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REVIEW

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An update on the role of free radicals and antioxidant defense in human disease

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Abstract Mounting clinical and experimental evidence indicates that free radicals play important roles in many physiological and pathological conditions. The wider application of free radical measurement has increased awareness of functional implications of radical-induced impairment of the oxidative/antioxidative balance. In the following review, the role of oxygen free radicals in some human and experimental pathological conditions is described, with particular emphasis on the mechanisms by which they produce oxidative damage to lipids, proteins, and nucleic bases. The role of free radicals and the activation of the antioxidant systems in arteriosclerosis and ageing, diabetes, ischemia/reperfusion injury, ethanol intoxication, and liver steatosis is discussed. Therapeutic approaches to the use of antioxidants have been described and prospects for clinical use have been considered.

Key words Antioxidants · Free radicals · Glutathione · Oxidized proteins · Oxidative stress · Vitamin E

Introduction

In the last few years there has been growing interest in the role played by oxidative reactions in human disease. A number of cell functions appear to be upregulated by the release of oxygen free radicals, such as DNA expression [1] and mitochondrial energy production [2]. Several experimental [3–5] and human pathological [6–8] conditions have been closely related to an overproduction of free radicals or to an impairment of the oxidative/antioxidative balance, which seems to be involved in the cell differentiation process, activation of specific metabolic pathways, and liver regeneration [9–11].

Oxygen free radicals are unstable compounds which exert their toxic effect by reacting with lipids, proteins, and nucleotides to produce oxidized compounds. Under particular conditions, some cofactors are involved in the mediation and amplification of these oxidative events. In the case of ethanol intoxication, for example, transition metals (Cu^{2+}, Fe^{2+}) are mobilized by free radicals from the cell storage pool and actively participate in the genesis of lipid and protein oxidation [12]. Other clinical conditions characterized by intracellular accumulation of iron (hemochromatosis) or copper (Wilson's disease) show pathogenic mechanisms of damage involving lipid and protein oxidation and also mitochondrial structures [13, 14]. Under similar experimental conditions, concomitant treatment with antioxidants (vitamin E) delays and lowers the extent of hepatic injury [15]. Most oxidative products are also intrinsically toxic and can cause damage to sites distant from the site of production after diffusion [16].

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Mechanisms of oxidative injury

Lipid peroxidation was the first oxidative phenomenon to be investigated. It was initially documented during drug and ethanol intoxication [17, 18]. Peroxidation of lipids occurs when a pro-oxidant compound reacts with unsaturated fatty acids of biological membranes; their oxidative modification causes changes in the physical and chemical properties of the membranes, thus altering their fluidity and permeability, with swelling of intracellular organelles and increased risk of membrane rupture. This is the mechanism responsible for the hemolysis in alcoholics [19, 20]. Modification of the lipid redox state may also affect specific properties of the membranes, such as signal transduction and ion exchange [16]. Moreover, lipid peroxides are reported to be mediators of fibroblastic cell activation in the presence of inflammation [21] and remodelling after tissue damage [22].

More recently, the protein oxidation process has been extensively investigated and has been assigned an important role in ageing [23], cataractogenesis [24], and radiation cell injury [25]. In all these conditions an increased

accumulation of carbonyl proteins (characterized by the oxidation of the carboxylic group into the carbonyl group) has been demonstrated. But, while in the case of ageing, the intracellular accumulation of carbonyl proteins seems to depend on a reduced capacity of the cell to degrade the oxidatively damaged proteins, in other conditions the accumulation of damaged proteins appears to be the consequence of an increased free radical production and/or decreased antioxidant protection. The sulfhydryl (–SH) group of the proteins, which is generally the first to be attacked and sacrificed when an increased production of pro-oxidant molecules occurs, is also involved in protein oxidation. The radical ·S-proteins react immediately with free glutathione (GSH) to form GS-S-proteins, which, in turn, are susceptible to be reconverted to the reduced form. Proteins containing an elevated number of –SH groups, namely sulfhydryl proteins (P-SH), play important roles in the cell (carriers, respiratory complexes, enzymes, histones, cytoskeleton) [26]. Thus, their oxidation may dramatically compromise cell function and survival.

The most recent to be investigated among the oxidative processes has been that of nucleic bases, and in particular of DNA. Increased levels of 8-hydroxy-deoxyguanosine have been found in aged animals [27] and in rats with chronic ethanol and iron intoxication [28]. These findings seem to have particular importance because of the association of the above conditions with DNA mutations and the high risk of cancer development. Oxidative alterations of mitochondria DNA bases and RNA bases are likely to be even more important, because of the lack of repair systems at these two levels. Damage occurring to these molecules may result in irreversible transcription or translation errors.

Arteriosclerosis and ageing

It is well known that high cholesterol levels associated with smoking and hypertension represent primary risk factors for cardiovascular disease, and that the pathogeneses of arteriosclerosis and ageing show several similarities. A major aspect is the oxidative modification of low-density lipoproteins (LDL), which promote arterial wall alterations [29]. Endothelial cells are able to oxidize LDL which successively accumulate in macrophages (foam cells) in the form of cholesterol and cholesterol esters [30]. Oxidized LDL can exert a strong chemotactic effect on monocytes and may also promote the activation of myofibroblasts [31]. Oxidized LDL may also activate circulating platelets with consequent thrombophilia effect [32]. The combination of these events can lead to partial or even complete occlusion of vessels, with a consequent dramatic reduction of blood supply to the relative tissues.

Under normal conditions, the reduced state of LDL is maintained by vitamin E [33], a circulating lipophilic compound which is also contained within the lipoprotein complex. Conditions such as smoking, diabetes, and hyperlipidemia, which alter the balance between unsaturated fatty acids and the vitamin E content within lipoproteins, expose LDL to the risk of oxidation.

A new interesting aspect of the free radical hypothesis of ageing is the oxidation of proteins. Increased levels of oxidized proteins have been detected in glial cells obtained from the brains of aged animals [34], and increased oxidation of the protein component of LDL has also been observed in the eldery [35]. The oxidation of cell and mitochondrial proteins in aged animals has been directly related to mitochondrial DNA oxidation and inversely related to the cell antioxidant capacity [36]. These observations clearly support the hypothesis of free radical-mediated intracellular oxidative damage in the ageing process. Furthermore, the increased oxidative damage of DNA bases observed in several cell lines derived from old people may correlate with the increased risk of malignancies in the elderly.

Finally, diet seems to play an important role in the protection against oxidative alterations. A Mediterranean diet, and especially its component olive oil, which contains large amounts of vitamins and antioxidant substances in an appropriate ratio with unsaturated fatty acids, has been shown to be protective against coronary heart disease [37], mainly by lowering the level of circulating oxidized LDL. Based on these observations, supplementation of diet with vitamin E and antioxidants may represent a preventive approach to degenerative diseases.

Diabetes

Diabetes mellitus represents a typical chronic degenerative disease associated with an early onset of atherosclerotic alterations. The high incidence of arteriosclerosis in diabetics has been associated with increased intracellular oxidative stress: an increased accumulation of protein and lipid oxidative products has been noted in the tissues of diabetic subjects [38, 39]. This is related to the impaired antioxidant capacity of both serum and cells of diabetic patients [40, 41]. This imbalance is thought to be involved in the genesis of diabetic complications [42], and originates from impairment of the pentose phosphate pathway, which is due to the deficiency of insulin. This metabolic impairment results in a decreased availability of reduced substrates, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), and consequently of GSH, because of the decreased oxidized glutathione (GSSG) reductase activity, which utilizes NADPH [43]. The consequence is an increased susceptibility to oxidation of proteins and membrane lipids. All these events are of particular importance to blood vessels, where diabetics show a pattern of alterations very similar to those described in arteriosclerotic patients and the eldery [44].

The eye is also affected in diabetic subjects [45]. It is hypothesized that the retina, which is very rich in polyunsaturated fatty acids, is repeatedly exposed to hypoxic

conditions and produces a large amount of lipid peroxides [46]. These, in turn, are able to migrate and extend the damage to protein structures, thus producing alterations of the protein redox status, with depletion of –SH groups and accumulation of carbonyl derivatives which are less soluble and consequently precipitate. In this way the damage extends from the retina to other ocular compartments, such as the vitreous humor and the lens, with the formation of opacities and cataract [47]. Based on recent investigations performed both in humans and animals by our group and others [46–48], it has been noted that the oxidative eye alterations in diabetic subjects are preceded by depletion of antioxidant agents, in particular GSH, vitamin E, and ascorbic acid. The antioxidant depletion and the oxidative alterations are related to the age of onset of diabetes and to the degree of glycometabolic control. Moreover, the presence of proliferative retinopathy seems to increase the ocular oxidative damage, as evidenced by measurements performed in the subretinal fluid from diabetic patients with retinal detachment in the presence or absence of proliferative retinopathy [49]. All these structural modifications seem to be consequential to the original oxidative alteration of the retina.

Other ocular diseases also show an impairment of the redox balance. Senile cataract, for example, is formed as a consequence of the accumulation of oxidative products, thus resembling myopic cataract [50]. Uveitis and episcleritis are typical inflammatory processes in which activated macrophages release oxygen free radicals with consequent oxidative damage to ocular structures [51].

Experimental treatment of the oxidative eye complications in diabetic patients by systemic administration or local application of antioxidants, such as GSH and vitamin E, did not produce appreciable results, probably because of the difficulties in reaching the inner eye compartments. However, the essential role of GSH in the maintenance of lens transparency is confirmed by the efficacy of GSH ester in the prevention of cataract in GSH-deprived new born mice [52], even if, at present, the best means of treating or avoiding the onset of diabetic oxidative eye complications in humans remains good metabolic control.

Ischemia/reperfusion

Reperfusion injury of post-ischemic tissue is the classic model for investigating the mechanisms of free radical-induced oxidative cell damage [53]. Ischemia and reperfusion occur when the blood supply to an organ, or part of it, is suddenly interrupted due to an intrinsic (thrombotic occlusion) or extrinsic (surgical clamp) cause. Every tissue may theoretically be subjected to ischemia and reperfusion injury and the pathophysiological mechanisms of cell damage are substantially identical. Because of the social impact of coronary heart disease, myocardial ischemia was the first condition to be investigated and represents a typical clinical example. Reperfusion injury of the liver, which occurs during major surgery of this organ or after liver transplantation, is another. However, it shows some pathogenic differences compared with the heart.

It is well known that during the anoxic period of ischemia important metabolic changes occur in the cell. Firstly, anaerobiosis lowers the intracellular pH (acidosis) which, if particularly prolonged, may favor irreversible damage during reperfusion [54]. The longer the period of ischemia, the more serious the damage during reperfusion. Reperfusion is characterized by the release of molecules (i.e., nitric oxide), probably by endothelial cells [55], which are responsible for the activation of pro-inflammatory cells, which subsequently deliver cytokines (interleukins, tumor necrosis factor, etc.) and adhesion molecules (intercellular adhesion molecule-1) to the vascular compartment [56]. These cytokines are involved in the propagation of inflammation and are directly responsible for cell damage. During the early phase of reperfusion after warm ischemia, endothelial cells of the hepatic sinusoids release factors which activate the resident macrophages (Kupffer cells) which, when activated, are able to produce an enormous amount of oxygen free radicals [57]. At this time conspicuous amounts of GSH are oxidized and consumed in the vascular compartment to conteract free radical attack [58]. The intracellular GSH is consumed because it is used as a source of extracellular GSH [59]. After a few hours (second or late phase), circulating neutrophils are attracted and activated with consequent release of toxic and necrotizing factors [60]. Most of these events have also been described when livers are subjected to cold ischemia and subsequent reperfusion, as in the case of organ storage for transplantation [61].

Alcohol

Ethanol intoxication is one of the most widely investigated pathologies characterized by increased free radical production and oxidative alterations. Ethanol is an exogenous substance which is mainly metabolized in the liver by a cytosolic dehydrogenase and a microsomal enzymatic complex (P-450) [62]. These metabolic pathways transform ethanol into acetaldehyde, which is responsible for the formation of acetaldehyde-protein adducts [63] and for the main toxic effects attributed to ethanol [64]. Within hepatic cells, during ethanol metabolism, ethyl radicals [65] are also released, with the promotion of oxidative damage and GSH consumption [66].

There are several ways in which ethanol and its metabolic products damage the cell. A major one is the interference with some metabolic pathways (i.e., transulfuration) and the competition with other exogenous and endogenous compounds for the P-450 metabolic site, with enhancement of the toxic effects and production of metabolic disturbances and cell alterations. One of these is the fat infiltration of hepatocytes which results in increased lipid peroxidation because of the relative decrease in the antioxidant protection [67]. Acetaldehyde

interferes with some mitochondrial functions, thus reducing the capacity for respiration and the production of reduced substrates (reduced nicotinamide adenine dinucleotide, NADH) and energy compounds (ATP) [68]. Mitochondria are selective targets of ethanol toxicity and are subjected to swelling with loss of cristae [69]. Recently, in both chronic and acute ethanol intoxication in the rat, a significant increase of protein and DNA oxidation has been demonstrated [28]. The former is certainly mediated by the Fenton reaction in which a metabolic activation of the iron storage pool represents a crucial mechanism for the amplification of the intracellular free radical damage [12]. The latter may be responsible for the increased risk of DNA mutation and cancer observed in alcoholics [70]. Peroxidative products of lipids have recently been indicated as promoters of Kupffer and Ito cell activation during chronic ethanol consumption, with consequent stimulation of hepatic fibrosis [21, 22]. Thus, interventions directed at conteracting the increased lipid peroxidation may also represent a protective measure against the onset of liver fibrosis.

Other areas are oxidatively damaged by ethanol. The stomach is the first organ to come into contact with ethanol after ingestion. The activity of alcohol dehydrogenase (ADH) in the stomach, an enzyme which is present in other tissues (pancreas, testis, brain) exposes these tissues to oxidative damage [71]. However, since the ADH content is lower in these organs than liver, this should expose them to less oxidative injury. The capacity to produce fatty acid ethyl esters by some kind of cells (pancreas, heart) represents an additional way by which ethanol induces cell injury [72]. Finally, ethanol also exerts its toxicity by stimulating the conversion of hypoxanthine to xanthine oxidase which, in turn, is able to release free radicals into the vasculature with consequent oxidative damage to circulating lipids and proteins [20]. The oxidative damage to erythrocyte membranes, where acetaldehyde-protein adducts have also been detected [73], results in a loss of fluidity and an increased risk of hemolysis.

Liver steatosis

Steatosis of the liver is considered an innocent and fully reversible condition [74] characterized by intracellular accumulation of triglycerides in vesicles. Causes of liver steatosis include metabolic disorders, hypothyroidism, alcoholism, drug intoxication, and viral infections. The recent observation that transplantation of fatty liver is associated with a high degree of primary non-function, caused by an increased susceptibility to ischemia/reperfusion injury [75], has prompted a series of studies to investigate the pathological mechanisms of reduced cell resistance to ischemic insult and to verify the hepatocyte antioxidant capacity.

By the use of animal models (choline-deficient diet, choline/methionine-deficient diet, drug or ethanol intoxication), it has been shown that steatosis of the liver is characterized by an increased peroxidation of unsaturated lipids, regardless of the etiology of fat accumulation, with the formation of diffusible products. Fatty livers are reported to have small amounts of antioxidant compounds, in particular vitamin E, ascorbic acid, and GSH, which could render cell structures more susceptible to free radical attack.

Of even greater importance seems the observation that mitochondria isolated from fat-infiltrating hepatocytes show impaired energy production, serious depletion of antioxidants, protein oxidation, and morphological changes such as mitochondrial swelling. These morphological/functional alterations of mitochondria are exaggerated by fasting [76], which per se represents a pro-oxidant condition because the cell is starved of metabolic nutrients. Thus, fatty livers from fasted rats have smaller amounts of ATP and are further damaged by ischemia/reperfusion than normal livers from fasted animals. Pre-treatment with infusions of glucose seems to attenuate this oxidative damage, probably by replenishing the cellular glycogen, which is also decreased in fasted rats.

Therapeutic approaches with antioxidants

Several lines of evidence suggest that free radical scavengers may represent a preventive measure or a cure for a large number of human conditions. Free radical scavengers may be used in all pathological conditions characterized by an increased formation of pro-oxidant compounds and/or by depletion of antioxidative capacity. The former typically occurs during ischemia/reperfusion, the latter in prolonged food deprivation. Moreover, high-risk situations occur when both mechanisms operate, such as when paracetamol overdose follows alcohol intoxication [77], in which paracetamol metabolites find hepatic cells depleted of sulfhydryl groups, which have previously been consumed by ethanol free radicals.

In most of the cases reported above, when associated with standard treatments, replenishment of antioxidant reserve may represent an important strategy to conteract early and late complications of diabetes and arteriosclerosis, as well as to delay the onset of degenerative phenomena which accompany ageing and cancer. However, despite the large number of compounds showing antioxidant properties in vitro, only a few may be of interest for humans. An antioxidant to be used in man should fulfil the following criteria: (1) it should be a biological compound naturally present in animal tissues; (2) it should be active in the protection of both lipid and protein molecules; (3) it should have good bioavailability after oral and parenteral administration; (4) it should have a long half-life; (5) it should act in both the extracellular and intracellular spaces; (6) it should be able to cross intact cell membranes; (7) it shouldn't have a high cost.

Antioxidant substances which satisfy all these criteria are few. Vitamin E is a lipid-soluble molecule contained

in natural foods (olive oil, fish,...), however it does not act directly in the protection of proteins, and no studies have been carried out with parenteral administration. Vitamin E shows a certain efficacy when administered per os in supplemented diets [15], and is reported to have some modulating activity on the immune system. Exogenous GSH shows a poor absorption after oral administration [78], has a very short half life (approximately 2 min), and is not able to cross intact cell membranes. Superoxide dismutase is active against oxygen free radicals (hydrogen peroxide), but not against free radicals generated during xenobiotic metabolism, and has never been tested in human with pharmacokinetic evaluations. Some other compounds are very costly or are not biological substances. In order to improve the characteristics of the most-interesting antioxidants, they have been conjugated to carriers or administered in the form of prodrug. Some have been used under experimental conditions: *N*-acetylcysteine (NAC), glutathione mono- (GSHE) and diethylester, γ-glutamylcysteinylethylester, S-acetyl and S-phenylacetyl-GSH.

Substrate molecules which may increase the synthesis of antioxidants have also been used. S-Adenosylmethionine, which is transformed within the cell in methionine, the amino acid precursor of cysteine, has been widely studied in many conditions of GSH depletion [79, 80]. NAC, which delivers free cysteine within the cell, is the universal antidote against paracetamol overdose [81].

Cysteine availability is the limiting step in the ex novo synthesis of GSH; however, administration of cysteine itself is not recommended, because it is rapidly transformed to cystine which is toxic and follows alternative routes. To overcome the block of GSH synthesis in conditions of poor ATP availability, GSH esters have been tested with promising results. GSHE has been reported to be a slow-release form of extracellular GSH under normal conditions [82]. Little is known about the ability of GSHE to cross membranes in conditions of oxidative stress, even if a protective effect of GSHE has been observed in livers subjected to ischemia/reperfusion and in acute pancreatitis [83, 84]. Diethyl esters of GSH disappear very soon from the circulation and release two molecules of ethanol per molecule of GSH, which shouldn't guarantee safety [85]. Hence at present, a cocktail of antioxidants, such as vitamin E, GSHE, and vitamin C, is the most-reasonable solution to the prevention or treatment of oxidative stress.

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