicals as well as the antioxidant interactions with various regulatory factors are also addressed.

2. Sources and reactions of ROS and RNS

Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons. The presence of unpaired electrons usually confers a considerable degree of reactivity upon a free radical. Those radicals derived from oxygen represent the most important class of such species generated in living systems [3].

ROS can be produced from both endogenous and exogenous substances. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation [6]. Mitochondria have long been known to generate significant quantities of hydrogen peroxide. The hydrogen peroxide molecule does not contain an unpaired electron and thus is not a radical species. Under physiological conditions, the production of hydrogen peroxide is estimated to account for about ~2% of the total oxygen uptake by the organism. However, it is difficult to detect the occurrence of the superoxide radical in intact mitochondria, most probably in consequence of the presence of high SOD activity therein. Generation of the superoxide radical by mitochondria was first reported more than three decades ago by Loschen et al. [7]. After the determination of the ratios of the mitochondrial generation of superoxide to that of hydrogen peroxide, the former was considered as the stoichiometric precursor for the latter.

Ubisemiquinone has been proposed as the main reductant of oxygen in mitochondrial membranes [6]. Mitochondria generate approximately 2–3 nmol of superoxide/min per mg of protein, the ubiquitous presence of which indicates it to be the most important physiological source of this radical in living organisms [6]. Since mitochondria are the major site of free radical generation, they are highly enriched with antioxidants including GSH and enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which are present on both sides of their membranes in order to minimise oxidative stress in the organelle [8]. Superoxide radicals formed on both sides of mitochondrial inner membranes are efficiently detoxified initially to hydrogen peroxide and then to water by Cu, Zn-SOD (SOD1, localised in the intermembrane space) and Mn-SOD (SOD2, localised in the matrix).

Besides mitochondria, there are other cellular sources of superoxide radical, for example xanthine oxidase (XO), a highly versatile enzyme that is widely distributed among species (from bacteria to man) and within the various tissues of mammals [9]. Xanthine oxidase is an important source of oxygen-free radicals. It is a member of a group of enzymes known as molybdenum iron–sulphur flavin hydroxylases and catalyzes the hydroxylation of purines. In particular, XO catalyzes the reaction of hypoxanthine to xanthine and xanthine to uric acid. In both steps, molecular oxygen is reduced, forming the superoxide anion in the first step and hydrogen peroxide in the second [3].

Additional endogenous sources of cellular reactive oxygen species are neutrophils, eosinophils and macrophages. Activated macrophages initiate an increase in oxygen uptake that gives rise to a variety of reactive oxygen species, including superoxide anion, nitric oxide and hydrogen peroxide [10]. Cytochrome P450 has also been proposed as a source of reactive oxygen species. Through the induction of cytochrome

P450 enzymes, the possibility for the production of reactive oxygen species, in particular, superoxide anion and hydrogen peroxide, emerges following the breakdown or uncoupling of the P450 catalytic cycle.

In addition, microsomes and peroxisomes are sources of ROS. Microsomes are responsible for the 80% H_2O_2 concentration produced in vivo at hyperoxia sites [11]. Peroxisomes are known to produce H_2O_2, but not $O_2^{\bullet -}$, under physiologic conditions. Although the liver is the primary organ where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these H_2O_2-generating mechanisms. Peroxisomal oxidation of fatty acids has recently been recognised as a potentially important source of H_2O_2 production as a result of prolonged starvation.

The release of the biologically active molecules such as cytokines and others, from activated Kupffer cells (the resident macrophage of the liver) has been implicated in hepatotoxicological and hepatocarcinogenic events. Recent results indicate that there is a close link between products released form activated Kupffer cells and the tumour promotion stage of the carcinogenesis process [12].

Reactive oxygen species can be produced by a host of exogenous processes. Environmental agents including non-genotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells. The induction of oxidative stress and damage has been observed following exposure to various xenobiotics. These involve chlorinated compounds, metal (redox and non-redox) ions, radiation and barbiturates. For example 2-butoxyethanol is known to produce ROS indirectly, which causes cancer in mice [13].

2.1. Chemistry and biochemistry of ROS

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-catalyzed processes [14]. Superoxide radical ion does not react directly with polypeptides, sugars, or nucleic acids, and its ability to peroxidise lipids is controversial. Superoxide is depleted undergoing a dismutation reaction [15]:

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (1)$$

SOD enzymes accelerate this reaction in biological systems by about four orders of magnitude. It should be noted that SOD enzymes work in conjunction with H_2O_2-removing enzymes, such as catalases and glutathione peroxidases [16].

The generation of various free radicals is closely linked with the participation of redox-active metals [17]. The redox state of the cell is largely linked to an iron (and sometimes 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 [18]. The released Fe(II) can participate in the Fenton reaction, generating highly reactive hydroxyl radical ($Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^-$). 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(II) available for the Fenton reaction [19–22]. 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(III) by superoxide, yielding Fe(II) and oxygen ($Fe(III) + O_2^{\bullet -} \rightarrow Fe(II) + O_2$) [22].

The hydroxyl radical is highly reactive with a half-life in aqueous solution of less than 1 ns [23]. Thus when produced in vivo it reacts close to its site of formation. It can be generated through a variety of mechanisms. Ionising radiation causes decomposition of H_2O, resulting in the formation of ${}^{\bullet}OH$ and hydrogen atoms. ${}^{\bullet}OH$ is also generated by photolytic decomposition of alkylhydroperoxides. Production of ${}^{\bullet}OH$ close to DNA could lead to this radical reacting with DNA bases or the deoxyribosyl backbone of DNA to produce damaged bases or strand breaks. It has been proposed that the extent of DNA strand breaking by ${}^{\bullet}OH$ is governed by the accessible surface areas of the hydrogen atoms of the DNA backbone.

As mentioned above, the majority of the hydroxyl radicals generated in vivo comes from the metal-catalyzed breakdown of hydrogen peroxide, according to the Fenton reaction M^{n+}(=Cu^+, Fe^{2+}, Ti^{3+}, Co^{2+}) + $H_2O_2 \rightarrow M^{(n+1)+}$(=$Cu^{2+}$, Fe^{3+}, Ti^{4+}, Co^{3+}) + ${}^{\bullet}OH + OH^-$ where M^{n+} is a transition metal ion [22,24]. The most realistic in vivo production of hydroxyl radical according to Fenton reaction occurs when M^{n+} is iron, copper, chromium, cobalt and certain other metals. However, O'Halloran 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 [25]. 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 [26]. 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 [26]. LIP is defined as a low-molecular weight pool of weakly chelated iron that rapidly passes through the cell. Most probably, it consists of both forms of iron ions (Fe(II) and Fe(III)) chelated by a variety of chelators such as citrate, phosphate, carboxylates, nucleotides and others. Experiments, carried out in a human K 562 cell line, have suggested that the concentration of Fe in this pool is 0.2–0.5 μM and that it is composed primarily of Fe(II) [27].

Typical of additional radicals derived from oxygen that can be formed in living systems are peroxyl radicals ($ROO^{\bullet}$). Peroxyl radicals are high-energy species, with a reduction potential ranging from +0.77 to +1.44 V, depending on the R group [28]. The simplest peroxyl radical is the dioxyl (hydroperoxyl) radical $HOO^{\bullet}$, which is the conjugate acid of superoxide, $O_2^{\bullet -}$. The chemistry of this type of molecule varies according to the nature of the R group, the local environment, and the concentration of oxygen and of other reactants [29]. Perhaps the most interesting feature of peroxyl radicals is the diversity of those biological reactions in which they participate. The detection and measurement of lipid peroxidation is most frequently cited as evidence to support the involvement of peroxyl radical reactions in human disease and toxicology [30,31]. Peroxyl radicals are involved in DNA cleavage and protein backbone modification. Peroxyl radicals synergistically enhance the induction of DNA damage by superoxide.

2.2. Chemistry and biochemistry of RNS

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 [32–35]. This small molecule 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 [36]. Due to its extraordinary properties, in 1992 was $NO^{\bullet}$ acclaimed as the "molecule of the year" in Science Magazine [37]. Overproduction of reactive nitrogen species is called nitrosative stress [38]. 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.

$NO^{\bullet}$ has a half-life of only a few seconds in an aqueous environment [39]. $NO^{\bullet}$ 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. $NO^{\bullet}$ 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.

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 an oxidising free radical that can cause DNA fragmentation and lipid oxidation [40]:

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^- \quad (2)$$

Reaction (2) 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 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(II)-haem groups in the enzyme *guanylate cyclase:*

$$Fe(II) + NO^{\bullet} \rightarrow Fe(II)\text{–}NO \quad (3)$$

Nitric oxide reacts fast with many radicals, e.g. with tyrosyl radical. By contrast, nitric oxide is generally unreactive with most non-radicals.

If cellular responses to nitrosative stress are similar to responses for oxidative stress, protein *S*-nitrosothiols (protein-SNO) might occur according to the reaction:

$$\text{protein-SH} + RSNO \rightarrow \text{protein-SNO} + RSH \quad (4)$$

and indeed, protein *S*-nitrosothiols have been detected in animal blood and involve *S*-nitrosoalbumin, nitrosohaemoglobin and the least stable *S*-nitrosocysteine.

3. Metal-induced oxidative stress and cancer

Many studies have focused on metal-induced toxicity and carcinogenicity, emphasising their role in the generation of reactive oxygen and nitrogen species in biological systems, and the significance of this therein [17,19–21,41–43]. Metal-mediated formation of free radicals may cause various modifications to DNA bases, enhanced lipid peroxidation, and changes in calcium and sulphydryl homeostasis.

3.1. Iron

From a compilation of biochemical, animal and human data, links have been proposed between increased levels of iron in the body and an enhanced risk of a variety of diseases including vascular disease, cancer and certain neurological conditions [44,45]. Iron-mediated formation of ROS leading to DNA and lipid damage appears to result from an exaggeration of the normal function of iron, which is to transport oxygen to tissues. Iron-induced free radical damage to DNA appears to be important for the development of cancer and cancer cells are known grow rapidly in response to iron [46]. Correspondingly, pre-menopausal women and children are believed to have a lower risk of common diseases because amounts of iron in the body are unlikely to be excessive at these times.

Nelson and Babbs proposed that intestinal exposure to ingested iron may be a principal determinant of human colorectal cancer in highly developed, meat-eating countries [47,48]. A dose-dependent relationship for serum ferritin level and colon adenoma risk was found. We have proposed an alternative mechanism in which the bile acids (deoxycholic acid), the K vitamins, iron(II) complexes and oxygen interact to induce an oncogenic effect in the colon by the generation of free radicals [49].

Genetic hemochromatosis is associated with an increased risk for hepatocellular carcinoma. The association between elevated body iron stores and the development of hepatocellular carcinoma in subjects with iron overload unrelated to genetic hemochromatosis along with the experimental evidence of a co-carcinogenic role of iron strongly support the contention that iron is involved in the development of hepatocellular carcinoma [50,51].

Occupational exposure to asbestos containing about 30% (weight) of iron is related to an increased risk of asbestosis — the second most important cause of lung cancer after smoking [52]. It is generally accepted that asbestos-induced carcinogenesis is linked with the free radicals.

Animal studies of iron-induced carcinogenesis are well documented. Intramuscular injections of an iron–dextran complex, frequently used for the treatment of anemia in humans, caused spindle cell sarcoma or pleomorphic sarcoma in rats at the site of injection [53].

Nitrilotriacetic acid (NTA), synthetic aminotricarboxylic acid forms water-soluble chelate complexes with metal cations and is used in household detergents. The Fe–NTA complex, however, induced renal carcinogenesis. Surprisingly, this complex, regardless of number of saturated binding sites on iron by NTA, works as efficiently as “free iron” in vitro at physiological pH catalyzing the breakdown of hydrogen peroxide via Fenton reaction [54].

3.2. Copper

Because copper is an essential component of several endogenous antioxidant enzymes, and that free radicals have been proposed to play a role in the process of carcinogenesis, the effects of dietary copper levels on the development of cancer have been investigated [55].

The weight of evidence from in vitro and in vivo assays indicates that copper (as the copper salts) is not genotoxic [56]. However, in vitro studies have shown that cancer cells in a high copper environment find it easy to proliferate into tumour [57,58]. Therefore, it has been proposed that copper-lowering drug may stabilise advanced cancer. Brewer and his group tested a drug known as tetrathiomolybdate (TM), which binds up dietary copper before it can be absorbed by the body, to see if they could reduce the spread of tumours in patients with different types of metastatic cancer [59]. In five of six patients kept at 80% of normal copper levels for more than 90 days, existing tumours did not grow and new tumours did not form for more than 1 year. This suggests the use of TM either as the sole therapy for cancer or in conjunction with other treatments, such as surgery, chemotherapy, radiation therapy and others. Similarly to iron, copper is a well-known pro-oxidant and may participate in metal-catalyzed peroxidation of lipids (see below).

3.3. Chromium

Chromium(III), which occurs in nature, is an essential trace element that plays an important role in regulating blood levels of glucose. Chromium(VI) is poten-

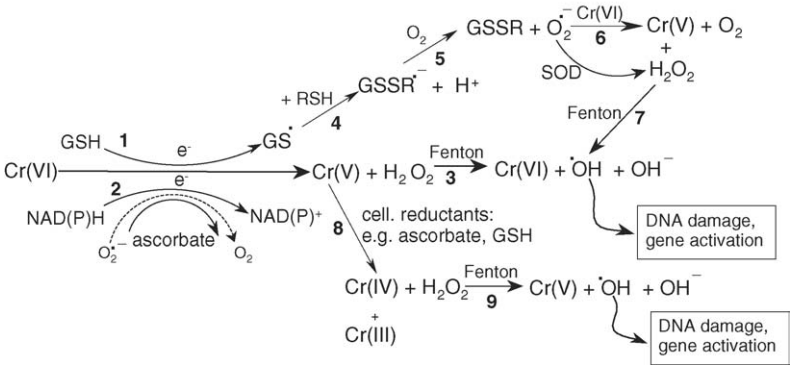

Fig. 1. Biological reductants of Cr(VI) and its reactions (for description, see the text).

tially toxic and carcinogenic at high doses [60–62]. All chromates, Cr(VI), can actively enter the cells through channels for the transfer of isoelectric and isostructural anions, such as those for SO_4^{2-} and HPO_4^{2-} [63]. Insoluble chromates are absorbed by cells via phagocytosis. Until recently, transport of chromium through the cell membrane was thought to concern exclusively the Cr(VI) species. However, very recent models have also considered the uptake of reduced Cr species generated by extracellular redox mechanisms. Certain extracellularly generated Cr(V) and Cr(III) complexes also have high permeabilities through the cell membrane. Once inside the cell, chromates are able to generate free radicals [64].

Only chromium(VI) does not react with DNA in vitro, or in isolated nuclei. However, once inside the cell, in the presence of cellular reductants, it causes a wide variety of DNA lesions including Cr–DNA adducts, DNA–protein crosslinks, DNA–DNA crosslinks and oxidative damage [17]. Within the cell, glutathione rapidly forms a complex with Cr(VI), followed by a slow reduction of Cr(VI) to yield Cr(V) (reaction (1), Fig. 1) [65]. Using an EPR spin-trapping technique, it was possible to demonstrate the formation of Cr(V) species (most probably the Cr(V)–glutathione complex) and the glutathione-derived thiyl radical ($GS^{\bullet}$) (reaction (1), Fig. 1) [65]. Once formed, Cr(V) species were found to alter the DNA conformation. In addition to GSH, a number of in vitro studies have confirmed that various other substances were capable of reducing Cr(VI). These include ascorbate, cystein, lipoic acid, NAD(P)H, fructose, ribose and others [65]. While pretreatment of the animals with ascorbate and GSH decreased formation of Cr(V), pretreatment with NAD(P)H augmented it. Based on these studies it was suggested that the in vivo one-electron reductant of Cr(VI) is most probably NAD(P)H flavoenzymes (reaction (2), Fig. 1). Once formed, Cr(V) can react via the Fenton reaction (reaction (3), Fig. 1) with H_2O_2 forming a hydroxyl radical which is capable of causing DNA damage [65]. In addition to the cellular damaging effect of the $GS^{\bullet}$ radical, it can further react with other thiol molecules in oxygenated tissues to give the superoxide radical (reactions (4) and (5), Fig. 1). Superoxide can further reduce Cr(VI) to Cr(V) (reaction (6), Fig. 1) which can then catalyze the decomposition of H_2O_2 thus creating the DNA damaging hydroxyl radical (reaction (7), Fig. 1). Cr(V) can also be reduced by cellular reductants (e.g. ascorbate, GSH) to Cr(IV) (reaction (8), Fig. 1), again participating in Fenton chemistry to generate a hydroxyl radical (reaction (9), Fig. 1).

A series of detailed studies advocating a Cr(III)-dependent pathway in Cr(VI) carcinogenicity and mutagenicity was presented by Zhitkovich and his group who presented the evidence that intracellular reduction of Cr(VI) results in the extensive formation of Cr–DNA adducts, among which Cr(III)-mediated DNA cross-links of glutathione, cysteine, histidine and ascorbate represent a major class of DNA modifications [66]. Several further studies from the same laboratory disproved the existence and genotoxic/mutagenic effect of the Cr(V) species and the hydroxyl radical. Reduction of carcinogenic Cr(VI) by physiological concentrations of Vitamin C has been shown to generate ascorbate–Cr(III)–DNA crosslinks, binary Cr(III)–DNA adducts, both potential sources of oxidative DNA damage by intermediate reaction products [66]. The results show that Cr–DNA adducts are responsible for both the mutagenicity and genotoxicity of Cr(VI).

Hexavalent chromium is known to cause lung cancer in humans [60]. For example, workers exposed to hexavalent chromium in workplace air had much higher rates of lung cancer than workers who were not exposed, and chromium was recently implicated as causing an increased rate of breast cancer [67].

3.4. Cobalt

Various studies have investigated the possibility that cobalt-mediated free radical generation contributes to the toxicity of cobalt. Hanna et al. performed EPR spin-trapping studies to detect the generation of oxygen-free radicals from the reaction of hydrogen peroxide with various Co(II) complexes under physiological conditions [68]. It was found that the superoxide radical was generated by the reaction of H_2O_2 with Co(II), but this was inhibited when Co(II) was chelated with adenosine 5′-diphosphate or citrate. An EDTA Co(II) complex also did not produce the detectable formation of free-radicals when H_2O_2 was added; however, visible absorption spectra indicated that the Co(II) had been oxidised to Co(III) in this case. The nitrilotriacetate cobalt(II) complex was found to catalyze the decomposition of H_2O_2 with the formation of hydroxyl radicals and was accompanied by the slow oxidation of Co(II). Another detailed study by Leonard et al. revealed that, in the presence of SOD, cobalt in suspension (cobalt metal particles) is able to react with dissolved oxygen to generate $^{\bullet}OH$, as monitored by EPR spin-trapping experiments [69].

Cobalt is known to be toxic to the heart and suspected to be carcinogenic in animals when given in large quantities. Exposure to cobalt sulphate by inhalation resulted in increased incidence of alveolar/bronchiolar neoplasms and a spectrum of inflammatory, fibrotic, and proliferative lesions in the respiratory tracts of male and female rats and mice [70]. Injection of Co(II) into rats lead to a pattern of oxidative DNA base damage characteristic of hydroxyl radical attack via the Fenton reaction [71]. In addition, cobalt was found to interfere with DNA repair processes [72]. However, trace amounts of cobalt are needed in the diet because cobalt is an integral metal of vitamin B12 [73].

3.5. Vanadium

Vanadium, is a transition metal element which occurs in various oxidative states and may participate in reactions involving formation of free radicals [74]. As mentioned above, vanadium(V) in plasma is rapidly reduced to vanadium(IV) by both enzymatic (e.g. NADPH) and non-enzymatic (ascorbic acid) plasmatic antioxidants and is then transported and bound to plasma proteins. The following reactions may take place inside the cell [75]:

$$V(V) + NADPH \rightarrow V(IV) + NADP^{+} + H^{+} \quad (5)$$

$$V(IV) + O_2 \rightarrow V(V) + O_2^{\bullet -} \quad (6)$$

$$V(V) + O_2^{\bullet -} \rightarrow [V(IV)\text{-}OO^{\bullet}] \quad (7)$$

leading to formation of peroxovanadyl radicals [V(IV)–$OO^{\bullet}$] and vanadyl hydroperoxide [V(IV)–$OH^{\bullet}$] [76]. The superoxide generated is further converted, by the dismutation reaction with SOD, into H_2O_2. In vitro EPR studies have confirmed that one-electron reduction of vanadium(V) to vanadium(IV), mediated by non-enzymatic ascorbate together with phosphate may represent an important vanadium(V) reduction pathway in vivo [77]. It has been proposed that the resulting reactive species generated by vanadium(IV) from H_2O_2 and lipid hydroperoxide via a Fenton-like reaction may play a significant role in the mechanism of vanadium(V)-induced cellular injury under physiological conditions [78]:

$$V(IV) + H_2O_2 \rightarrow V(V) + OH^{-} + {}^{\bullet}OH \quad (8)$$

In addition, the use of vanadium compounds as inhibitors of tyrosine phosphatases in studies of signal transduction points to their potential to induce oxidative stress [79].

3.6. Cadmium

Cadmium is a highly toxic metal. Cadmium itself is unable to generate free radicals directly, however, indirect generation of various radicals involving the superoxide radical, hydroxyl radical and nitric oxide has been reported [80]. Some experiments also confirmed the generation of (non-radical) hydrogen peroxide which itself in turn may be a significant source of radicals via Fenton chemistry [81].

An interesting mechanism explaining the indirect role of cadmium in free radical generation was presented some years ago [82]. In this mechanism it was proposed that cadmium can replace iron and copper in various cytoplasmic and membrane proteins (e.g. ferritin, apoferritin), thus increasing the amount of unbound free or chelated copper and iron ions which then participate in oxidative stress via Fenton reactions [83]. Similar findings were very recently presented by Watjen and Beyersmann [84].

Cadmium is a potent human carcinogen and occupational exposure to it has been associated with cancers of the lung, the prostate, pancreas and kidney [85]. Because of its characteristics as a lung carcinogen, cadmium has been classified as a category #1 human carcinogen by the International Agency for Research on Cancer and the National Toxicology Program of the USA. It has also been suggested that cadmium might also be implicated in the pathogenesis of human pancreatic cancer and renal carcinoma [85].

3.7. Arsenic

Arsenic (usually as arsenic trioxide, As_2O_3) is well known as a poison and has been discovered to be a carcinogen in humans. Many studies confirmed the generation of free radicals during arsenic metabolism in cells [86]. Interestingly, some recent reports have provided experimental evidence that arsenic-induced generation of free radicals can cause cell damage and death through activation of oxidative sensitive signalling pathways [87]. Arsenic-mediated generation of reactive oxygen species is a complex process which involves the generation of a variety of ROS including superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), the peroxyl radical ($ROO^{\bullet}$), nitric oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), dimethylarsinic peroxyl radicals ($[(CH_3)_2AsOO^{\bullet}]$) and also the dimethylarsinic radical $[(CH_3)_2As^{\bullet}]$. The exact mechanism responsible for the generation of all these reactive species is not yet clear, but some workers have proposed the formation of intermediary arsine species [86]. Another route to the production of H_2O_2 was suggested, involving the oxidation of As(III) to As(V) which, under physiological conditions, results in the formation of H_2O_2:

$$H_3AsO_3 + H_2O + O_2 \rightarrow H_3AsO_4 + H_2O_2 (\Delta_r G^{\ominus} = -40.82\ \text{kcal/mol}) \quad (9)$$

The above reaction is spontaneous and exergonic with an estimated standard reaction free energy change for H_2O_2 formation of −40.82 kcal/mol (−170.87 J/mol). H_3AsO_3 (arsenious acid) has the ability to generate a dioxygenated complex because it is a Lewis acid and may accept unpaired electrons that could originate either from water or from oxygen.

In recent studies concerning the mechanism of arsenite toxicity in the brain it was reported that some of its effects have been traced to the generation of the hydroxyl radicals [88]. The time-evolution of the formation of the hydroxyl radical in the striatum of both female and male rats who underwent a direct infusion of different concentrations of arsenite was investigated. The treatment with arsenite induced significant increases of hydroxyl radical formation. These results support the participation of hydroxyl radicals in arsenic-induced disturbances in the central nervous system.

Arsenic is a well-established human carcinogen [89]. Arsenic compounds bind to −SH groups and can inhibit various enzymes, including glutathione reductase. Studies support the hypothesis that arsenic may act as a co-carcinogen-not causing cancer directly, but allowing other substances, such as cigarette smoke and UV radiation, to cause DNA mutations more effectively [90]. Arsenic is one of the few species besides vinyl chloride that causes angiosarcoma, which provides a good indication of the potency of arsenic as a cancer-causing agent.

3.8. Nickel

Nickel is a human carcinogen that can alter gene expression by enhanced DNA methylation and compaction, rather than via mutagenic mechanisms [91]. The nickel compounds implicated as potential carcinogens are insoluble dusts of nickel subsulphides and nickel oxides, the vapor of nickel carbonyl, and soluble aerosols of nickel sulphate, nitrate, or chloride [92]. Almost all cases of acute nickel toxicity result from exposure to nickel carbonyl. Patients with severe poisoning develop intense pulmonary and gastrointestinal toxicity.

The lung is the primary target organ for nickel toxicity in humans. A very recent epidemiologic study of workers in nickel refineries has been conducted in Norway [93]. The results have shown that there was a substantial association between cumulative exposure to water-soluble nickel and the risk of developing lung cancer. Exposure to water-soluble nickel remained the most likely explanation for the excess lung cancer risk in the cohort. Other occupational exposures did not confirm the strong dose-related effect of nickel to any appreciable degree.

Some other studies have shown that workers' inhalation of nickel refinery dust, which contains nickel subsulphide, has resulted in increased numbers of deaths from nasal cavity cancers, and possibly cancer of the larynx [94]. In addition, nickel may interfere with DNA repair processes and toxic doses of nickel are found to induce lipid peroxidation and protein carbonyl formation in animals.

Very small amounts of nickel have been shown to be essential for normal growth and reproduction in some species of animals; therefore, small amounts of nickel may also be essential to humans.

4. Oxidative damage to biomolecules

4.1. Oxidative nuclear and mitochondrial DNA damage

Reactive oxygen species are formed through a variety of events and pathways. It has been estimated that one human cell is exposed to approximately 1.5×10^5 oxidative hits a day from hydroxyl radicals and other such reactive species [95]. The hydroxyl radical is known to react with all components of the DNA molecule: dam-

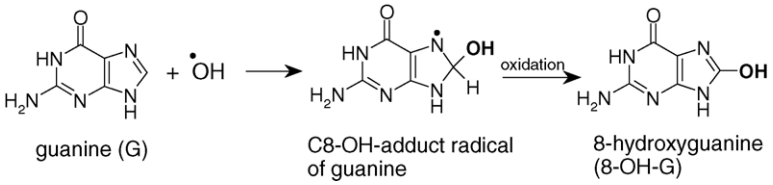

Fig. 2. Reaction of guanine with hydroxyl radical.

aging both the purine and pyrimidine bases and also the deoxyribose backbone [96]. Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis and ageing. In fact, as is well established, in various cancer tissues free radical-mediated DNA damage has occurred. To date, more than 100 products have been identified from the oxidation of DNA. ROS-induced DNA damage involves single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis [97,98].

The hydroxyl radical is able to add to double bonds of DNA bases at a second-order rate constant in the range of $(3–10) \times 10^9\ M^{-1}\ s^{-1}$ and it abstracts an H-atom from the methyl group of thymine and each of the five carbon atoms of 2′ deoxyribose at a rate constant of approximately $2 \times 10^9\ M^{-1}\ s^{-1}$ [96]. While OH-adduct radicals of DNA bases are generated via an addition reaction, the allylic radical derived from thymine and carbon-centred sugar radicals arise by abstraction reactions [96]. Further reactions of base and sugar radicals generate a variety of modified bases and sugars, base-free sites, strand breaks and DNA–protein cross-links.

An example illustrating the mechanisms of 8-hydroxyguanine (8-OH-G) formation is given in Fig. 2. The presence of 8-OH-G in human urine was first reported by Ames and co-workers [99]. This oxidised DNA product is important because it is both relatively easily formed and is mutagenic and carcinogenic. It is a good biomarker of oxidative stress of an organism and a potential biomarker of carcinogenesis. We note that 8-hydroxyguanine undergoes keto-enol tautomerism and therefore 8-OH-G is often called 8-oxoguanine or 8-oxo-G, however, 8-oxo-G and 8-OH-G are equivalent.

This base modification occurs in approximately one in 10^5 guanidine residues in a normal human cell. Ionising radiation, a carcinogenic and exogenous source of ROS, induced both urinary and leukocyte biomarkers of oxidative DNA damage [1]. Tobacco smoking, another carcinogenic source of ROS, increases the oxidative DNA damage rate by 35–50%, as estimated from the urinary excretion of 8-oxo-G, and the level of 8-oxo-G in leukocytes by 20–50%.

Measurements by Kasai et al. demonstrated that factors such as hard physical labour, day–night shift work, smoking and low meat intake significantly increased the 8-oxo-G level, while moderate physical exercise, such as sports reduced its level [100]. These findings were comparable with previous data obtained from studies on rats and suggest that our lifestyle may significantly affect the level of oxidative damage we sustain.

Multiple methods for measuring oxidative DNA damage exist, of which one of the more popular employs enzymatic digestion of DNA [101]. This method liberates oxidised DNA products for analysis by HPLC, usually with electrochemical detection (HPLC–EC). Another method employs acidic hydrolysis of DNA, which liberates the free bases, since the glycosidic bonds are cleaved by acid. Detection is made via HPLC or, after conversion to volatile derivatives, by gas chromatography mass spectrometry (GC–MS).

In addition to ROS, reactive nitrogen species (RNS), such as peroxynitrites and nitrogen oxides, have also been implicated in DNA damage [102]. 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 the mitochondrial oxidative DNA damage in the carcinogenesis process [6]. 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. The following points account for the fact that mitochondrial DNA is more susceptible to oxidation than nuclear DNA [6]: (i) under physiological conditions, the mitochondria

convert ∼5% of oxygen consumed into superoxide anion and subsequently hydrogen peroxide; (ii) mitochondrial DNA repair capacity is limited, since they lack entirely the feature of nucleotide excision repair; (iii) mitochondrial DNA is not protected by histones.

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 [103]. 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.

In conclusion, as observed with oxidative genomic DNA modification, oxidative damage and the induction of mutation in mitochondrial DNA may participate at multiple stages of the process of carcinogenesis, involving mitochondria-derived ROS, induction of mutations in mitochondrial genes, and possibly the insertion of mitochondrial genes into nuclear DNA [103].

As described above, oxygen radicals may induce a number of DNA base alterations that can lead to mutagenesis. However, there are specific and general repair mechanisms that can repair DNA base modifications [104,105]. Of interest is the fact, that the efficiency of repair mechanisms may be enhanced following exposure to reactive oxygen species because expression of many DNA repair enzymes is upregulated following oxidative stress.

Since in nuclear DNA, ∼90% of oxidised bases are repaired by single nucleotide repair mechanisms and the remaining 10% by long-patch base excision repair, the single nucleotide base excision repair is the primary pathway for repair of 8-OH-G. The first evidence of a repair mechanism for the 8-OH-G lesion was observed in irradiated mouse liver, where levels of this lesion were found to decrease with time [106]. A repair enzyme was partially purified from *E. coli* and was later found to be identical to the cloned DNA repair enzyme, formamidopyrimidine–DNA glycosylase FPG protein, previously isolated from *E. coli* [107]. While significant knowledge of the DNA repair mechanisms in nuclear DNA exists, much less is known about the repair systems in the mitochondria. However, compared with nuclear DNA repair mechanisms, DNA repair capacity in the mitochondrion appears to be rather low [108]. The impaired repair capacity may lead to mitochondrial dysfunction and the onset of degenerative diseases.

4.2. Lipid peroxidation

It is known that metal-induced generation of oxygen radicals results in the attack of not only DNA in the cell nucleus, but also other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation [109,110].

Whilst the ability of the hydroxyl radical (generated via Fenton chemistry) to initiate lipid peroxidation is unquestionable, it is necessary to also consider the diffusion-limited reactivity of the hydroxyl radical toward sugars, nucleotides, or proteins. The mechanism, proposed more than two decades ago by Bucher et al., involves the formation of a Fe(II):Fe(III) complex (or a Fe(II)–O_2–Fe(III) species) [111]. The maximal rates of lipid peroxidation are observed when the ratio of Fe(II):Fe(III) is 1:1. Bucher et al. also demonstrated that ADP–Fe(II) promoted the peroxidation of phospholipid liposomes, but only after a lag phase [111]. Catalase, superoxide dismutase and hydroxyl radical scavengers did not extend the lag phase or inhibit the subsequent rate of lipid peroxidation.

Several experimental models of iron overload in vivo, confirmed increased polyunsaturated fatty acids (PUFA) oxidation of hepatic mitochondria, as well as lysosomal fragility. Bacon et al. observed that, following oral intake of carbonyl iron in rats, mitochondrial lipid peroxidation occurred [112]. Experiments also showed that this was accompanied by substantial decrements in mitochondrial metabolism. These observations suggest that mitochondrial PUFA are a preferential target for iron-driven peroxidation. The deleterious process of the peroxidation of lipids is also very important in arteriosclerosis, cancer and inflammation.

The overall process of lipid peroxidation consists of three stages: initiation, propagation and termination and is presented in Fig. 3 [113,114]. 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) (Fig. 3) [110]. The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE) (Fig. 3). 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. In addition, HNE has powerful effects on signal transduction pathways, which in turn have a major effect on the phenotypic characteristics of cells. Per-

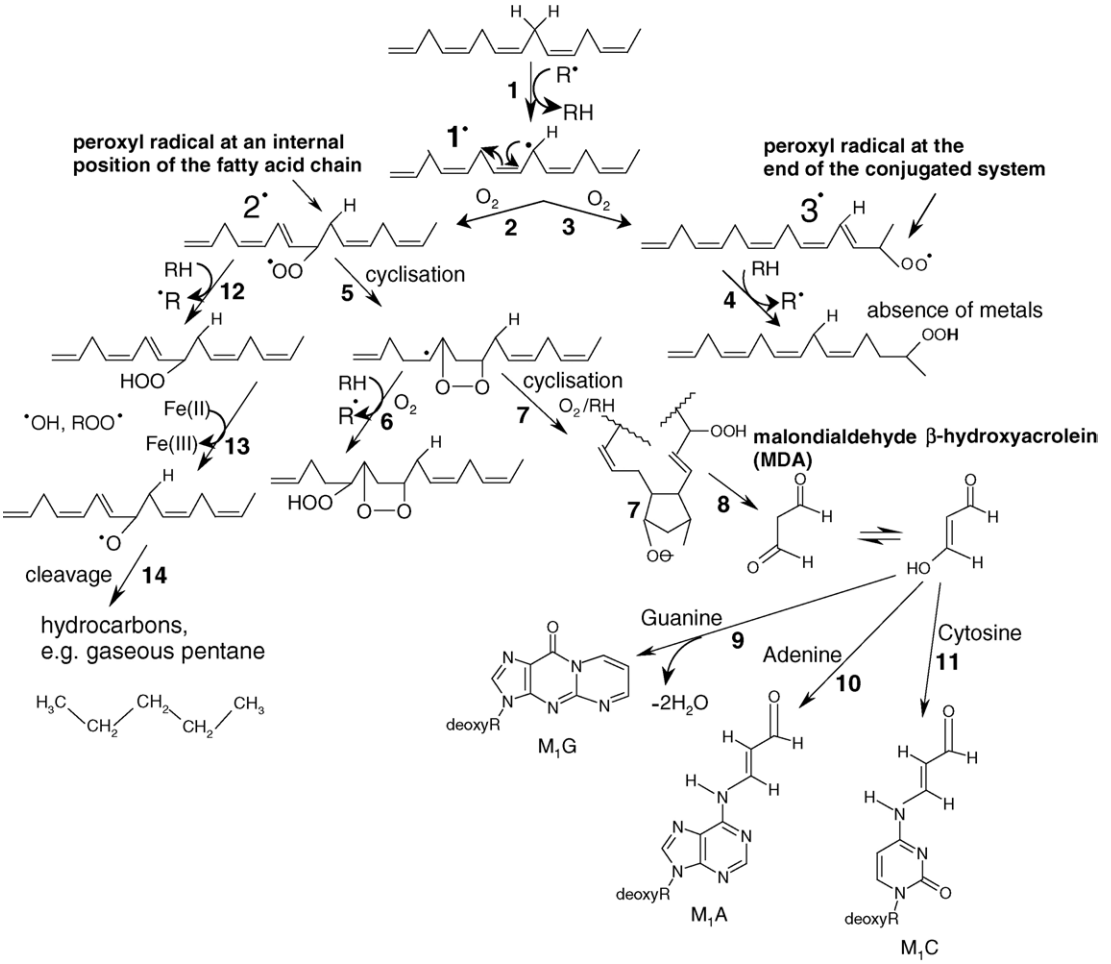

Fig. 3. Various pathways of lipid peroxidation. The methylene groups of polyunsaturated fatty acids are highly susceptible to oxidation and their hydrogen atoms, after the interaction with radical R•, are removed to form carbon-centred radicals 1• (reaction (1)). Carbon-centred radicals react with molecular dioxygen to form peroxyl radicals (reactions (2) and (3)). If the peroxyl radical is located at one of the ends of the double bond (3•) it is reduced to a hydroperoxide which is relatively stable in the absence of metals (reaction (4)). A peroxyl radical located in the internal position of the fatty acid (2•) can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical (reaction (5)). This can then either be reduced to form a hydroperoxide (reaction (6)) or through reaction (7) 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. Compound 7 is an intermediate product for the production of malondialdehyde (reaction (8)). Malondialdehyde can react with DNA bases G, A, and C to form adducts M_1G, M_1A and M_1C (reactions (9)–(11)). Peroxyl radicals located in the internal position of the fatty acid (2•) can, besides cyclisation reactions, (reaction (5)) also abstract hydrogen from the neighbouring fatty acid molecule, creating thus lipid hydroperoxides (reaction (12)). They can further react with redox metals (e.g. iron) to produce reactive alkoxyl radicals (RO•) (reaction (13)) which, after cleavage (reaction (14)) may form, e.g. gaseous pentane; a good marker of lipid peroxidation.

oxidation of lipids is an autocatalytic process which is terminated for example by the recombination of radicals ($R^\bullet + R^\bullet \rightarrow$ non-radical product) or depletion of the substrate.

MDA can react with DNA bases G, A and C to form adducts M_1G, M_1A and M_1C, respectively (Fig. 3) [110]. 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). M_1G has also been detected in human breast tissue by ^{32}P-post-labeling as well as in rodent tissues [115]. Site-specific experiments confirmed that M_1G is mutagenic in *E. coli*, inducing transversions to T and transitions to A [116]. The mutation frequencies are comparable with those reported for 8-oxo-G in similar systems. M_1G is repaired by both bacterial and mammalian nucleotide excision repair pathways and is also repaired in *E. coli* by mismatch repair. Studies employing NMR spectroscopy indicate that M_1G undergoes rapid and quantitative ring-

opening to form N^2-oxopropenyl-G when it is present in duplex DNA; however, not when it is present in single-stranded DNA [117]. While the reactive functionality of M_1G is present in the major groove, the reactive functionality of N^2-oxo-propenyl-dG is present in the minor groove of DNA. The interconversion of M_1G and N^2-oxo-propenal-dG within the DNA may lead to the formation of DNA–DNA interstrand crosslinks or DNA–protein crosslinks.

4.2.1. Etheno adducts

There are also other exocyclic DNA adducts that arise from lipid peroxidation. For example etheno-dA, -dC and -dG have been detected by both 32P-post-labeling and GC–MS [118]. While the precise pathway of their formation in DNA is unknown, the adducts can readily be generated in vitro by exposure of DNA to a peroxidising lipid. The biological activity of etheno adducts involves transitions to A (induced by *N*2,3-etheno-dG) and transversions to T in *E. coli* (induced by 1,*N*2-etheno-dG) [119]. It has been demonstrated that etheno-dA and -dC are strongly genotoxic but weakly mutagenic when introduced on single-stranded vectors in *E. coli*. Etheno-dA induces predominantly transitions to G whereas etheno-dC induces transversions to A and transitions to T. Studies dealing with the repair of etheno adducts have shown that etheno-dA is removed by the action of 3-methyladenine glycosylase and its mammalian homolog AAG [120]. In addition to efficient removal by glycosylases, other repair pathways should also be considered.

4.2.2. Propano adducts

It has been demonstrated that hydroxypropanodeoxyguanosines (HO-PdGs) are present in human DNA [121]. 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. However, the mutagenic potency of HO-PdGs has not been evaluated by site-specific approaches, due to the instability of these adducts, which renders their incorporation into oligonucleotides unviable. Therefore, a novel post-oligomerization strategy for the synthesis of oligonucleotides containing the acrolein-derived HO-PdG was reported recently which should make it possible to construct the requisite adducted vectors [122]. Experiments with unsubstituted adduct PdG revealed that this induces base pair substitution mutations in *E. coli* with high efficiency. To date little is known about the repair of HO-PdGs. There may be a possibility that PdG or HO-PdGs are substrates for base excision repair enzymes, however, this needs to be evaluated in more detail.

4.3. Proteins

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 [123]. The results of these studies demonstrated that reactions with hydroxyl radicals lead to abstraction of a hydrogen atom from the protein polypeptide backbone to form a carbon-centred radical, which under aerobic conditions reacts readily with dioxygen to form peroxyl radicals [124]. The peroxyl radicals are then converted to the alkyl peroxides by reactions with the protonated form of superoxide ($HO_2^{\bullet}$). In the absence of ionising radiation the same reactions can be initiated by hydroxyl radicals produced under in vivo conditions by the Fenton reaction (see above). Thus in the absence of radiation, proteins are resistant to damage by H_2O_2 and by other simple oxidants unless transition metals are present. Metal-catalyzed damage to proteins involves oxidative scission, loss of histidine residues, bityrosine crosslinks, the introduction of carbonyl groups, and the formation of protein-centred alkyl, $R^{\bullet}$, alkoxyl, $RO^{\bullet}$, and alkylperoxyl, $ROO^{\bullet}$, radicals [125–129]. The alkoxyl radical derivatives of proteins are capable of undergoing peptide bond cleavage. Peptide bond cleavage can also occur by hydroxyl radical-initiated attack of the glutamic acid and proline residues of proteins to form a mixture of various products. Protein damage is likely to be repairable and is a known non-lethal event for a cell, however, evidence has been reported that two mitochondrial proteins – aconitase and adenine nucleotide – translocase may be important targets of long-term oxidative damage [123].

The side chains of all amino acid residues of proteins are susceptible to oxidation by ionising radiation and by the action of ROS/RNS [123]. Since proline, histidine, arginine, lysine, and cysteine residues in proteins are highly sensitive to oxidation by redox metals, redox metal (iron, copper)-mediated oxidation of a protein may be a site-specific process. It is believed that the iron(II) binds both to high- and low-affinity metal-binding sites on the protein, most probably involving the above-mentioned amino acids. The Fe(II)–protein complex reacts with H_2O_2 via the Fenton reaction to yield an active oxygen species, e.g. $^{\bullet}OH$, ferryl ion, etc., at the site. While it has been proposed by many authors that the hydroxyl radical represents the major species responsible for the oxidation of proteins, clear experi-

mental evidence is still missing. A recent study by Welch et al. demonstrated the site-specific modification of ferritin by iron which involved the oxidation of cysteine, tyrosine, and also some other residues [130]. Whilst the hydroxyl radical scavenger HEPES protected the protein against oxidation, catalase did not, confirming the site-specific oxidation of ferritin. The oxidation of myoglobin by H_2O_2 yields ferrylmyoglobin, which contains two oxidising equivalents: the ferryl complex and an amino acid radical.

Using EPR spectroscopy Giulivi and Cadenas showed that the spectra of the amino acid radicals consisted of a composite of three signals attributable to a peroxyl radical, a tyrosyl radical and radicals in an aromatic amino acid-containing peptide [131]. The aromatic amino acid radical was observed to be relatively long lived and in close proximity to the heme iron. Hence, it has been proposed that this is the first site of the protein radical. Reduction of the ferryl complex by Tyr is described by the reaction:

$$\mathrm{Fe(IV){=}O + Tyr{-}OH + H^+ \rightarrow Fe(III) + H_2O + Tyr{-}O^\bullet} \quad (10)$$

and alternatively by other amino acids leads to the subsequent formation of other amino acid radicals via an electron-transfer process that occurs throughout the protein. This view suggests that the protein radical(s) is highly delocalised within the globin moiety in a dynamic process which encompasses electron tunnelling through the backbone chain, or H-bonds, leading to the formation of secondary radicals.

The amino acid residue side chains that are most vulnerable to attack by various ROS and RNS lead to the formation of the following products: arginine → glutamic semialdehyde; glutamate → 4-hydroxy-glutamate; histidine → 2-oxo-histidine; tyrosine → 3,4-dihydroxy phenylalanine, Tyr–tyr cross-linked proteins, 3-nitro-tyrosine; valine → 3,4-hydroxy valine; cysteine → cys–S–S–cys, cys–S–S–R disulphied; proline → glutamic semialdehyde, 2-pyrrolidone-4-hydroxy-proline; methionine → methionine sulphone and sulphoxide [123].

In view of the fact that protein carbonyl groups are generated by many different mechanisms and a number of highly sensitive methods have been developed for the assay of protein carbonyl groups, the concentration of protein carbonyl groups is a good measure of ROS-mediated protein oxidation.

Nitric oxide reacts rapidly with superoxide radical to from the highly toxic peroxynitrite anion $ONOO^-$ (see the reaction (2) above) which is able to nitrosate the cysteine sulphydryl groups of proteins, to nitrate tyrosine and tryptophan residues of proteins and to oxidise methionine residues to methionine sulphoxide. However, the process of modification of proteins by peroxynitrite is strongly inhibited by physiological concentration of CO_2 since peroxinitrite is known to react rapidly with CO_2 to form the $ONOOCO_2^-$:

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \quad (11)$$

The nitration of tyrosine residues, which is irreversible process, may prevent the phosphorylation or adenylylation of tyrosine residues of regulatory proteins.

When superoxide is depleted from the biological environment via a dismutation reaction, $NO^\bullet$ interacts directly with the biological substances (for example with the hem iron of guanylate cyclase) to form a complex:

$$\mathrm{Fe(II)\{guanylate\ cyclase\} + NO^\bullet \rightarrow Fe(II)\{guanylate\ cyclase\} - NO^\bullet} \quad (12)$$

Protein oxidation by ROS is associated with the formation of many different kinds of inter- and intra-protein cross-linkages, including those formed, (i) by addition of lysine amino groups to the carbonyl group of an oxidised protein; (ii) by interaction of two carbon-centred radicals obtained by the hydroxyl radical-driven abstraction of hydrogens from the polypeptide backbone; (iii) by the oxidation of sulphydryl groups of cysteine residues to form –S–S– crosslinks; (iv) the oxidation of tyrosine residues to form –tyr–tyr– cross-links.

Cysteine and methionine residues of proteins are particularly susceptible to oxidation by ROS [132]. However, oxidation of the sulphur amino acids is reversible. The oxidised products of cysteine, intra-molecular (P1–S–S–P1) and inter-molecular (P1–S–S–P2) protein cross-linked derivatives can be repaired by disulphide exchange reactions catalyzed by thiol transferases.

The oxidation of methionine (Met) residues of proteins leads to the formation of a mixture of the S– and R– isomers of methionine sulphoxide, Met–(S)–SO and Met–(R)–SO:

$$\mathrm{Met + ROS \rightarrow Met{-}(S){-}SO + Met{-}(R){-}SO + products} \quad (13)$$

Because almost all forms of ROS are able to oxidise methionine residues of proteins to methionine sulphoxide, it was proposed that the cyclic oxidation and reduction of methionine residues of proteins serves an important antioxidant function to protect cells from oxidative damage.

Oxidation of proteins is associated with a number of age-related diseases and ageing [133,134]. The concept of the role of protein oxidation in ageing is supported

by animal studies showing that the process of ageing is often associated with the accumulation of oxidised forms of proteins. The accumulation of oxidised proteins in living systems may be: (i) due to an increase in the steady state level of ROS/RNS and/or to a decrease in the antioxidant capacity of an organisms; (ii) a decrease in the ability to degrade oxidised proteins due to either a decrease in the protease concentrations and/or to an increase in the levels of protease inhibitors.

5. Oxidative stress, cell signalling and cancer

Cells communicate with each other and respond to extracellular stimuli through biological mechanisms called cell signalling or signal transduction [4,135]. 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 [136]. 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 [137].

While ROS are predominantly implicated in causing cell damage, they also play a major physiological role in several aspects of intracellular signalling and regulation [138]. It has been clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways [135]. Because ROS are oxidants by nature, they influence the redox status and may, according to their concentration, cause either a positive response (cell proliferation) or a negative cell response (growth arrest or cell death). As already mentioned above, while high concentrations of ROS cause cell death or even necrosis, the effects of ROS on cell proliferation occurred exclusively at low or transient concentrations of radicals. Low concentrations of superoxide radical and hydrogen peroxide in fact stimulate proliferation and enhanced survival in a wide variety of cell types. ROS can thus play a very important physiological role as secondary messengers [139]. Other examples include regulation of the cytosolic calcium concentration (which itself regulates the above-mentioned biological activities), regulation of protein phosphorylation, and activation of certain transcription factors such as NF-κB and the AP-1 family factors [140].

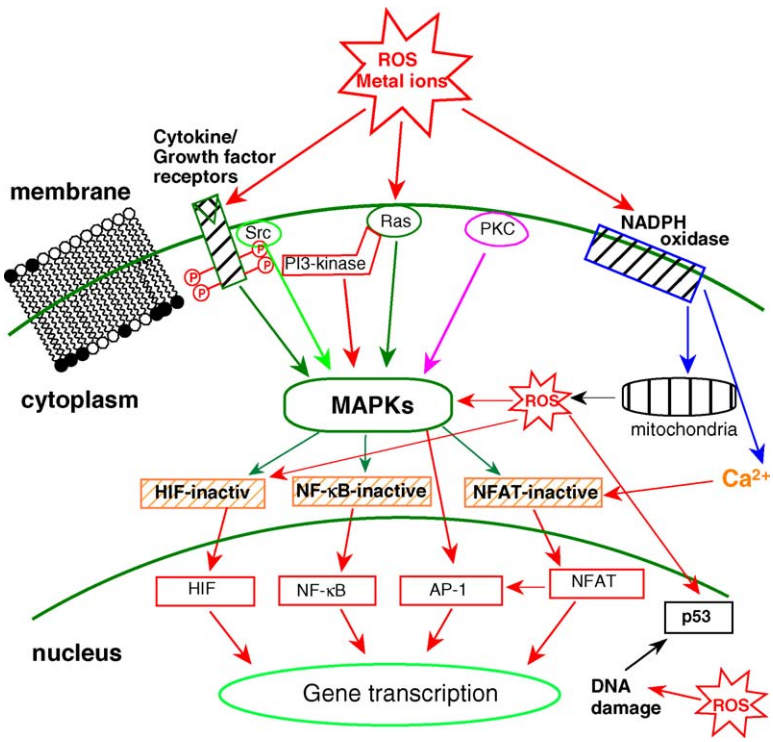

Fig. 4. ROS and metal ions-induced signalling pathways.

ROS and metal ions primarily inhibit phosphor-serine/threonine-, phosphotyrosine- and phospholipid-phosphatases, most probably by interacting with sulphydryl groups on their cystein residues, which are oxidised to form either intramolecular or intermolecular disulphide bonds [4,135]. These structural changes alter protein conformation which leads to the upregulation of several signalling cascades, most importantly growth factor kinase-, src/Abl kinase-, MAPK- and PI3-kinase-dependent signalling pathways. These signalling cascades lead to the activation of several redox-regulated transcription factors (AP-1, NF-κB, p53, HIF-1, NFAT). Fig. 4 summarises ROS-induced signalling pathways.

5.1. 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 [141]. The information is transmitted via the activation of mitogen-activated protein kinases (MAPKs) signalling pathways [142–149].

ROS production as a result of activated growth factor receptor signalling includes epidermal growth factor (EGF) receptor [150], platelet-derived growth factor (PDGF) receptor [151], vascular endothelial growth factor (VEGF) [141]. Further examples involve cytokine receptors (TNF-α and IFN-γ) or interleukin receptors (IL-1β) [152]. Cytokines receptors fall into a large and heterogenous group of 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 [153]. 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.

Abnormalities in growth factor receptor functioning are closely associated with the development of many cancers [154]. Several growth factor receptors (EGF, PDGF, VEGF) are affected by carcinogenic metals such as nickel, arsenic, cobalt and beryllium [155]. The EGF receptor is associated with cell proliferation in normal cells. Nickel has been found to increase expression of the EGF receptors and overexpression of the EGF receptor has been observed in lung and urinary cancers [156]. Exogenous oxidative stress appears to stimulated secretion of heparin-binding EGF. VEGF is involved in proliferation and angiogenesis and also is induced by carcinogenic metals (Co, Ni and As) and hypoxia. Arsenic-induced VEGF expression appears to be associated with p38 [157]. Activation of both EGF and VEGF results in increases in cellular Ca(II). A similar effect was observed in a various cell types following treatment with Ni(II), Cd(II) and Be(II) compounds. The VEGF is probably most strongly activated by hydrogen peroxide. The PDGF is found in endothelial cells, fibroblasts and mesenchymal cells; the overexpression of PDGF has been found in lung and prostate cancers.

5.2. Non-receptor tyrosine kinases

In addition to receptor tyrosine kinases (see above), several non-receptor protein kinases (PTKs) belonging to the Src family (Src kinases) and Janus kinase (JAK) are also activated by ROS [158,159]. 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. It is noteworthy that Src has been activated by As(III) and organic Cr(III) compounds and by UV radiation as well as by various ROS [155]. Overexpressed Src has been found in colorectal, breast, pancreas, bladder and head/neck cancers. Activated Src binds to cell membranes by myristilation and initiates MAPK, NF-κB and PI3K signalling pathways (Fig. 4).

5.3. Ras

Ras gene products are membrane-bound G proteins whose main function is to regulate cell growth and oppose apoptotic effects. Ras is activated by UV radiation, ROS, metals and mitogenic stimuli. Ras genes were found to be mutated in 30% of lung, skin, liver, bladder and colon cancers [160]. As, Ni, Fe and Be are linked with ras mutations (in two ras genes, H- and K-ras) and cancer [161]. H-ras mutations were observed in rats following dimethylarsinic acid administration in drinking water. Ni(II) compounds induced K-ras mutations and kidney tumours in rats. Mutations to ras caused by metals have not been reported in human cancers.

5.4. 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 [162]. Cystein residues are most susceptible to oxidative damage by hydrogen peroxide and other oxidants, producing sulphenic acid intermediates, which can further react with thiols to form catalytically inactive PTP disulphides. Superoxide radical was also shown to regulate the activity of PTPs very efficiently, in particular PTP-1B via cysteine residues [163].

5.5. Serine/threonine kinases

All receptor serine/threonine kinases described in mammalian cells are members of TGF-β superfamily. TGF-β1 is the prototype of this large family of polypeptide growth factors. Unlike RTKs-linked growth factors, TGF-β1 typically inhibits the growth of most target cells. The TGF-β1 has been shown to stimulate ROS production in a variety of cells [164,165]. 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 [166]. 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. In addition, ROS and metals can directly activate some serine/threonine phosphorylation processes. 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 [167]. Both regulatory and catalytic domains of the PKC are susceptible to H_2O_2-induced oxidative modification; in fact treatment of different cell types using e.g. H_2O_2 leads to stimulation of its activity. 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 [168].

The group of proteins termed mitogen-activated protein kinases (MAPKs) 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 [169].

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 (MAPKs): 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 [170]. Disregulation of MAPK function has been reported for skin, breast and cervival cancers in humans. 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 upregulation 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 [171]. Conversely, exogenous H_2O_2 activates p38 kinase, but not ERK in rat alvedor macrophages [172]. 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 is required for the induction of apoptosis.

Carcinogenic metals have been shown to activate MAPKs [19]. Arsenic(III) and chromium(VI) activates ERK1and ERK2, JNK and p38 in the human cells. Beryllium difluoride activates ERK1, JNK and p38 in human breast cancer cells. BMAPK-1 is not activated directly by metal ions; however, indirect activation by hydrogen peroxide has been well documented.

5.6. Nuclear transcription factors

Probably the most significant effect of metals and ROS on signalling pathways has been observed in the mitogen-activated protein kinase (MAPK) pathways. 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 [173]. The p53 protein guards a cell-cycle checkpoint, and 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.

5.6.1. 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 [174]. 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. In addition, the in vitro transcriptional activity of AP-1 is regulated by the redox state of a specific cysteine64 located at the interface between the two c-Jun subunits, highlighting the importance of redox status on gene transcription; however, recent in vivo experiments demonstrated that cysteine64/65 is not required for redox regulation of AP-1 DNA binding in vivo [175].

The induction of AP-1 by H_2O_2, metals, cytokines, and other stressors is mediated mainly by JNK and p38 MAP kinase cascades [176]. It is known that stressors can activate MAP kinases and thereby AP-1 in several ways. One involves a MAP kinase, the apoptosis signal-regulating kinase (ASK1). The other mechanism involves oxidant-mediated inhibition of MAP kinase phosphatases, which leads to increased MAP kinase activation. Whichever mechanism prevails, activation of MAP kinases leads directly to increased AP-1 activity. The role of cellular oxidants and AP-1 activation in the cancer process is now well documented by a number of experiments [177]. 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) [178]. 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.

5.6.2. NF-κB

A number of reports published during recent years indicate that some metals are able to affect the activation or activity of NF-κB transcription factors [179]. NF-κB is an inducible and ubiquitously expressed transcription factor for genes involved in cell survival, differentiation, inflammation and growth [179].

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 [179]. 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 localisation 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 [180]. 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 benzo(a)pyrene are among the external stimuli that activate NF-κB [181]. 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 [182]. The mechanism for activation of NF-κB by metals and reactive oxygen species is not yet clear. Reactive oxygen species have been implicated as second messengers involved in the activation of NF-κB via tumour necrosis factor (TNF) and interleukin-1 [183,184]. The importance of metals

and other reactive oxygen species on NF-κB activation is further supported by studies demonstrating 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.

5.6.3. p53

The nuclear factor plays a key role in protecting a cell from tumourigenesis [185]. 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 [186]. 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. Since the reduction or oxidation of disulphide bonds often occurs at a posttranslational level, p53 is considered as one of the oxidative stress response transcription factors. Many studies have been devoted to mutations in p53 caused by direct action of ROS or by carcinogenic metals [187].

ROS have been correlated with p53-mediated apoptosis [188]. Upon overexpression of p53, levels of ROS rise, and inhibition of ROS by antioxidants inhibits apoptosis in smooth muscle cells. 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.

The effect of antioxidants on p53 is not unambiguous [189]. While SOD, which converts $O_2^{\bullet -}$ to H_2O_2, was found to increase p53 activity, catalase, a scavenger of H_2O_2, inhibited p53 activation. Interestingly, aspirin, a scavenger of $^{\bullet}OH$, suppressed activation of p53 [190]. Increased formation of $^{\bullet}OH$ enhanced p53 activation in A549 cells through Cr(VI) reduction by NAD(P)H followed by Cr(V) catalyzed decomposition of H_2O_2. Wang and Shi also studied the mechanism of Cr(VI)-induced p53 activation [191]. They found that the activation of p53 was at the protein level instead of the transcriptional level. In response to Cr(VI) treatment, protein p53 becomes phosphorylated and acetylated at Ser15 and Lys383, respectively. Cr(VI) can indirectly induce p53-mediated apoptosis in multiple ways: by causing DNA damage, via DNA binding by Cr(VI) reduction products, by activation of MAPKs upstream of p53, through the direct activation of p53, or by enhancing the effect of other carcinogens.

As already noted, mutations in p53 are where most of the transformations were observed, e.g. these are the common genetic alterations found in human cancers; however, several conflicting results have been reported. Maehle et al. found altered *p53* gene structure and expression in human epithelial cells after exposure to nickel, however, in contrast, a low incidence of point mutations were detected in the *p53* tumour supressor gene from nickel induced rat renal tumours [192]. These studies raise questions as to whether p53 mutations are really involved in nickel-induced transformation.

The effect of arsenic on p53 is not fully understood. The problem is that various studies have reported conflicting results spanning the range of no effect of arsenic on p53, through an induced p53 phosphorylation and ultimately to a decrease of p53 expression. For example, Huang et al. reported no effect for various concentrations of arsenic on the p53-dependent transcription in p53 promoter transfected in C141 JB6 cells, which suggests no involvement of p53 in the arsenic-induced apoptosis [193]. In contrast, other groups have reported overexpression of *p53* gene in patients suffering from arsenic-related skin disease [194].

In conclusion, the role of p53 in arsenic-stimulated cellular effects is not yet clear. The experimental results suggest both p53-dependent and p53-independent induction of apoptosis, and also both an increased and decreased expression of the protein. Another mechanism by which metals affect p53 is through zinc substitution which is essential for the binding of p53 to DNA. Metals substituting zinc can inactivate p53 without mutation or oxidation.

Several studies confirmed mutations in p53 following exposure to $NO^{\bullet}$ [195]. Experiments have also indicated that exposure of cells to a high level of $NO^{\bullet}$ and its derivatives during chronic inflammation in the absence of wild-type p53 – and therefore negative iNOS regulation – might increase the susceptibility to cancer. There is an association between increased iNOS expression and G:C to A:T transition mutations in p53 in stomach, brain and breast cancers. $NO^{\bullet}$ and its derivatives are therefore capable of causing mutations in cancer-related genes and therefore act as both an endogenous initiator and a promoter in human carcinogenesis.

5.6.4. NFAT

The NFAT family of nuclear transcription factors regulates muscle growth and differentiation, cytokine formation, angiogenesis and other processes [174,196]. Four of five NFAT proteins are calcium dependent. NFAT

is activated by phosphatase calcineurin, which is in turn activated by high intracellular calcium levels [197]. Various metals are known to increase intracellular calcium and this may represent a probable mechanism by which metals activate NFAT. Similarly to NF-κB, inactive NFAT components are present in the cytoplasm and translocate to the nuceleus upon activation. NFAT interacts with both NF-κB and AP-1.

Vanadium, nickel and iron have been documented to activate NFAT [19]. Recent studies revealed that vanadium induces NFAT activation not only through a calcium-dependent and cyclosporin A-sensitive pathway, but also through H_2O_2 generation.

5.6.5. HIF-1

HIF-1 is a heterodimer and is composed of two bHLH proteins, HIF-1α and -1β. HIF-1α is expressed and HIF-1β accumulated only in hypoxic cells [198]. 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 [199]. VEGF as one of the HIF-1-regulated proteins plays an important role in tumour progression and angiogenesis.

The major ROS responsible for the induction of HIF-1 and VEGF expression is hydrogen peroxide [200]. Also carcinogenic metals have been shown to activate HIF-1. The HIF-1 was found to be elevated in nickel-treated cells [19]. HIF-1 is very sensitive to hypoxia stimulus and precise regulation of oxygen homeostasis. One explanation for the nickel-induced activation of the HIF-1 transcription factor is based on the assumption that nickel replaces iron in the oxygen carrier, Fe(II)-hybrid hemoglobin. Substitution of iron by nickel switches the signal to permanent hypoxia, which in turn activates the HIF-1 factor. HIF-1 is also involved in the regulation of numerous genes, which also involve glucose transport and glycolysis. Several experiments on various cells confirmed activated glucose metabolisms and glycolysis after nickel exposure. Also vanadate was found to induce expression of hypoxia-inducible factor 1 alpha (HIF-1α) in DU145 cells [201].

6. The three stages model and mechanisms of carcinogenesis

Carcinogenesis is a complex multi-sequence process leading a cell from a healthy to a precancerous state and finally to an early stage of cancer [202]. There are several theories of carcinogenesis. The old theories described cancer as a “disease of cell differentiation” or “stem cell disease”. These theories seem to point to a “single cell origin” of the cancer [203].

Two key mechanisms have been proposed for the induction of cancer. In one, an increased DNA synthesis and mitosis by nongenotoxic carcinogens may induce mutations in dividing cells through misrepair. Mutations may then clonally expand from an initiated preneoplastic cell state to a neoplastic cell state [204–206]. Another mechanism accounts for an equilibrium between cell proliferation and cell death. If the damage to DNA is too great, there exists an important process that eliminates altered cells selectively. This process is called apoptosis. During apoptosis, which is a normal physiological process, cells initiate a programmed suicide mechanism leading to many morphological changes [207]. During cell proliferation, protein p53 plays a primordial role, checking the integrity of the DNA [208]. It triggers mechanisms that eliminate, for instance, the oxidised DNA bases that cause mutations. When cell damage is too great, p53 triggers cell death by apoptosis. Uncontrolled apoptosis can be harmful to an organism, leading to destruction of healthy cells [209]. There thus exists a subtle regulatory system consisting of pro-apoptotic factors (e.g. p53) and anti-apoptotic factors. More than half of cancers have defects in upstream or downstream genes of p53 function. The carcinogenic process can be described as an imbalance between cell proliferation and cell death shifted towards cell proliferation.

In addition to these mechanisms, gap junctional intercellular communication (GJIC) (or gap Junctions) has been proposed to play an important role in the regulation of cell growth control, differentiation and apoptosis of progenitor cells [210]. The results of molecular biology have shown that there are genes, which when “activated” (oncogenes) or “inactivated” (tumour suppressor genes) contribute to the clonal expansion of an initiated stem cell. Many experiments have shown that tumour cells with activated oncogenes have dysfunctional GJIC. It points to the fact that activated oncogenes and GJIC are functionally linked by the signalling pathways affected by oncogenes. These findings suggest the role of gap junctional intercellular communication in the molecular biology of cancer and provide promising target in chemoprevention/chemotherapy of cancer.

Epidemiological clues and animal experiments have shown that the process of carcinogenesis consists of multiple and distinct stages, each characterised by different underlying mechanisms, i.e. the multi-stage and -mechanism hypothesis or the “initiation–promotion–progression” model of carcinogenesis [12,202]. Recent discoveries in molecular biology have helped to generate the “oncogene/tumour sup-

pressor gene" theory of carcinogenesis. Most recent is the integrative theory or the theory of "cancer as a disease of homeostasis" [211].

As mentioned above, cancer development is a multi-stage process. Chemical carcinogens interfere with various stages of this process and function through modifications of cellular and molecular events. Chemicals, participating in the process of carcinogenesis may function differently; to help to better understand the mechanism through which a carcinogen acts, terms "genotoxic" and "epigenetic" (non-genotoxic) have been introduced. Genotoxic agents are usually chemicals that directly damage DNA, which in turn leads to mutation and/or clastogenic changes. A second category of carcinogenic compounds (non-genotoxic) function through non-DNA or indirect-DNA reaction mechanisms. These compounds modulate cell growth and cell death; however, their mode of action is not yet fully understood.

A multi-stage process such as cancer development is characterised by cumulative action of multiple events occurring in a single cell and can be described by three stages: initiation, promotion and progression. ROS can act in all these stages of carcinogenesis [12]. The three stages model of carcinogenesis is shown in Fig. 5.

Initiation involves a non-lethal mutation in DNA that produces an altered cell followed by at least one round of DNA synthesis to fix the damage (e.g. 8-OH-G) produced during the initiation (Fig. 5); if dividing cells are damaged for whatever reason, they are able to interrupt temporarily their cell cycle at stage G1, S, or G2 ("check-points"), repair the damage, and resume division [212]. Oxidative DNA damage can occur via action of ROS, e.g. hydroxyl radicals, formed through the Fenton-type mechanism, along with other species (see above). Several studies on benign tumours revealed an interesting correlation between the size of tumour and the amount of 8-OH-G adduct formation; the level of 8-OH-G may thus determine the transformation from benign to malignant tumour [212]. The process of initiation further proceeds through oxidative stress-induced Ca(II) changes leading to increase in intracellular free calcium as a result of its release from intracellular Ca(II) stores and through the influx of extracellular Ca(II) [213].

The promotion stage is characterised by the clonal expansion of initiated cells by the induction of cell proliferation and/or inhibition of programmed cell death (apoptosis). This process results in the formation of an identifiable focal lesion. This stage dose-dependently requires the continuous presence of the tumour promotion stimulus and therefore it is a reversible process [212]. Many tumour promoters have a strong inhibiting effect on cellular antioxidant defence systems such as SOD, catalase, glutathione, etc. While a high level of oxidative stress is cytotoxic to the cell and halts proliferation by inducing apoptosis or even necrosis, a low level of oxidative stress can in fact stimulate the cell division in the promotion stage and thus stimulate the promotion of tumour growth [213]. This implies that production of ROS during this stage of carcinogenesis is the main line of ROS-related tumour promotion.

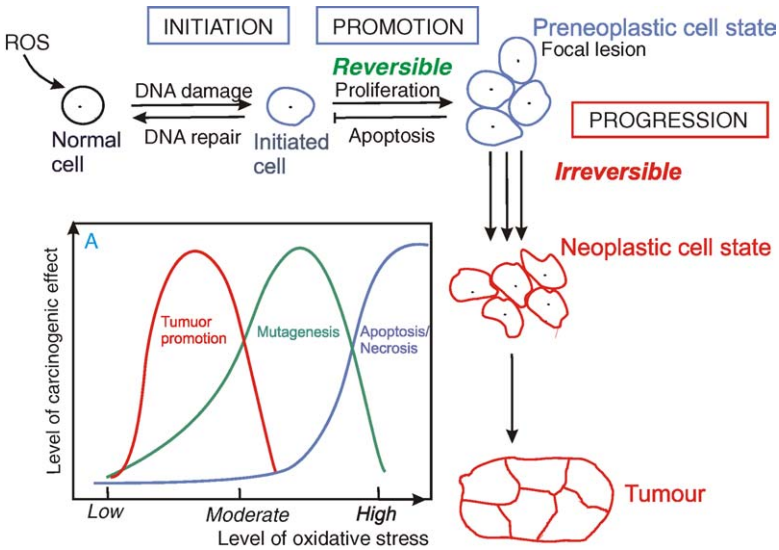

Fig. 5. Three stages model of carcinogenesis and the level of carcinogenic effect vs. level of free radicals at various stages of carcinogenic process (inset A).

Progression is the third and final stage of the carcinogenic process [12]. This stage involves cellular and molecular changes that occur from the preneoplastic to the neoplastic state. This stage is irreversible and is characterised by accumulation of additional genetic damage, leading to the transition of the cell from benign to malignant. This stage is characterised by genetic instability and disruption of chromosome integrity.

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 [214]. 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.

7. Antioxidant defence mechanisms in carcinogenesis

The effect of reactive oxygen and nitrogen species is balanced by the antioxidant action of non-enzymatic antioxidants, as well as by antioxidant enzymes. Such antioxidant defences are extremely important as they represent the direct removal of free radicals (prooxidants), thus providing maximal protection for biological sites. A good antioxidant should: (i) specifically quench free radicals; (ii) chelate redox metals; (iii) interact with (regenerate) other antioxidants within the "antioxidant network"; (iv) have a positive effect on gene expression; (v) be readily absorbed; (vi) have a concentration in tissues and biofluids at a physiologically relevant level; (vii) work in both the aqueous and/or membrane domains.

The most efficient enzymatic antioxidants involve superoxide dismutase, catalase and glutathione peroxidase [215]. Non-enzymatic antioxidants involve Vitamin C, Vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), natural flavonoids, a hormonal product of the pineal gland, melatonin and other compounds [216]. Some antioxidants act in a hydrophilic environment, others in a hydrophobic environment, and some act in both environments of the cell. For example, Vitamin C reacts with superoxide in the aqueous phase while Vitamin E does so in the lipophilic phase. In contrast, α-lipoic acid is both water and fat-soluble and therefore can operate both in cellular membranes and in cytosol.

Certain antioxidants are able to regenerate other antioxidants and thus restore their original function. This process is called an "antioxidant network" [217]. The redox cycles of vitamins E and C form such an antioxidant network. The capacity to regenerate one antioxidant by another is driven by the redox potentials of the [Red/Ox] couple. There is a link between increased levels of ROS and disturbed activities of enzymatic and non-enzymatic antioxidants in tumour cells.

7.1. Enzymatic antioxidants

7.1.1. Superoxide dismutase (SOD)

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD) (EC 1.15.1.1). Superoxide dismutase is the antioxidant enzyme that catalyzes the dismutation of $O_2^{\bullet -}$ to O_2 and to the less-reactive species H_2O_2. While this enzyme was isolated as early as 1939, it was only in 1969 that McCord and Fridovich proved the antioxidant activity of SOD [218].

Superoxide dismutase exists in several isoforms, differing in the nature of the active metal centre and amino acid constituency, as well as their number of subunits, cofactors and other features. In humans there are three forms of SOD: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) [219]. SOD destroys $O_2^{\bullet -}$ with remarkably high reaction rates, by successive oxidation and reduction of the transition metal ion at the active site in a "Ping-Pong" type mechanism [215].

Cu, Zn-SOD is an enzyme with a molecular weight of about 32 kDa and is composed of two identical subunits (homodimer) [215]. Cu, Zn-SOD specifically catalyzes the dismutation of the superoxide anion to oxygen and water. Each subunit contains as the active site, a dinuclear metal cluster constituted by copper and zinc ions. Enzyme activity is relatively independent of pH in the range of 5–9.5.

Mitochondrial Mn-SOD is a homotetramer (96 kDa) containing one manganese atom per subunit [215]. This enzyme cycles from Mn(III) to Mn(II) and back to Mn(III) during the two step dismutation of superoxide.

Mn-SOD is one of the most effective antioxidant enzymes that has anti-tumour activity. A set of studies on different cell lines has confirmed that overexpression of Mn-SOD leads to tumour growth retardation [220]. However, the general statement of Mn-SOD as a tumour suppressor protein is far from clear. Though Mn-SOD activity was found to be rather low in many cancers, some experiments confirmed markedly increased Mn-SOD expression [220]. The decreased level of Mn-SOD

in some kind of tumours is related to a defect in the gene expression rather than to its deletion. In line with this is the fact that the concentrations of transition metal ions have been found to be significantly reduced in some tumours. For certain tumour cells the activity of total SOD (Cu, Zn-SOD and Mn-SOD) has also been found to be reduced [221]. Interestingly, a marked overexpression of Mn-SOD has been detected in cancers of the gastrointestinal tract. High Mn-SOD expression correlates with poor prognosis, advanced stages of progression and an invasive and metastatic phenotype. These data indicate that abnormally high levels of Mn-SOD, while suppressing cell growth, increase the invasive potential of cancer cells.

Of interest were findings that overexpression of Mn-SOD results in the activation of enzymes of the zinc-dependent matrix metalloproteinase family (MMP), namely MMP-1 and -2. The MMPs play various roles in cellular remodelling processes and some members of the MMP family have been reported to play a critical role in tumour invasion. The activation of MMPs occurs most probably via activation of the redox-sensitive transcription factor AP-1 and the nuclear factor NF-κB by elevated levels of hydrogen peroxide induced by Mn-SOD activity [222]. In conclusion it may be hypothesised that an imbalance between superoxide radical formation and hydrogen peroxide degradation occurring in cells with overexpressed Mn-SOD might activate the metastatic potential of cancer cells. The role of Mn-SOD as a prospective inducer of the loss of matrix functions in metastasis has to be clarified.

Extracellular superoxide dismutase (EC-SOD) is a secretory, tetrameric, copper and zinc containing glycoprotein, with a high affinity for certain glycosaminoglycans such as heparin and heparin sulphate [215]. Its regulation in mammalian tissues occurs primarily in a manner coordinated by cytokines, rather than as a response of individual cells to oxidants.

A completely distinct SOD class that contains Ni (Ni-SOD) was recently discovered in *Streptomyces* and cyanobacteria. Ni-SOD is a small 117 amino acids protein with no sequence homology to other SODs [223].

7.1.2. Catalase

Catalase (EC 1.11.1.6) is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria [215]. Catalase is located in a cell organelle called the peroxisome. The enzyme very efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert ~6 million molecules of hydrogen peroxide to water and oxygen each minute:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \quad (14)$$

The significantly decreased capacity of a variety of tumours for detoxifying hydrogen peroxide is linked to a decreased level of catalase.

7.1.3. Glutathione peroxidase

There are two forms of the enzyme glutathione peroxidase, one of which is selenium-independent (glutathione-*S*-transferase, GST, EC 2.5.1.18) while the other is selenium-dependent (GPx, EC 1.11.1.19) [215]. These two enzymes differ in the number of subunits, the bonding nature of the selenium at the active centre and their catalytic mechanisms. Glutathione metabolism is one of the most essential of antioxidative defence mechanisms.

Humans have four different Se-dependent glutathione peroxidases [215]. All GPx enzymes are known to add two electrons to reduce peroxides by forming selenoles (Se-OH). The antioxidant properties of these selenoenzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction. GPx acts in conjunction with the tripeptide glutathione (GSH), which is present in cells in high (micromolar) concentrations. The substrate for the catalytic reaction of GPx is H_2O_2, or an organic peroxide ROOH. GPx decomposes peroxides to water (or alcohol) while simultaneously oxidising GSH:

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O \quad (15)$$

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O \quad (16)$$

Significantly, GPx competes with catalase for H_2O_2 as a substrate and is the major source of protection against low levels of oxidative stress.

7.2. Non-enzymatic antioxidants

7.2.1. Vitamin C

Vitamin C (ascorbic acid) is a very important, and powerful, antioxidant that works in aqueous environments of the body, such as are present in the lungs and in the lens of the eye. Its primary antioxidant partners are Vitamin E and the carotenoids, as well as working along with the antioxidant enzymes. Vitamin C cooperates with Vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins (see also below) [224,225].

Ascorbic acid has two ionisable hydroxyl groups and therefore is a di-acid ($AscH_2$) (Fig. 6). At physiological pH, 99.9% of Vitamin C is present as $AscH^-$, and only

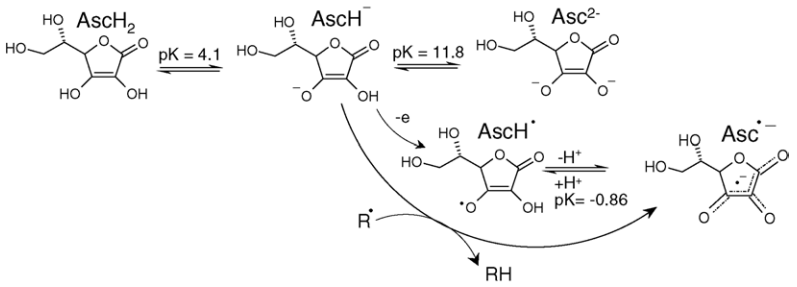

Fig. 6. Various forms of ascorbic acid (Vitamin C) and its reaction with radicals ($R^{\bullet}$).

very small proportions as $AscH_2$ (0.05%) and Asc^{2-} (0.004%). The antioxidant chemistry of Vitamin C is thus the chemistry of $AscH^-$. $AscH^-$ is a donor antioxidant and reacts with radicals to produce the resonance stabilised tricarbonyl ascorbate free radical ($AscH^{\bullet}$). $AscH^{\bullet}$ has a $pK = -0.86$, and hence it is not protonated but is present in the form of $Asc^{\bullet -}$ (Fig. 6). Thus the product of ascorbate oxidation by many ROS is the semidehydroascorbate radical ($Asc^{\bullet -}$) a poorly reactive radical that is considered to be a terminal, small molecule antioxidant and the level of this radical is a good measure of the degree of oxidative stress in biological systems [226,227].

It is acknowledged that Vitamin C protects membranes against oxidation [228]. The intake of very high doses of Vitamin C, suggested initially by Linus Pauling, has been a subject of intense debate for many years [229]. While intake of high doses of Vitamin C (up to 2000 mg/day) has not been consistently reported to result in side effects, the benefit of a high intake of Vitamin C has never been established. However, it has been reported that low serum levels of Vitamin C in high-risk population may contribute to the increased risk of gastric metaplasia or chronic gastritis, which are both precancerous lesions [230]. The positive effect of Vitamin C in reducing the incidence of stomach cancer is most probably due to the inhibitory action in the generation of *N*-nitroso compounds by interrupting the reaction between nitrites and amine groups. A consistent protective effect of Vitamin C has also been found in lung and colorectal cancer [231].

Some in vitro and animal supplementation studies explored the pro-oxidant properties of ascorbate [232]. The pro-oxidant effect of ascorbate was attributed to the release of metal ions from damaged cells. In addition, it has been reported that Vitamin C induces lipid hydroperoxide decomposition to the DNA-reactive bifunctional electrophiles 4-oxo-2-nonenal, 4,5-epoxy-2(E)-decenal and 4-hydroxy-2-non-enal [233]. The compound 4,5-epoxy-2(E)-decenal is a precursor of etheno-2′-deoxyadenosine a highly mutagenic lesion found in human DNA. Recent in vitro and ex vivo studies revealed that Vitamin C in plasma increases dose-dependently resistance to-lipid peroxidation, even in the presence of redox-active iron or copper and H_2O_2 [234]. Overall, in vitro studies have shown that Vitamin C either has no effect or inhibits transition metal (Fe, Cu)-ion dependent lipid peroxidation in plasma and other biological fluids. In contrast, Vitamin C may be able to promote metal ion-dependent hydroxyl radical formation in biological fluids, but only under unphysiological conditions [235].

Majority of in vivo studies showed a reduction in markers of oxidative DNA, lipid and protein damage after supplementation with Vitamin C. Even in the presence of iron, Vitamin C predominantly reduces in vivo oxidative damage, despite its well known pro-oxidant properties in vitro in buffer systems containing Fe. Studies reporting a pro-oxidant effect for Vitamin C in human supplementation studies should be taken with caution as to their choice of biomarkers, methodology and experimental design to rule out any oxidation effects [236].

Recent studies indicate the ability of ascorbic acid to regulate factors that may influence gene expression, apoptosis and other cellular functions [230]. In many studies Vitamin C protects against cell death triggered by various stimuli and a major proportion of this protection has been linked with its antioxidant ability. Studies of the anti-apoptotic acivity of Vitamin C have revealed a role of Vitamin C in modulation of the immune system. Several studies reported the mechanisms by which Vitamin C regulates the AP-1 complex, including the Fos and Jun superfamilies. Ascorbate treated cells exposed to UV-B irradiation led to a 50% decrease in JNK phosphorylation (which activated AP-1), therefore inhibiting the JNK/AP-1 signalling pathways.

7.2.2. Vitamin E

Vitamin E is a fat-soluble vitamin that exists in eight different forms. α-Tocopherol is the most active form of vitamin E in humans and is a powerful biological antiox-

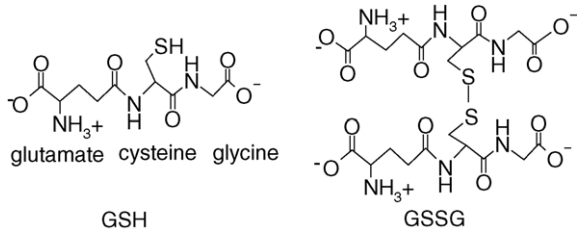

Fig. 7. Structures of reduced (GSH) and oxidised (GSSG) glutathione.

idant which is considered to be the major membrane-bound antioxidant employed by the cell [237]. Its main antioxidant function is protection against lipid peroxidation [238]. As mentioned above, ascorbic acid is regarded as the major aqueous phase antioxidant. Recent evidence suggests that α-tocopherol and ascorbic acid function together in a cyclic-type of process. During the antioxidant reaction, α-tocopherol is converted to an α-tocopherol radical by the donation of a labile hydrogen to a lipid or lipid peroxyl radical. The α-tocopherol radical can thus be reduced to the original α-tocopherol form by ascorbic acid [224].

Several epidemiological trials reported the effect of the intake of Vitamin E supplements. It has been demonstrated that the intake of Vitamin E [200 IU (international units)/day] reduced the incidence of coloretal cancer by triggered apoptosis of cancer cells by inducing p21wafi/cip1, a powerful cell cycle inhibitor [239]. Generally, the protective effect of Vitamin E is a result of the inhibition of free radical formation and activation of endonucleases. Other study reported negative results for Vitamin E in combination with Vitamin C and beta-carotene to prevent colorectal cancer adenoma over a period of 4 years [240]. Since Vitamin C regenerates Vitamin E, it has been proposed that addition of Vitamin E hinders the protective effect of Vitamin C against oxidative damage [213].

Of great surprise was a recent trial which revealed that daily Vitamin E doses of 400 IU or more can *increase* the risk of death and should be avoided [241]. According to the analysis, there is no increased risk of death with a dose of 200 IU per day or less, and there may even be some benefit.

7.2.3. Thiol antioxidants — glutathione

The major thiol antioxidant is the tripeptide, glutathione. Glutathione (GSH) (Fig. 7) is a multifunctional intracellular non-enzymatic antioxidant. It is considered to be the major thiol-disulphide redox buffer of the cell [242]. 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 [242]. The reduced form of glutathione is GSH, glutathione, and the oxidised form is GSSG, glutathione disulphide.

GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression. An oxidative environment leads to rapid modification of protein sulphydryls (protein-SH): two-electron oxidation yields sulphenic acids (protein-SOH) and one-electron oxidation yields thiyl radicals (protein-S$^\bullet$) [243]. These partially oxidised products react with GSH and form *S*-glutathiolated protein (protein-SSG), which is reduced further by the glutathione cycle through glutathione reductase and small proteins such as glutaredoxin and thioredoxin, to restore protein sulphydryls (protein-SH). However, if the process of oxidation of protein sulphydryls is not trapped by GSH, further oxidation leads to the formation of irreversibly oxidised forms such as sulphinic (protein-SO_2H) and sulphonic (protein-SO_3H) acids (Fig. 8).

Generally, the antioxidant capacity of thiol compounds is due to the sulphur atom which can easily accommodate the loss of a single electron [244]. In addition the lifetime of sulphur radical species thus generated, i.e. a thiyl radical (GS$^\bullet$), may be significantly longer than many other radicals generated during the stress. The reaction of glutathione with the radical R$^\bullet$ can be described:

$$GSH + R^\bullet \rightarrow GS^\bullet + RH \quad (17)$$

Thiyl radicals generated may dimerise to form the non-radical product, oxidised glutathione (GSSG):

$$GS^\bullet + GS^\bullet \rightarrow GSSG \quad (18)$$

Oxidised glutathione GSSG is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organisms [245]. Too high a concentration of oxidised glutathione GSSG may damage many enzymes oxidatively. GSSG can react with protein sulphydryl groups to produce protein–glutathione-mixed disulphides:

$$GSSG + \text{protein-SH} \leftrightarrow \text{protein-SSG} + GSH \quad (19)$$

The mixed disulphides (protein-SSG) have a longer half-life than GSSG, most probably due to protein folding.

The main protective roles of glutathione against oxidative stress are [242]: that (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione-transferase 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

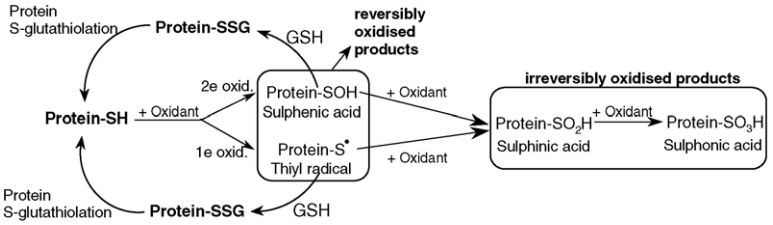

Fig. 8. Role of GSH in oxidation of protein sulphydryl groups.

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. 9).

The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide–glutathione couple (GSSG/2GSH) [246]. This, in turn, has a high impact on the overall redox environment of the cell. The values of the half-cell reduction potential for the GSSG/2GSH

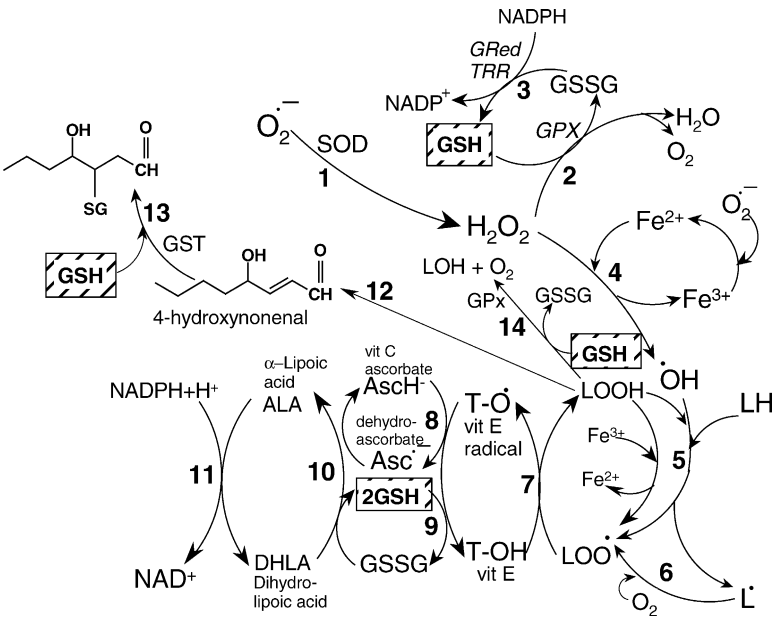

Fig. 9. The various pathways of glutathione (GSH) and other antioxidants (Vitamin E, Vitamin C, lipoic acid) in the management of oxidative stress. (Equations are not balanced.) Reaction (1): superoxide is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. Reaction (2): hydrogen peroxide is most efficiently scavenged by the enzyme glutathione peroxidase (GPx) which requires GSH as the electron donor. Reaction (3): the oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (Gred) which uses NADPH as the electron donor. Reaction (4): some transition metals (e.g. Fe^{2+}, Cu^{+} and others) can breakdown hydrogen peroxide to the reactive hydroxyl radical (Fenton reaction). Reaction (5): the hydroxyl radical can abstract an electron from polyunsaturated fatty acid (LH) to give rise to a carbon-centred lipid radical ($L^{\bullet}$). Reaction (6): the lipid radical ($L^{\bullet}$) can further interact with molecular oxygen to give a lipid peroxyl radical ($LOO^{\bullet}$). Reaction (7): 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 (8): 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 (9): the regeneration of Vitamin E by GSH: the oxidised Vitamin E radical ($T\text{-}O^{\bullet}$) is reduced by GSH. Reaction (10): 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 (11): the regeneration of DHLA from ALA using NADPH. Reaction (12): lipid hydroperoxides can break down into aldehydes, such as the strong oxidant 4-hydroxynonenal. Reaction (13): 4-hydroxynonenal is rendered into an innocuous glutathiyl adduct (GST: glutathione *S*-transferase). Reaction (14): lipid hydroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor.

couple depend on the redox environment in which the couple is functioning. For example, the redox potential is −180 mV in the endoplasmatic reticulum, whilst in the cytosol it is −232 mV [246]. Thus the compartmentalisation of GSH is linked with the different redox environments in these compartments. We note that for the GSSG/2GSH couple the reduction potential is dependent not only on the GSH/GSSG ratio, but also on the absolute concentration of GSH. An increase in the concentration of GSH shifts the half-cell reduction potential towards higher values. The intracellular content of glutathione is responsive to environmental factors and is a function of the balance between use and synthesis. Exposure to ROS (involving H_2O_2)/RNS or to compounds that can generate ROS including 4-hydroxy-2-nonenal (HNE) can increase the content of GSH by increasing the rate of GSH synthesis. HNE is removed from many cells by reactions with GSH, catalyzed by glutathione *S*-transferases (GSTs, see below) that have relative specificity for alkenals. HNE has been demonstrated to activate the JNK pathways, which in turn activate the activator protein-1 (AP-1) transcription complex. This suggests that HNE was signalling for increased GSH biosynthesis via the JNK pathway.

Various pathways for the management of oxidative stress by GSH are shown in Fig. 9.

GSH has been reported to regulate redox signalling by alterations in both the level of total GSH and in the ratio of its oxidised (GSSG) to reduced (GSH) forms [246]. Cellular GSH depletion has been found to be associated with decreased cell proliferation in vascular endothelial cells and increased proliferation of fibroblasts. It has been shown that autophosphorylation of the PDGF receptor is inhibited by low cellular GSH levels. GSH is involved in regulating the activation of various transcription factors, including nuclear factor NF-κB and activator protein AP-1. In the nucleus, the GSH redox couple appears to regulate DNA binding of the Sp-1 transcription factor.

GSH protects cells against apoptotis — the protective role originates from multifactorial mechanisms that involve detoxification and modulation of cellular redox state and the subsequent redox-sensitive cell-signalling pathways and interaction with pro- and anti-apoptotic signals [242]. Since the level of GSH is an important factor in the protection against apoptosis, the efficacy of anticancer drug-induced apoptosis requires depletion of GSH, thus facilitating tumour treatment.

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. 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 to -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 [247]. This has been explained by an increased level of oxidised glutathione GSSG, especially in advanced stages of cancer progession. 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.

7.2.4. Thiol antioxidants — the thioredoxin system, TRX, TRX-S_2/TRX-$(SH)_2$

Thioredoxin (TRX) is a small multifunctional, disulphide-containing redox protein possessing two redox-active cysteins within a conserved active site (Cys–Gly–Pro–Cys) [248]. Thioredoxin contains two adjacent –SH groups in its reduced form that are converted to a disulphide unit in oxidised thioredoxin which is udergoing redox reactions with multiple proteins:

$$\text{thioredoxin-(SH)}_2 + \text{protein-S}_2 \rightarrow \text{thioredoxin-S}_2 + \text{protein-(SH)}_2 \quad (20)$$

The reduction of the disulphide back to the dithiol form is catalyzed by thioredoxin reductase (TR), the source of electrons being NADPH:

$$\text{TR-S}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{TR-(SH)}_2 + \text{NADP}^+ \quad (21)$$

$$\text{thioredoxin-S}_2 + \text{TR-(SH)}_2 \rightarrow \text{thioredoxin-(SH)}_2 + \text{TR-S}_2 \quad (22)$$

Thioredoxin also provides control over a number of transcription factors affecting cell proliferation and death through a mechanism referred to as redox regulation. Thioredoxin levels are 100- to 1000-fold less than GSH, however, thioredoxin and GSH may have overlapping as well as compartmentalised functions in activation and regulation of transcription factors, including NF-κB and AP-1. The mechanisms of TRX functioning is as follows: (i) Trx is reduced into its active state, TR-$(SH)_2$ by the enzyme thioredoxin reductase; (ii) TR-$(SH)_2$ enters

the nucleus to regulate transcription factor activity (factors which affect DNA replication); (iii) TR-$(SH)_2$ is excreted out of cell where it operates with other growth factors to stimulate cell growth.

The results have also shown that TRX has a different role in regulating NF-κB in the cytoplasm and in the nucleus [249]. TRX was found to inhibit NF-κB activity in cytoplasm and upregulate the ability of NF-κB to bind to DNA in the nucleus. In addition, TRX can regulate HIF-1 and cytochrome P450. Recent evidence suggests that TRX can regulate the activity of some proteins by directly binding to them. TRX is able to translocate from the cytosol to the nucleus in response to oxidative stress to regulate gene expression through Ref-1. Other transcription factors regulated by TRX involve p53 and polyomavirus enhancer binding protein 2/core binding factor (PEBP2/CBF).

There exists significant experimental and clinical evidence connecting thioredoxin to cancer which suggests the following: (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).

7.2.5. Thiol antioxidants — lipoic acid

α-Lipoic acid (ALA), a disulphide derivative of octanoic acid, is a natural compound also referred to as thiothic acid and has the full chemical name 1,2-dithiolane-3-pentanoic acid ($C_8H_{14}O_2S_2$) (Fig. 10). α-Lipoic acid is both water and fat-soluble and therefore is widely distributed in both cellular membranes and the cytosol. α-Lipoic acid is readily absorbed from the diet and is converted rapidly in many tissues to its reduced dithiol form, dihydrolipoic acid (DHLA) [250].

Both ALA and DHLA (Fig. 10) are powerful antioxidants [251]. Their antioxidant functions involve: (i) quenching of reactive oxygen species; (ii) regeneration of endogenous and exogenous antioxidants involving vitamins C and E and glutathione; (iii) chelation of redox metals including Cu(II) and Fe(II); (iv) repair of oxidised proteins. ALA is a possible chelator for Cd(II), but much less effectively than DHLA.

The DHLA/ALA couple has a redox potential of −320 mV, and so DHLA has one of the highest antioxidant potentials known in biological systems [252]. DHLA acts synergistically with other antioxidants, indicating that it is capable of regenerating other antioxidants from their radical, or inactive, forms (Fig. 9, reaction (10)).

The ability of DHLA to regenerate oxidised thiols in proteins is very important. Endogenously supplied ALA influences the overall redox state of proteins, such as thioredoxin and transport proteins.

The positive effect of ALA has been associated with being beneficial to cardiovascular ailments, HIV infections and several different neurodegenerative diseases [253]. Lipoic acid has a long history in Germany as being safe to use in the treatment of complications arising from diabetes mellitus. ALA prevents β-cell destruction, stimulates glucose uptake, protects against atherosclerosis and cataracts, and decreases symptoms from diabetic neuropathy. In ischemia-reperfusion injury, ALA has also been shown to prevent, or ameliorate, the damage that occurs from ROS, produced when the ischemic tissue is re-oxygenated. ALA also possesses radio-protective properties and furthermore minimises the pathological consequences of cigarette smoke [252]. In Ukrainian children, following the 1986 Chernobyl nuclear reactor disaster, lipoic acid was found to markedly reduce the ill effects of radiation on liver and kidney function, even though it was administered for only 1 month. Lipoic acid is very effective in the treatment of heavy metal poisoning and lipoic acid infusions have been used successfully in the treatment of *Aminata* mushroom poisoning [253].

7.2.6. Carotenoids

Carotenoids (Car) are pigments that are found in plants and microorganisms. There are over 600 carotenoids occurring in nature. Various studies have indicated that carotenoids may prevent or inhibit certain

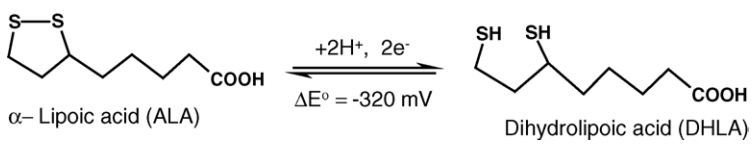

Fig. 10. Structures of ALA and DHLA.

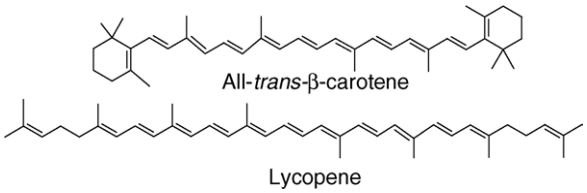

Fig. 11. Carotenoid structures.

types of cancer, artherosclerosis, age-related muscular degeneration, and other diseases.

The antioxidant activity of carotenoids arises primarily as a consequence of the ability of the conjugated double-bonded structure (Fig. 11) to delocalise unpaired electrons [254]. This is primarily responsible for the excellent ability of β-carotene to physically quench singlet oxygen without degradation, and for the chemical reactivity of β-carotene with free radicals such as the peroxyl ($ROO^{\bullet}$), hydroxyl ($^{\bullet}OH$), and superoxide radicals ($O_2^{\bullet -}$). At sufficiently high concentrations, carotenoids can protect lipids from peroxidative damage.

Generally three mechanisms are proposed for the reaction of free radicals ($ROO^{\bullet}$, $R^{\bullet}$) with carotenoids: (i) radical addition, (ii) hydrogen abstraction from the carotenoid and (iii) electron-transfer reaction [255].

Recently a growing body of evidence has accumulated which suggests that the scavenging of lipid $ROO^{\bullet}$ (where R is an aliphatic group), by β-carotene may not proceed via an electron-transfer mechanism reaction, but rather by adduct formation and/or hydrogen abstraction [255].

Burton and Ingold were among the first to propose that β-carotene might participate in lipid peroxidation as a pro-oxidant [256]. The term 'pro-oxidant activity' involves the 'ability' of β-carotene to increase the total radical yield in the system. In support of this hypothesis is the recent alpha-tocopherol/beta-carotene (ATBC) trial carried out by the U.S. National Cancer Institute [257]. In this trial a supplemental dose of 20 mg/day of β-carotene was administered to 29,133 50–69 years old male smokers in Finland for 5–8 years. The dosage of 20 mg/day was substantially higher than that typically contained in the Finnish diet. The results of trial were very surprising. There was a significant, 18% increase in the incidence of lung cancer, which contributed to an 8% excess in total mortality! The ATBC trial results imply that β-carotene or, more specifically, the all-*trans* isomer of β-carotene in a water-soluble beadlet, is not associated with the reduced risk of cancer associated with the intake of fruit and vegetables. However, these results do not entirely rule out a protective role for β-carotene; for example, if it is given in higher dosages or during the earlier stages of the process of lung carcinogenesis. The key factors, which determine the switch of carotenoids from antioxidants to pro-oxidants are the partial pressure of dioxygen (pO_2) and the carotenoid concentration [258]. At higher pO_2 a carotenoid radical, $Car^{\bullet}$, (generated through the hydrogen abstraction) can react with dioxygen to generate a carotenoid-peroxyl radical, Car-$OO^{\bullet}$ [259]:

$$Car^{\bullet} + O_2 \rightarrow Car\text{-}OO^{\bullet} \quad (23)$$

which Car-$OO^{\bullet}$ can act as a pro-oxidant by promoting oxidation of unsaturated lipid (RH):

$$Car\text{-}OO^{\bullet} + RH \rightarrow Car\text{-}OOH + R^{\bullet} \quad (24)$$

In conclusion, carotenoids (in particular β-carotene) exhibit antioxidant behaviour at low oxygen partial pressures, usually below 150 Torr, but they may lose antioxidant properties, or even become pro-oxidants, at high pressures of oxygen. The concentration of carotenoids also influences their anti/pro-oxidant properties in a similar manner; at high carotenoid concentrations there is a propensity for pro-oxidant behaviour.

Carotenoids and retinoic acid may regulate transcription factors [260]. For example retinoic acid is able to inhibit cell proliferation and enhance cell differentiation. Cells exposed to oxidative stress and simultaneously treated with β-carotene exhibited suppressed activation of NF-κB and production of interleukin-6 (IL-6) and TNF-alpha inflammatory cytokines, suggesting a protective effect of β-carotene. Carotenoids may influence the process of apoptosis in healthy cells; while the pro-apoptotic BAX protein was downregulated after induction of external stimuli, the anti-apoptotic protein Bcl-2 was upregulated by β-carotene supplementation [261].

Carotenoids have antiproliferative effect on various cancer cell lines; lycopene has been shown to inhibit cell cycle progression in breast, lung and prostate cell lines. Lycopene has also been shown to regulate transcription factors. Mammary cancer cells treated with lycopene have shown inhibited AP-1 binding and reduced the insuline-like growth factor-I induction, suggesting an inhibitory effect of lycopene on mammary cancer cell growth. In addition, β-carotene exhibits a pro-apoptotic effect in colon and leukemic cancer cells; the mechanisms has been shown to proceed via a redox dependent mechanism, increased ROS and GSSG/GSH ratio linked with increased NF-κB binding ability, inhibition of cell growth and enhanced pro-apoptotic activity in tumour cells [262]. β-Carotene has been shown to inhibit the expression of anti-apoptotic protein Bcl-2 in cancer cells, reducing thus growth of cancer cells.

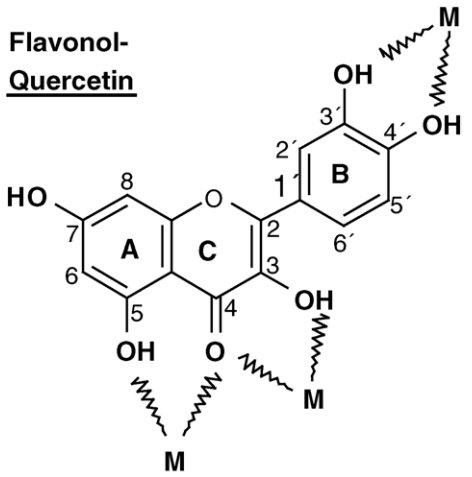

Fig. 12. Structure of a flavonoid quercetin. (M: coordinated metal ion).

7.2.7. Flavonoids

Polyphenolic compounds constitute one of the most commonly occuring and ubiquitous groups of plant metabolites and represent an integral part of human diet [263–265]. Flavonoids constitute the most important single group of polyphenols; with more than 4000 compounds described, and which can be subdivided into 13 classes. Their common structural feature is the diphenylpropane moiety, which consists of two aromatic rings linked through three carbon atoms that together usually form an oxygenated heterocycle (Fig. 12).

Recent interest in phenolic compounds in general, and flavonoids in particular, has increased greatly owing to their antioxidant capacity and their possible beneficial implications in human health [265]. These include the treatment and prevention of cancer, cardiovascular disease and other pathological disorders [264].

Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalysing lipid peroxidation [265]. Phenolic antioxidants (PhOH) interfere with the oxidation of lipids and other molecules by the rapid donation of hydrogen atom to radicals $ROO^{\bullet} + PhOH \rightarrow ROOH + PhO^{\bullet}$. The phenoxy radical intermediates are relatively stable so they do not initiate (propagate) further radical reactions. They even act as terminators of the reaction chain by interacting with other free radicals. However, under certain conditions, e.g. a high concentration of phenolic antioxidants, the presence of redox-active metals (copper, iron) and a high pH, they may behave as pro-oxidants.

The key factors affecting the biological activity of flavonoids (both in vivo and in vitro) are the extent, nature, and position of the substituents and the number of hydroxyl groups [265]. All these factors influence whether a flavonoid will act as an antioxidant or as a modulator of enzyme activity, or whether it possesses antimutagenic or cytotoxic properties.

One of the most actively studied properties of flavonoids is their protection against oxidative stress [264,266]. For example, flavonoids are ideal scavengers of peroxyl radicals due to their favourable reduction potentials relative to alkyl peroxyl radicals and thus, in principle, they are effective inhibitors of lipid peroxidation. Of particular importance is the hydrogen (electron) donating ability of a flavonoid molecule which acts to scavenge a reactive radical species, and is primarily associated with the presence of a B-ring catechol group (dihydroxylated B-ring) [265]. One important structural feature which is partly responsible for the antioxidant properties of flavonoids involves the presence of 2,3 unsaturation in conjugation with a 4-oxo group in the C-ring. In addition, the presence of functional groups involving both hydroxyl groups of ring-B and the 5-hydroxy group of ring-A are all important contributors in the ability of flavonoids to chelate redox-active metals and thus prevent catalytic breakdown of hydrogen peroxide (Fenton chemistry). A novel proposed mechanism for the action of flavonoids as modulators of cell signalling is currently under investigation [265].

The nutritional benefit of flavonoids is generally linked with their healing potential [266]. A regular intake of flavonoids, such as polyphenols and quercetin, is linked to lower rates of stomach, pancreatic, lung and possibly breast cancer [267]. Some studies indicate that a diet high in flavonoids, particularly quercetin, may help prevent blood clots and blocked arteries, significantly reducing the chance of death from stroke or heart.

It should be noted, however, that while most flavonoids and phenolic compounds possess antioxidant properties and are considered safe, there have been reports of toxic flavonoid-drug interactions, as well as liver failure, contact dermatitis, hemolytic anemia and oestrogenic-related concerns, including male reproductive health and breast cancer associated with dietary flavonoid intake [268]. In fact, the very features that underlie antioxidant activity can also promote cytotoxicity. Indeed, the mechanism of flavonoid cytotoxicity may relate to their pro-oxidant properties. Thus, while the beneficial effect of many flavonoids on human health is clear, further work on the potential toxic effects linked with flavonoids as dietary supplements is necessary.

7.2.8. Selenium

Selenium appears to function as an antimutagenic agent, preventing the malignant transformation of normal cells. These protective effects of Se seem to be primarily associated with its presence in the glutathione peroxidases (GSH-Pxs) and thioredoxin reductase, which are known to protect DNA and other cellular components from oxidative damage [202,269]. Generally, seleno-enzymes are known to play roles in the control of cell division, oxygen metabolism, detoxification process, induction of apoptosis in cancer cells and the functioning of the immune system. Other modes of actions involve inactivation of oncogenes.

Selenium and GSH-Px levels have been found to be decreased in patients with carcinoma of the uterine cervix. Two clinical intervention trial on the protective effect of selenium against cancer performed in the US have shown that selenium intake (200 μg/day) did not reduce the risk of skin cancer, however, significant reductions in the secondary end points in lung, colon and prostate cancer incidence were observed [270].

A clinical trial conducted in China has shown that a daily intake of 50 μg of selenium combined with β-carotene and α-tocopherol reduced the mortality rate from stomach cancer significantly [271]. Additionally, in vitro studies have shown that selenium compounds are able to inhibit oxidative stress-induced DNA damage and carcinogen-induced covalent DNA adduct formation. Further in vitro studies have shown that selenium is able to induce apoptosis and inhibits cell growth in transformed cells [272]. This implies that selenium compounds are able to induce the *p53* gene.

Selenium is involved in signal transduction via activation of MAPKs and transcription factors such as AP-1, NF-κB which influence gene expression and cell growth [273]. Selenium has been shown to regulate p53. In addition, it has been suggested that the activity of p53 is selenium dependent and redox-influenced. The molecular basis for selenium in inducing apoptosis in cancer cells occurs via mediation of cell-signalling targets. Pre-malignant human breast cancer cells incubated with methylselenic acid exhibited growth inhibition and induction of apoptosis. The effect of selenium on molecular processes involving cell signalling and apoptosis occurs mainly via a redox-dependent mechanism.

8. Oxidative stress and redox environment of a cell

Oxidation and reduction reactions in biological systems are called redox reactions and represent the basis for numerous biochemical mechanisms. When discussing redox reactions in biological systems, instead of the terms reductant and oxidant, it is more appropriate to use the terms antioxidant and pro-oxidant, respectively [114]. A reductant, or reducing agent, is a substance which donates electrons; an oxidant, or oxidising agent, is a substance that accepts electrons. A chemical process during which a loss of electrons occurs is called an oxidation process (oxidation reaction). Conversely a reduction process (reduction reaction) is characterised by the gain of electrons. The theory describing this phenomenon is called the redox (reduction/oxidation) theory of cellular functioning.

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 differentiation [271–279]. The redox state of a biological system 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 [276].

The intracellular “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 [276]. The intracellular concentration of glutathione is 500 times higher than the extracellular concentration and is considered to function in detoxification processes for the cell. Of interest is the question how does the ratio 2GSH/GSSG translate into the process of redox signalling. In the endoplasmic reticulum, where the ratio 2GSH/GSSG is rather low, mixed disulphide formation and disulphide exchange is an important component o protein folding. However, under ehanced oxidative stress conditions, GSSG content increases through the reaction (15), which in turn will increase 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 thiol systems counteract intracellular oxidative stress by reducing both hydrogen peroxide and lipid peroxides, reactions that are catalyzed by peroxidases. For example, GSH peroxidase catalyzes the reaction $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$.

In addition to antioxidant functioning in the cell, GSH and TRX are involved in cell-signalling process. Other major players affecting redox regulation involve extrinsic molecules such as ascorbic acid, carotenoids and selenium.

In recent years the term "redox state" has not only been used to describe the state of a redox pair, e.g. GSSG/2GSH, $Asc^{\bullet -}/AscH^{-}$ and others, but also to describe more generally the redox environment of a cell [276]. It is evident that the origin of cellular oxidants as well as the balance of cellular redox state involves complex regulatory pathways. In homogeneous fluids such as plasma, the determination of the molar concentrations of major redox couples is relatively easy, thus the evaluation of the redox environment is straightforward. On the other hand, in cells or tissues, compartmentalisation and non-equilibrium state of a particular redox couple may be a serious problem in determining the molar concentrations. Since cytosol, extracellular space, mitochondria and nucleus represent large compartments of a cell without significant gradients of various redox couples involving glutathione, we can estimate the overall concentration of glutathione in each compartment [276].

The cellular redox environment plays an important role in signal transduction, enzyme activation, DNA and RNA synthesis, cell proliferation, differentiation and apoptosis [278,279]. Generally, while cell death is initiated by an oxidising environment of the cell, a reducing environment is an important factor for increased cell proliferation. An example of increased cell proliferation involves stimulated proliferation of some tumour cells exposed to high concentrations of thiols. In accord with this feature, antioxidants have been shown to prevent apoptosis [280].

It has been shown that the cell cycle is characterised by fluctuations in the redox environment of a cell, mediated, in particular by intracellular changes in concentration of glutathione [276]. Oxidising molecules, such as H_2O_2 and thioredoxin are present outside the cell; H_2O_2 can cross the plasma membrane and enter the cells, leading to changes in the intracellular redox environment. In addition, membrane proteins, such as NADPH oxidase on neutrophil membranes, can produce H_2O_2. Induction or inhibition of cell proliferation seems to be dependent on levels of oxidants/antioxidants in the cell. 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 highly oxidising environment in the cell leads to apoptosis and necrosis. Apoptosis is induced by moderate oxidising stimuli and necrosis by an intense oxidising effect [280]. In various cell lines apoptosis was induced by low levels of hydrogen peroxide (5–35 μM), whereas a high concentration of hydrogen peroxide above 100 μM induced necrosis. Thus each stage of the cell cycle has a tightly controled redox state which is characterised by a particular value of the cellular reduction potential.

In agreement with these findings, reduced glutathione (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. The loss of GSH was due to extrusion of GSH outside the cell through specific membrane translocators. It was also demonstrated that the release of mature *Cytochrome c* from mitochondria is a cellular response to the depletion of glutathione. It should be noted, that the depletion of intracellular glutathione is not the only factor involved in the commitment to undergo apoptosis [276]. Regardless of this, the redox environment is the critical determinant for the trigger of 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.

Extensive evidence has shown that redox balance is impaired in cancer cells compared with normal cells, which may be related to oncogenic stimulation. Altered levels of antioxidant enzymes (SOD, catalase, glutathione peroxidase) and non-enzymatic antioxidants (GSH, Vitamin C, thioredoxin) as well as changes in the related signal pathways are evident in many human cancers [281]. The cumulative production of ROS typical for many cancer cells is linked with altered redox regulation of signalling cascades. The reducing intracellular environment in the nucleus and in mitochondria (maintained by elevated levels of glutathione and thioredoxin) not only facilitates escape from apoptosis but also produces a proliferation potential through activation of cell survival signals mediated by redox-sensitive nuclear transcription factors [282].

The human DNA repair enzyme APE/Ref-1 is a dual function protein that plays an important role in transcriptional response to oxidative stress and in DNA base excision repair. In addition, APE/Ref-1 facilitates the DNA-binding activity of several transcription factors (AP-1, NF-κB, p53 and others) via redox-dependent and redox-independent mechanisms [283]. Many studies have documented that upregulation of Ref-1 protects cells from various apoptosis stimuli, involving also oxidative stress and radiation. Conversely, down-regulation of Ref-1 is linked with apoptosis and sensitisation of cells. Ref-1 is involved in different stages of carcinogenesis (initia-

tion, promotion and progression) mainly through maintenance of intracellular redox balance and activation of a cell survival signal and repair of damaged DNA lesions. Elevated expression of Ref-1 compared to normal cells has been found in cervical, prostate, ovarian cancers. Ref-1 exhibits both cytoplasmic and nuclear enzymatic activities [284].

Apoptosis is closely tied to the Bcl-2 [285]. Bcl-2 can block *Cytochrom c* release which in turn inhibit a decrease in glutathione concentration, shifting thus the redox environment of the cell away from apoptosis. Cancer cells are characterised by overexpressed Bcl-2 which may enhance resistance against oxidative stress (ROS)-induced apoptosis.

Interestingly a redox regulation of progression from G1 to S in nonmalignant cells has been found to be defective in cancer cells. While thiol antioxidant-induced modulation of the intracellular redox state results in G1 arrest in nonmalignant cells, tumour cells continue to cycle.

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 [286].

9. Conclusions

The generation of reactive oxygen species (ROS) is a consequence of aerobic life and is unavoidable. ROS and RNS represent a constant source of assaults upon our genetic material which can be either enhanced or partly reduced by nutritional, hormonal and environmental influences. Overproduction of ROS and RNS through either endogenous or exogenous insults is harmful to living organisms and is termed oxidative and nitrosative stress. ROS are known not only to attack DNA, but additional cellular components such as proteins and lipids, leaving behind reactive species that can, in turn, couple to DNA bases. 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 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. The extent to which oxidative DNA damage contributes to the process of carcinogenesis is not yet clear, however, it appears that the DNA damage is predominantly linked with the initiation process.

Besides the role of oxidants in the induction of mutations, it is apparent that ROS mediate cell-signalling pathways that are involved in cell growth regulatory pathways and are thus instrumental in the process of carcinogenesis. 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. Thus DNA damage, mutations and altered gene expression are all key players in the process of carcinogenesis. The involvement of oxidants appears to be the common denominator to all these events.

While the involvement of oxidants at various stages of the malignant transformation is evident, many details regarding the detailed role of ROS-induced damage in the etiology of multifactor diseases such as cancer are yet to be discovered. The effect of oxidative stress at a certain stage of carcinogenesis is directly related to the type, the reactivity and the concentration of the radicals involved. To determine with confidence which type and what level of oxidative damage is really a valid biomarker for cancer incidence, requires measuring the DNA of healthy subjects over a few decades to map the individuals who develop cancer.

The harmful effect of oxidative stress is counteracted by the antioxidant action of both antioxidant enzymes and non-enzymatic antioxidants. Mn-SOD is considered as one of the most effective antioxidant enzymes with anti-tumour activity. Experimental studies indicate that abnormally high levels of Mn-SOD, while suppressing cell growth, also increase the invasive potential of cancer cells. It may be hypothesised that the imbalance between superoxide radical formation and hydrogen peroxide degradation occuring in cells with overxepressed Mn-SOD might activate the metastatic potential of cancer cells. Generally, antioxidants are involved directly in the conversion of ROS to less-reactive species. However, antioxidant protection therapy against free radicals should be used only with caution since its effects depend on the stage at which it is introduced. When used during the progression stage of cancer, it might actually stimulate growth of tumours through the enhanced survival of tumour cells. Another important issue which should be taken into consideration is a pro-oxidant character of some antioxidants which may occur depending on the concentration and environment (oxygen pressure) in which they act.

Thus to prevent oxidative stress-related cancer, the most pivotal action would seem to be to minimise exposure to endogenous and exogenous sources of oxidative

stress, by the elimination of environmental carcinogens including carcinogenic metals insofar as this is possible. "Prevention," as in all threatening aspects of life, being "better than cure."

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