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REVIEW ARTICLE

REACTIVE OXYGEN SPECIES AND OXIDATIVE DNA DAMAGE

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Abstract : Reactive oxygen species (ROS) such as the superoxide anion radical ($0_2^{\cdot -}$) hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) have been implicated in the pathophysiology of various states, including ischemia reperfusion injury, haemorrhagic shock, atherosclerosis, heart failure, acute hypertension and cancer. The free radicals, nitric oxide (NO) and $0_2^{\cdot -}$ react to form peroxynitrite ($ONOO^-$), a potent cytotoxic oxidant. A potential mechanism of oxidative damage is the nitration of tyrosine residues of protein, peroxidation of lipids, degradation of DNA and oligonucleosomal fragments. Several mechanisms are responsible for the protection of the cells from potential cytotoxic damage caused by free radicals. Cells have developed various enzymatic and nonenzymatic defense systems to control excited oxygen species, however, a certain fraction escapes the cellular defense and may cause permanent or transient damage to nucleic acids within the cells, leading to such events as DNA strand breakage and disruption of Ca^{2+} metabolism. There is currently great interest in the possible role of ROS in causing DNA damage that leads to cancer and spontaneous mutations. A high rate of oxidative damage to mammalian DNA has been demonstrated by measuring oxidized DNA bases excreted in urine after DNA repair. The rate of oxidative DNA damage is directly related to the metabolic rate and inversely related to life span of the organism.

Key words : reactive oxygen species free radicals oxidative DNA damage antioxidants carcinogenesis

INTRODUCTION

On earth twenty one percent of the gaseous atmosphere is oxygen (O_2). Because of its ability to readily accept electrons (e^-), O_2 is a powerful oxidizing agent. A free radical is any species which is capable of independent existence and contains one or more unpaired electrons (1). Oxygen qualifies as a free radical because it possesses two unpaired e^-, each in a different orbital and both spinning in the same direction. The unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding non radical (2). There is considerable current interest in the role of reactive oxygen species (ROS) as

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mediators of tissue injury in human disease. The oxidative properties of oxygen play a vital role in diverse biological phenomena such as utilization of nutrients, electron transport to produce ATP and the removal of xenobiotics. However, oxygen has double edged properties. While it is essential for life, it can also provoke damaging oxidative events within cells. Oxygen, mainly via its transformation to more reactive forms i.e., $O_2^{\cdot -}$, $^{\cdot}OH$ and H_2O_2 can nick DNA (3), can damage essential enzymes and structural proteins and can also provoke uncontrolled chain reactions, such as lipid peroxidation or autoxidation reactions (e.g. the polymerization of catecholamines) (4, 5).

Peroxidation of membrane lipids is likely to lead to a disturbance of the membrane integrity (6, 7). ROS can interact with proteins directly, especially their sulfhydryl groups (8). ROS have been put to good use by phagocytes. NADPH oxidase is located in the plasma membrane of neutrophils (9). ROS may sometimes function as intracellular signaling molecule (10). ROS are known to interfere with actions of NO, which has been recognized as a messenger with widespread actions. Interactions between $O_2^{\cdot -}$ and NO regulate vascular tone or inflammatory processes. A harmful situation might arise from overproduction of $O_2^{\cdot -}$ or NO (11–13).

How are reactive oxygen species generated?

Reactive oxygen species (ROS) is a collective term which is used by biologists to include not only oxygen radicals ($O_2^{\cdot -}$,$^{\cdot}OH$) but also some derivatives of oxygen that do not contains unpaired electrons such as H_2O_2, singlet oxygen ($^1O_2$, hypochlorous acid (HOCL) and peroxynitrite ($ONOO^-$). ROS are produced continuously in living cells as by-products of normal metabolism, during metabolism of xenobiotics (14–16) and during exposure to high temperature (17, 18) or radiation (19). Several sources of ROS in the cells are proposed. ROS are generated from leakage of electrons on to oxygen from mitochondrial electron transport chains, microsomal cytochrome P–450 and their electron donating enzyme and other systems (20–22). For useful purposes, ROS, e.g.$O_2^{\cdot -}$, HOCL and H_2O_2 are produced from activated phagocytes (23, 24). The univalent reduction of O_2 forming $O_2^{\cdot -}$ also occurs from other normal biochemical oxidation-reductions, both enzymatic (e.g. xanthine oxidase) and non enzymatic reactions (such as autoxidation of catecholamines). Transfer of a single electron connects O_2, $O_2^{\cdot -}$, H_2O_2 and $^{\cdot}OH$. $O_2^{\cdot -}$ is metabolized by metalloenzymes, superoxide dismutases (SODs), to form H_2O_2 and O_2 (reaction 1).

$$O_2 + e^- \xrightarrow[+2H]{+1e} O_2^{\cdot -} \xrightarrow{SOD} H_2O_2 + O_2 \quad \text{.....(1)}$$

H_2O_2 is additionally generated *in vivo* by several oxidase enzymes, viz. monoamine oxidase (MAO), tyrosine hydroxylase and L-amino oxidase (25, 26). H_2O_2 can be safely decomposed by glutathione peroxidase (GPx) and catalase (CAT). At low concentrations, H_2O_2 is removed by reacting with reduced glutathione (GSH) to form oxidized glutathione (GSSG) and H_2O, catalyzed by the GPx (reaction 2). GSH is regenerated by the action of glutathione reductase (GRD) (reaction 3). At high concentrations, H_2O_2 is removed by the enzyme catalase (reaction 4).

$$2GSH + H_2O_2 \xrightarrow[GPX]{} GSSG + 2H_2O \quad \text{.....(2)}$$

$$GSSG + NADPH + H^+ \xrightarrow[GRD]{} 2GSH + NADP^+ \text{..(3)}$$

$$2H_2O_2 \xrightarrow[CAT]{} O_2 + 2H_2O \text{.....(4)}$$

H_2O_2 can react nonenzymatically with Fe^{2+} and Cu^{1+} or chelates in Fenton type reactions (27), thereby being converted into reactive ·OH radical (reaction 5).

$$H_2O_2 + Fe^2 \dashrightarrow {\cdot}OH + {}^-OH + Fe^{3+} \text{.....(5)}$$

The Fenton reaction can be augmented by the reduction of Fe^{3+} by $O_2{\cdot}^-$, regenerating Fe^{2+} (reaction 6). The net result is the production of ·OH as in the iron catalyzed Haber-Weiss type of reaction (28) (reaction 7).

$$H_2O_2 + Fe^{2+} \dashrightarrow {\cdot}OH + {}^-OH + Fe^{3+} \text{.....(5)}$$

$$Fe^{3+} + O_2{\cdot}^- \dashrightarrow Fe^{2+} + O_2 \text{.....(6)}$$

$$O_2{\cdot}^- + H_2O_2 \dashrightarrow O_2 + {\cdot}OH\ {}^-OH \text{.....(7)}$$

·OH is also formed by the decomposition of $ONOO^-$ (reaction 9). $ONOO^-$ is formed in cell when $O_2{\cdot}^-$ reacts with nitric oxide radical (NO·) in a radical addition reaction (reaction 8).

$$O_2{\cdot}^- + NO{\cdot} \dashrightarrow ONOO^- \text{.....(8)}$$

$$ONOO^- + H^+ \dashrightarrow {\cdot}OH, NO_2, NO_2{}^+ \text{.....(9)}$$

The ·OH is the most reactive form of the oxygen radical. It is highly reactive and hence no enzyme systems involving it as a substrate exist (29).

Evidences have shown that controlled generation of these highly reactive molecules has important roles in blastocyst implantation (30), disintegration of the structural elements of the sperm cells (31), iodination of tyrosine in the thyroxine biosynthesis (32, 33) and secretion of mucous in goblet cells (Fig 1a) (17, 24). However, their uncontrolled production is generally considered to be an important factor in the etiology of pathological conditions such as myocardial infarction, rheumatoid arthritis, cardiovascular, neurodegenerative, ischaemia-reperfusion and cancer diseases (Fig. 1b) (34–38).

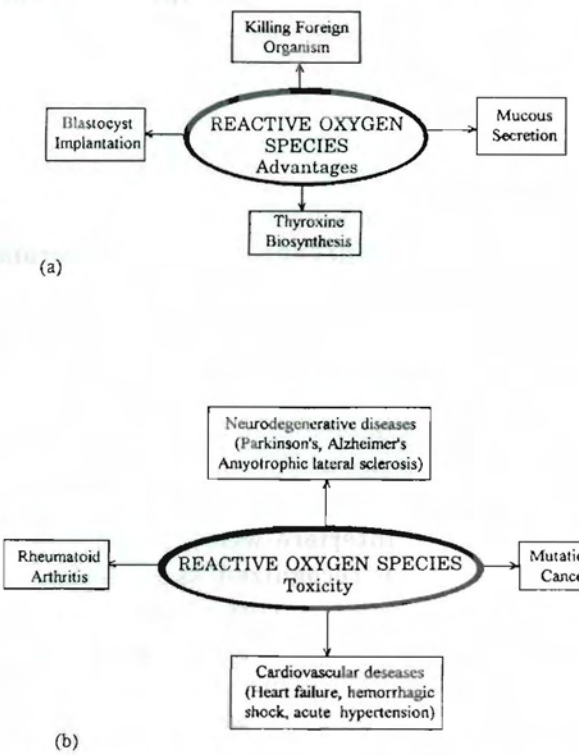

Fig. : (a) Potential advantages of reactive oxygen species (ROS).
(b) Potential toxicity of reactive oxygen species (ROS).

Oxidative stress

All aerobic organisms are continually exposed to oxidative stress. Normally there is an equilibrium between free radical

formation and antioxidant defense mechanisms. An imbalance leads to oxidative stress (39). A variety of critical biological molecules, including DNA, cellular protein and membrane lipids, are subjected to oxidative damage (40). The most reactive oxygen species is ·OH, which reacts at near diffusion limited rates. In contrast, $O_2^{\cdot -}$ can cross membranes and may be able to act at a distance; however, it is much less reactive than the ·OH. $O_2^{\cdot -}$ is normally converted by SOD to H_2O_2. H_2O_2 crosses cell membranes and can inactivate a few cell enzymes. However, H_2O_2 reacts with transition metals to generate ·OH. Oxidation reaction are, therefore, influenced by the regional concentrations of transition metals.

Another compound of recent interest is $ONOO^-$ which acts like an ·OH radical. The formation of $ONOO^-$ does not require transition metals. It may be able to produce cell damage by oxidizing lipids, proteins and DNA. $ONOO^-$ can react with Cu.Zn. SOD to form nitronium ion, which then nitrates tyrosine residues (41, 42). Recent findings have shown that ONOO reacts with carbon dioxide (CO_2) to form an unstable nitrosoperoxycarbonate adduct ($O=N-OOCO^{2-}$) that appears to rearrange to give a nitrocarbonate anion ($O_2N-OOCO^{2-}$) which may serve as the proximal oxidant in bilogical systems (43).

How is hydroxyl radical generated in the nucleus?

The formation of ROS is enhanced with an increased O_2 tension. However, neither $O_2^{\cdot -}$ nor H_2O_2 at physiological concentrations seem capable of producing DNA strand breakage (44, 45). All of the toxicity of $O_2^{\cdot -}$ and H_2O_2 *in vivo* arises by their metal ion dependent conversion into highly reactive ·OH radical (11, 46, 47). If the function of the radical is to destroy the molecules and tissue the ·OH is identified as radical's radical.

Several sources of ·OH production have been suggested. Background radiation may be one of its sources (48). Other sources of ·OH include the decomposition of $ONOO^-$ (49), the reaction of $O_2^{\cdot -}$ with HOCL (50) and via Fenton chemistry (12). Both Fe^{2+} and Cu^{2+} have been detected in the nucleus (51), facilitating the conversion of $O_2^{\cdot -}$ and H_2O_2 to ·OH, a species frequently proposed to initiate DNA damage (reaction 5).

$$Cu(II) + O_2^{\cdot -} \dashrightarrow Cu(I) + O_2 \quad \text{.....(18)}$$

$$Cu(I) + H_2O_2 \dashrightarrow Cu(II) + {}^{\cdot}OH + {}^{-}OH \quad \text{.....(19)}$$

$$2O_2^{\cdot -} + 2H^+ \dashrightarrow H_2O_2 + O_2 \quad \text{.....(20)}$$

The necessary iron (or other metals) might exist bound to the DNA *in vivo* or the oxidative stress might cause their liberation from intracellular storage sites. Subsequent binding to DNA could then make this molecule a target for DNA damage by oxidants. ·OH produces large number of sugar derived and base derived products in DNA as well as DNA protein cross-links (DPCs) (48). Both Fe^{2+} and Cu^{2+} markedly enhance the breakage of DNA strand (52, 53). In terms of DNA damage the ·OH can induce strand breaks as well as chemical changes in the deoxyribose and in the purine and pyrimidine bases (54).

Chemistry of oxidative DNA damage

DNA damage is the result of extrinsic and intrinsic processes including ionizing

radiation, toxic chemical ingestion, u.v. light exposure and oxygen-derived free radicals that are a normal consequences of the cellular metabolism of O_2 (55). These extrinsic factors generate free radicals which are, therefore, also the mechanism by which they inflict their damage on DNA. At the current time there is no means available to estimate the relative contribution of each factor to over all DNA damage. Several types of damage including base lesions, sugar lesions, protein and DNA crosslinks, single-strand breaks and double strand breaks are produced by free radical induced reactions (56–58).

The endogenous reactions that are likely to contribute ongoing DNA damage *in vivo* are oxidation methylation, depurination and deamination (59). Among more than 20 different products known to be formed by exposure of DNA bases to the ·OH, 8–hydroxy–2^1–deoxyguanosine (8–OHLG) is one of the major oxidized DNA bases and proposed to be an excellent marker for estimating oxidative damage to DNA (60–62). Some lesions in DNA are subjected to cellular repair processes and can be cleaved out, and the DNA is repaired *in vivo*. However, failure of repair can have serious biological consequences (63–64).

(i) DNA base damage

Reaction of ·OH and H atom with DNA bases is characterized by addition to the double bonds of these molecules to give adduct radicals of bases. An abstraction of the H atom by ·OH from the methyl group of thymine also occurs (48). In the presence of oxygen, pyrimidine radicals add oxygen to give corresponding peroxyl radicals. By contrast, evidence indicates that the majority of purine adduct radicals do not react with oxygen. Subsequent reactions of base radicals lead to a vareity of products from each of the DNA bases (48, 65, 66). When free radicals react with the sugar moiety of DNA, some sugar products and intact bases are released from DNA (67). However, the modified bases remain attached to the polynucleotide chain. These modified bases are released by hydrolysis for chemical characterization and quantitation of free radical induced products of all four DNA bases in isolated DNA as well as in isolated mammalian chromatin (68).

Fig.2 illustrates the structures of some of the free radical induced products of pyrimidines and purines that have been identified in isolated DNA, in mammalian chromatin, and in cellular DNA by the use of the gas chromatography/mass spectrometry with selected ion monitoring techniques (GC/MS–SIM). In living cells, DNA is not free but complexed with histones to form chromatin. This complex forms a number of substructures such as nucleosome, solenoid, loop-domain, miniband and chromatid which are present during the various phases of cell cycle (Nelson et al 1986) (69). Histones that are closely associated with DNA in nucleosomes may also react with free radicals, and may affect oxidation and reduction reactions of adduct radicals of DNA bases. Furthermore, DNA bases may participate in formation of DNA protein cross-links, which may also affect the types of DNA base products and their yields.

Evidence indicates the involvement of ·OH in the formation of DNA Protein Cross-

links (DPCs) induced by ionizing radiation and H_2O_2 (70, 71). Gas chromatography/ mass spectrometry with selected ion-monitoring (GC/MS–SIM) technique has been used to establish the chemical nature of large number of ·OH–induced DPCs in calf thymus nucleohistone (72, 73).

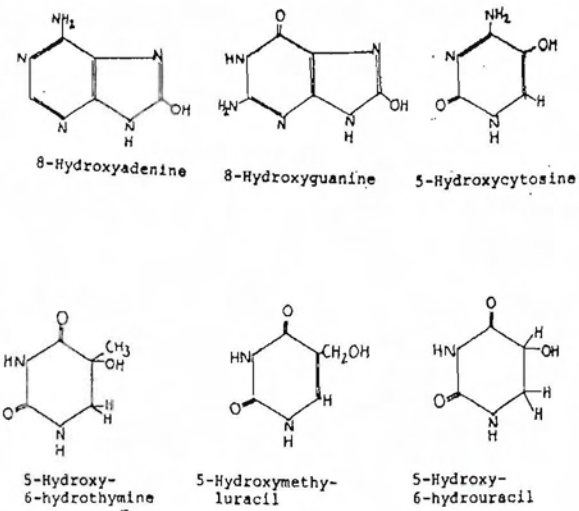

Fig. 2 : Free–radical induced modified DNA bases.

(iv) DNA–Sugar damage

A small amount of ·OH also reacts with the sugar moiety by abstracting an H atom leading to sugar radicals (48). Reactions of sugar radicals lead to the release of intact bases and result in alterations of the sugar moiety and strand breaks (74). In the DNA backbone, these altered sugar are released and some altered sugars which remain in the DNA backbone constitute the so called alkali-labile sites. By the use of GC/MS technique the ·OH induced sugar products of DNA have been identified. These include 2, 5–Dideoxypentos–4–ulose, 2, 3, dideoxypentose–4–ulose, 2–deoxypentos–4 ulose 2–deoxytetro–dialdose (74) (Fig. 3).

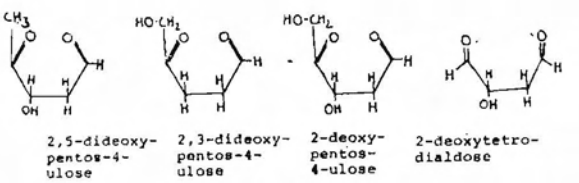

Fig. 3 : Free–radical–induced altered suger moiety in DNA

Role of metal ions in oxidative DNA damage

Data based on animal as well as epidemiologic studies provide strong evidence that transition metals are potential oxidative agents of biological macromolecules and therefore toxicities associated with these metals may be due atleast in part to oxidative tissue damage. Recent studies have shown that metals such as iron, copper, cadmium, chromium, lead, mercury, nickel and vanadium exhibit the ability to produce ROS, resulting in lipid peroxidation, DNA damage, depletion of sulfhydryls and altered calcium homeostasis (15, 75).

The two most commonly studied transition metals are the cations Fe^{2+} and Cu^{2+}. The basic mechanisms involving production of ROS are the same for transition metal ions (15, 76, 77). The mechanism by which Cu^{2+} and Fe^{2+} produce ROS via Fenton reaction is described earlier in this article (reactions 5, 18, 20). Similar mechanism involving the Fenton like production of $O_2^{\cdot -}$ and ·OH radicals appears to be true also for chromium (Cr) and vanadium (V).

$$Cr(VI) \text{ or } V(V)+O_2^{\cdot -} \rightarrow Cr(V) \text{or } V(IV)+O_2 \quad ...(21)$$

$$Cr(V)\text{or}V(IV)+H_2O_2 \rightarrow Cr(VI)\text{or}V(V)+ {}^{\cdot}OH + {}^{-}OH \quad ...(22)$$

Cadmium (Cd), Nickle (Ni) and lead (Pb) induced genotoxicity may also mediated by ROS (13, 78–80).

The DNA is likely to be a critical target for oxidative damage by the Fenton–type reaction with H_2O_2 which generates highly oxidative ·OH radicals immediately at the Cu^{1+} binding site (76, 77).

$$DNA + Cu^{1+} ========= DNA - Cu^{1+} \quad ...(23)$$

$$DNA–Cu^{1+}+H_2O_2 ---> DNA \cdot OH + Cu^{2+}+OH^- \quad ...(24)$$

Damage

A variety of endogenous reductants such as ascorbate, catechols and thiols have been shown to activate such DNA damage by copper ions and H_2O_2, and concern is growing about possible implications in various human diseases (81).

DNA Repair

DNA damage by ROS can cause multiple lesions, including single and double strand breaks, apurine/apyrimidine (AP) sites and modified purines and pyrimidines. Repair of these lesions occurs by the action of a series of enzymes (38,82). However, these enzymes do not achieve complete removal of modified bases perhaps because they operate at close to maximum capacity *in vivo* (37). DNA glycosylases exist for the repair of several DNA base lesions, including oxidized, methylated and deaminated bases. A repair system for a basic site produced by spontaneous depurination also exists. Role of poly–(ADP–ribose) polymerase(PARP) in the rejoining of DNA strand breaks, including those induced by ROS, is an area of current interest.

Occurrence of mutation and cancer caused by ROS–induced DNA damage:

It is currently beleived that there are two major mechanisms by which normal cells can be converted into malignant cells. These include the conversion of normal protooncogenes into activated oncogenes, and the inactivation of normal tumor suppresor genes. It is hypothesized that oxidants might contribute to carcinogenesis by causing oncogene activation or tumor suppressor gene inactivation (37, 38, 59, 64). Damage of DNA by ROS results in mutations which are associated with initiation and progression of cancer (83). There are several different pathways leading from initial DNA base damage by ROS to subsequent mutation. The first is the chemical modification of DNA bases causing a change in their hydrogen bonding specificity, e.g. 8–OHdG, thymine glycol and 2–hydroxy adenine (2–OHdA) (84). In addition 8–hydroxy–adenine ring opened purines and pyrimidine fragmentation products which can block replication in E.coli may be mutagenic. The contribution of oxidative damage to polymerase-specific 'hot spots' which is a likely major contributor to DNA polymerase mediated mutagenesis is a second possible mechanism (37).

A third mechanism is linked to a conformational change in the DNA template that diminishes the accuracy of replication by DNA polymerases (84).

DNA base damage could cause DNA base mispairing, that could result in point mutations and also lead to oncogene activation. Jackson (85) used K–*ras* protooncogene which is known to be activated into a transforming oncogene by point mutations (86). The studies clearly indicate that ·OH can cause point mutations and activate the K–*ras* protooncogene. ·OH induced DNA strand breaks can cause oncogene activation and it is likely that this oncogene activation could ultimately contribute to carcinogenesis (85). There exists another mechanism by which an oxidant might contribute to carcinogenesis. Studies have shown that cells containing the normal tumor suppressor gene p53 undergo DNA damage and G_1 cell cycle arrest when they are treated with gamma irradiation (87, 88). This G_1 cell cycle arrest allows the cells to repair their damaged DNA, and only after the damaged DNA is repaired, the cells resume their cell cycle. Therefore, this G_1 cell cycle arrest prevents cells containing damaged DNA from replicating their damaged DNA. Errors in replication can result in oncogene activation as well as inactivation of additional tumor suppressor genes. The accumulation of activated oncogenes and inactivated tumor suppressor genes ultimately results in carcinogenesis.

ROS–induced protein damage which is the major consequence of excess ROS generation *in vivo* can also cause mutation. It has been suggested that an alteration in the conformation of DNA polymerase could explain the frequency of close-proximity double mutations that occur secondarily to a wide range of genetic stresses (84). Oxidative protein damage could also affect the activity of DNA repair enzymes. Another possible mutagenic effect of ROS involves their attack on lipids to initiate lipid peroxidation.

Cellular reponse to DNA damage

Oxidant carcinogens interact with multiple cellular targets including membranes, proteins and nucleic acids. They cause structural damage to DNA and have the potential to mutate cancer-related genes. At the same time, oxidants activate signal transduction pathways and alter the expression of growth and differentiation-related genes. Cells adapt chemical and physical stresses by inducing expression of a variety of genes involved in growth arrest, DNA repair and defense against injury (89). One category of these genes is called DNA damage-inducible (DDI). One group of DDI genes, gadd genes, mediate growth arrest during the late G_1 phase of the cell cycle. This G_1 arrest theoretically enables the cell to undergo DNA repair before entering synthetic (S) phase. One example of gadd gene is gadd 45 (90). Increased expression of *gadd 45* depends on wild type p53 protein. Other DDI genes include early response genes, cytokines and enzymes such as collagenase (89).

Oxidants have the capacity to induce the transcription of growth competance–related protooncogenes C–*fos* and C–*jun* in several system. The induction of these immediate genes represents a prerequesite for the stimulation of cell proliferation (91).

Antioxidant intervention in oxidant DNA damage

Vitamins and related compounds provide significant protection against oxidative

damage (43, 92, 93). Evidence suggests that low molecular weight antioxidants, antioxidant enzymes, and antiinflammatory agents that inhibit arachidonic acid metabolism are anticarcinogenic (94). Changes in antioxidant defense enzymes such as SOD, GPx and CAT have been widely described in cancerous cells (91). Among nonenzymatic antioxidants epidemiological studies on serum antioxidants and diet suggest that an elevated level of vitamins E and ß–carotene reduce mortality due to cancer in the lung and colon (95–96). Main source of these antioxidants are fruits, grains and vegetables. Intake of fresh fruits and vegetables appears to be inversely correlated with several types of cancer (35, 93).

In addition to antioxidants, fruits and vegetables contain many vital micronutrients that may be protective. These include folic acid, which is required for the synthesis of DNA precursors and niacin, which is required for the NAD^+ used by PARP (poly-ADP-ribose polymerase). Among many other potentially protective substances anti-angiogenesis factors, inducers of carcinogen removing enzymes and dietary fibre are very important. However, to what extent dietary changes can decreases steady state and total body oxidative DNA damage in humans needs the attention of scientists.

The way forward

In short, it is likely that increase in steady state level of ROS is associated with DNA damage. A central theme of these proposals has been that mitochondria, acting as initiating sources of ROS, promote their own disarray and destruction (98, 99). The proximity of mitochondrial DNA to the respiratory chain, absence of protective histones, and limited DNA repair make it particularly vulnerable to oxidative damage. It is well established that the regulated expression of nuclear genes in response to environmental signals is a key mechanism for mediating changes in respiratory chain function (100). Numerous forms of oxidative DNA damage have been identified including strand breaks, intra– or inter–strand cross–links, DNA–protein crosslinks and various types of base damage. Overall studies indicate that DNA is an early target for oxidative stress which could contribute to the cascade of pathogenesis of cells including gene mutation and cancer. Although we do not understand the initiating events in cancer, it might be possible to direct therapeutic efforts at oxidative events in the pathway of carcinogenesis. Thus trials of antioxidant agents should be undertaken. It is possible that a combination of therapies that act at sequential steps in the DNA destruction process may be useful in minimizing its damage. The use of several agents together might make some compounds effective at lower dose levels than would otherwise be possible and thereby minimize adverse tissue side effects. Once effective treatment is established, future strategies will include the development of both genetic and biochemical markers which may be useful to identify patients at high risk for cancer so that one can take appropriate measures at a presymptomatic stage and hopefully prevent the disease.

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