Free Radicals and Antioxidants for Non-Experts

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Abstract

Oxidative stress is a phenomenon associated with the pathology of several diseases including atherosclerosis, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, cancer, diabetes mellitus, inflammatory diseases, as well as psychiatric disorders or aging process. Oxidative stress is defined as an imbalance between the production of free radicals and reactive metabolites, so called oxidants, and their elimination by protective mechanisms named antioxidative systems. Free radicals and their metabolites prevail over antioxidants. This imbalance leads to damage of important biomolecules and organs with plausible impact on the whole organism.

Oxidative and antioxidative processes are associated with electron transfer influencing the redox state of cells and organisms; therefore, oxidative stress is also known as redox stress.

At present, the opinion that oxidative stress is not always harmful has been accepted. Depending on its intensity, it can play a role in regulation of other important processes through modulation of signal pathways, influencing synthesis of antioxidant enzymes, repair processes, inflammation, apoptosis and cell proliferation, and thus process of a malignity. Therefore, improper administration of antioxidants can potentially negatively impact biological systems.

Keywords

Antioxidants • Oxidative stress • Reactive metabolites • Redox stress • Signaling

Introduction

On the one hand, free radicals are linked with the emergence of life on our planet, and on the other hand, they are considered destructive substances which are effective in the extinction of living organisms. Life originated spontaneously about 3.5 million years ago from amino acids, nucleotides, and other basic chemicals, which were created from simple substances present in the primitive atmosphere (Rutten 1971). Free radicals participated in these reactions. Their generation was initiated mainly by ionizing radiation from the sun. Ionizing radiation gave rise to free radicals and thus to the primitive molecules needed

as a food for survival and growth of the first protocells. On the other hand, this radiation was the cause of more or less random changes in protocells caused by free radicals. Some changes were useful for the protocells, and some were heritable and caused destruction of cells. Heritable changes caused the death of cells. Therefore, these changes were useful in terms of evolution. Dead cells were considered no competition for their offsprings in the race for nutrients. By such, a dual role of the free radicals took part in the early evolution of life on our planet (Freeman 1984). Life in the oxygen atmosphere when organisms started to develop the respiratory chain and other enzymatic systems utilizing oxygen required a parallel formation of protective systems for elimination of toxic effects of oxygen (Ďuračková 1998).

In the past, free radicals were not taken into account, either because there were doubts about their existence in a biological system or simply because they were considered to be an insignificant curiosity. Despite the fact that the issue of free radicals has been paid little attention in the basic textbooks of biochemistry or physiology, today their existence and importance is widely accepted. Knowledge of the effects of free radicals in biological systems began to develop rapidly in the 1960s and has been launched especially after the discovery of the enzyme superoxide dismutase (SOD) (McCord and Fridovich 1969). There is little doubt these days about free radical formation in the environment and this extends to their participation in several diseases in humans.

Free Radicals and Reactive Metabolites

Free radicals (*FR*) are atoms, molecules, or fragments of atom and molecules with one or more unpaired electrons, capable of short independent existence. They are either electroneutral or they have an anionic or cationic character. The simplest radical is a hydrogen atom with only one unpaired electron. In addition, we recognize free radicals derived from oxygen, nitrogen, or various organic compounds. Also ions of transition metals have a radical nature (Cu^{2+}, Fe^{2+}, and others).

Free radicals are mostly very reactive substances which can pair their unpaired electron with an electron taken from other compounds, causing their oxidation, and they become reduced. Therefore, they are called *oxidants*. The presence of an unpaired electron gives the radical another chemical possibility—the radical can get rid of the free electron by its transmission to another molecule (the radical becomes oxidized and electron acceptor becomes reduced). During such an electron movement, new radicals are formed from originally nonradical molecules and *chain reactions* may occur.

Of course, some concerns about free radicals and the underestimation of their role persist even today, probably due to difficulties in their tangible image and due to the short lifetime of most of them. The majority of free radicals have a very short half life, which means that it is very difficult, sometimes even impossible, to synthesize them, store them, prepare solutions at known

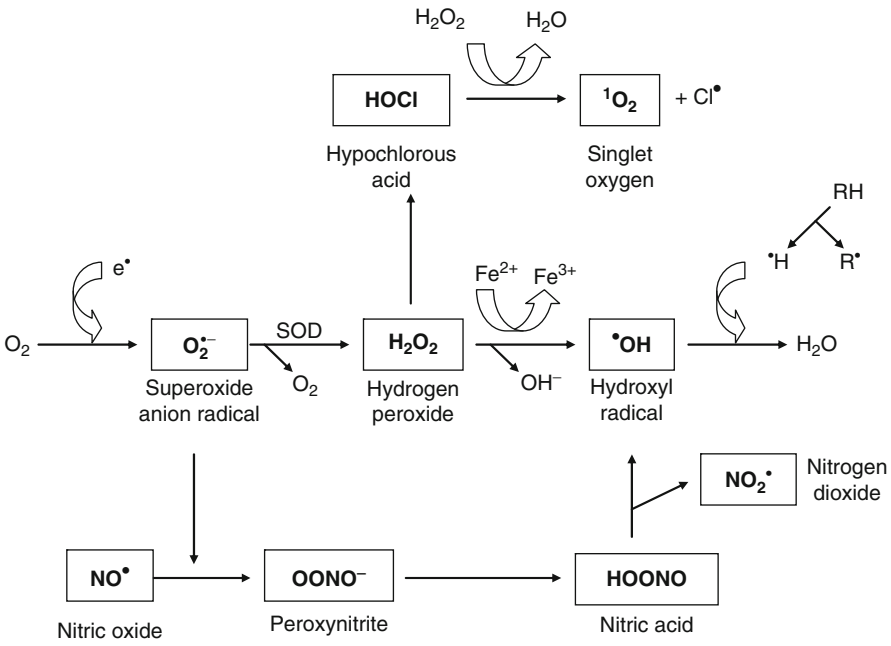


Fig. 1.1 Mutual associations between free radicals and their reactive metabolites

concentrations, use them in experimental or clinical practice, and quantify them. Anyone interested in free radicals, or trying to understand their participation in biochemical, physiological, and pathophysiological processes, should appreciate the specificity of free radicals and should expect continuing challenges with their mechanistic studies.

Nevertheless, free radicals are an area of intense study, not only for physicists, chemists, physiologists, biochemists, and pharmacologists but also for various clinical disciplines. In the medical field, they play a role mainly in the processes of aging, cancerogenesis, cardiovascular, various metabolic, neurodegenerative and endocrine diseases, in addition to their roles in several immune and autoimmune mechanisms. This realization of their roles in disease processes caused laboratory and clinical scientists to better understand the detailed chemistry of free radical induced damage, and to use this knowledge to design strategies to better protect the organism against pathophysiological consequences brought about by free radicals.

Free radicals participate in a large number of subsequent reactions (Fig. 1.1) in which other very reactive metabolites are formed. They are derived from basic radical molecules such as superoxide anion radical, abbreviated as superoxide $O_2^{\bullet -}$ or nitric oxide (nitroxide) $NO^{\bullet}$. Newly formed metabolites have a great oxidative ability and they are often more reactive than their maternal molecules. To such metabolites belong, for example, the most reactive hydroxyl radical, $HO^{\cdot}$, or

nonradical molecules such as hydrogen peroxide, singlet oxygen, peroxynitrite, or hypochlorous acid.

Free radicals and their reactive metabolites (RM) are designated collectively as "reactive species" (RS) (Ďuračková 2010).

Radicals Derived From Oxygen

Superoxide Anion Radical

The product of one-electron reduction of oxygen is the superoxide anion radical, superoxide in short, $O_2^{\bullet -}$, which is less reactive than, for example, hydroxyl radical $HO^{\bullet}$. Superoxide does not react with most biological molecules in aqueous solutions. Its reactivity with nonradical molecules is pH dependent.

Superoxide as a base can form its conjugate acid according to the reaction

$$O_2^{\bullet -} + H^+ \leftrightarrow HO_2^{\bullet} \quad pK = 4.8 \tag{1.1}$$

producing more powerful reductant, hydroperoxyl radical. The uncharged character of $HO_2^{\bullet}$ allows it to cross membranes and potentially cause damage in contrast to negatively charged superoxide. However, at the physiological pH in tissues (pH = 6.8), the ratio $\left[O_2^{\bullet -}\right]/[HO_2^{\bullet}] = 100/1$; therefore, the reactivity of $HO_2^{\bullet}$ is not as important.

Superoxide can cause damage to biologically important molecules by oxidative or reductive effects. Superoxide is a stronger reductant than oxidant (Gutteridge and Halliwell 2010). As a reducing agent, the superoxide reacts, for example, with cytochrome c and reduces Fe^{3+} to Fe^{2+}. The reducing properties of superoxide are also used in reactions with some tetrazolium dyes (NBT – nitro blue tetrazolium, INT – tetrazolium violet) for quantitative determination of superoxide in aqueous environments. As an oxidant, the superoxide can oxidize, for example, transition metal ions (Cu^{3+}, Fe^{4+}, Mn^{3+}), which are strong oxidizing agents able to oxidatively damage biomolecules or can oxidize dopamine, adrenaline, or ascorbate, leading to the formation of hydrogen peroxide.

However, the toxicity of superoxide is mainly ascribed to the formation of other reactive metabolites, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen. Superoxide is considered as the trigger of toxic reactions of oxygen. It can react with some other radicals, for example, with $NO^{\cdot}$, leading to the formation of more toxic nonradical molecule $OONO^-$.

Hydroxyl Radical

Hydroxyl radical can be generated by the reaction of hydrogen peroxide, H_2O_2, with metal ions (*Fenton reaction*) (Fenton 1894),

$$Fe^{2+} + H_2O_2 \rightarrow \text{ intermediate oxidizing species} \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \quad (1.2)$$

or by homolytical cleavage of hydrogen peroxide catalyzed by UV radiation

$$H - O - O - H \xrightarrow{UV} 2\,HO^{\bullet} \quad (1.3)$$

The reaction 1.3 might occur in skin exposed to sunlight. However, the course of the reaction 1.2 (*Fenton reaction*) is questionable in in vivo systems. Free (non-chelated) ion of metal (Fe^{2+}) does not exist due to the presence of many chelators in biological systems. The ionic form of iron can be chelated by different ligands (citrate, oxalate, ATP, phosphate) and its reactivity is dependent on the type of chelator, pH, concentration of metal ion, and H_2O_2, whereas the rate of hydroxyl radical formation may be inhibited or stimulated.

In addition to this main reaction, some other sources of hydroxyl radical formation can also be involved, for example, in reactions of hypochlorous acid (reaction 1.4), quinones (Q), and semiquinones ($SQ^{\bullet -}$) (reaction 1.5) or controversial ultrasonication of aqueous solutions (Riesz et al. 1990):

$$HOCl + O_2^{\bullet -} \rightarrow O_2 + Cl^{-} + HO^{\bullet} \quad (1.4)$$

$$SQ^{\bullet -} + H_2O_2 \xrightarrow{Fe^{3+}/Fe^{2+}} Q + HO^{\bullet} + OH^{-} \quad (1.5)$$

Reactions of $HO^{\bullet}$ with biomolecules are of three main types: hydrogen abstraction, addition, and electron transfer, leading to the formation of different new radicals (L – the lipid residue, Ph – phenyl):

$$LH + {}^{\bullet}OH \rightarrow L^{\bullet} + H_2O \quad (1.6)$$

$$Ph - {}^{\bullet}OH \rightarrow Ph - OH^{\bullet} \quad (1.7)$$

$$Cl^{-} + {}^{\bullet}OH \rightarrow Cl^{\bullet} + OH^{\bullet -} \quad (1.8)$$

Carbonate Radicals

Carbonate radicals (Augusto et al. 2002) are formed during the reaction of carbonate or bicarbonate with hydroxyl radical

$$CO_3^{2-} + {}^{\bullet}OH \rightarrow CO_3^{\bullet -} + OH^{-} \quad (1.9)$$

$$HCO_3^{-} + {}^{\bullet}OH \rightarrow CO_3^{\bullet -} + H_2O \quad (1.10)$$

Although the rate of the reaction 1.10 is lower than other reactions of $^{\bullet}OH$, it is important due to the high concentration of bicarbonate in blood (25 mmol/l). Carbonate radical reacts selectively at moderate rates with nonradicals but reacts readily with other radicals. For example, carbonate radical ($CO_3^{\bullet-}$) and nitrogen dioxide radical ($NO_2^{\bullet}$) constitute an efficient nitrating system that is pivotal to biological nitration of protein tyrosine residues. These reactions establish "cross talk" between reactive oxygen and reactive nitrogen species (Pryor et al. 2006).

The carbonate radical is a relatively strong one-electron oxidant, which can oxidize important biomolecules, such as hyaluronic acid, by abstraction of hydrogen from cysteine or tyrosine residues.

Alkoxyl and Peroxyl Radicals

Alkoxyl and peroxyl radicals ($RO^{\bullet}$, $ROO^{\bullet}$) are radicals related to peroxidation of fatty acids in lipids. $ROO^{\bullet}$ is formed from carbon-centered radicals in the presence of oxygen. Both organic radicals can be formed during decomposition of lipoperoxides (ROOH) by heating and UV radiation and at the presence of transition metal ions.

$$ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + Fe^{2+} + H^{+} \tag{1.11}$$

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + Fe^{3+} + OH^{-} \tag{1.12}$$

Organic peroxides can be also decomposed by protonated superoxide to peroxyl radical and hydrogen peroxide according to the reaction

$$HO_2^{\bullet} + ROOH \rightarrow ROO^{\bullet} + H_2O_2 \tag{1.13}$$

Both $ROO^{\bullet}$ and $RO^{\bullet}$ are relatively good oxidants with high electrochemical potential (higher positive potential of redox pair means better oxidant – stronger oxidant; more negative potential of redox pair means better reductant – better antioxidant) (Ďuračková 1998).

Alkoxyl and alkoperoxyl radicals can abstract H atom from other molecules (e.g., another fatty acid), leading to branching of lipoperoxidation. Other mutual reactions of FR can also proceed to generate new reactive species (e.g., singlet oxygen).

Sulfur and Nitrogen Radicals

On the one hand, thiols are compounds with significant biological properties in a living system. For example, reduced glutathione plays an important antioxidant role; others, for example, hydrogen sulfide (H–S–H), act as signaling molecules in vivo (Wang 2003). However, on the other hand, thiols can form free radicals and reactive species.

The most studied radical derived from sulfur is thiyl radical formed from thiol and carbon-centered radical according to the general reaction

$$\text{R-SH} + \begin{array}{c}|\\ -\text{C}^{\bullet}\\ |\end{array} \rightarrow \begin{array}{c}|\\ -\text{CH}\\ |\end{array} + \text{RS}^{\bullet} \qquad (1.14)$$

or also by other oxygen radicals such as $^{\bullet}OH$, $CO_3^{\bullet-}$, $RO^{\bullet}$, $ROO^{\bullet}$, and, very slowly. $O_2^{\bullet-}$ (Jones et al. 2002).

$RS^{\bullet}$ radical can react with oxygen, generating an unstable thiyl peroxyl radical $RSOO^{\bullet}$, or can be reduced with $NO^{\bullet}$, resulting in nitrosothiol RSNO formation. The cytotoxic thiyl and oxysulfur reactive species ($GS^{\bullet}$, $GSO^{\bullet}$, $GSOO^{\bullet}$, GSOH, GSO_3H) can be formed as end products of glutathione oxidation (it is present in cells at millimolar concentration) by ROS.

$GS^{\bullet}$ radical can be regenerated by ascorbate (AH^-), producing reduced glutathione and ascorbyl radical ($A^{\bullet-}$):

$$GS^{\bullet} + AH^- \rightarrow GSH + A^{\bullet-} \qquad (1.15)$$

Nevertheless, understanding the chemistry of sulfur radicals in vivo and its relation to different pathological states requires further extensive investigation.

Nitric Oxide

Nitric oxide ($NO^{\bullet}$, nitrogen monoxide) is a colorless gas moderately soluble in water. It has one unpaired electron and is a free radical. The lifetime of $NO^{\bullet}$ is dependent on its radical character as well as on the oxygen concentration. In an environment rich in oxygen, its half-life is less than 0.86 s (Czapski and Goldstein 1995); at the concentration of $NO^{\bullet}$ in 1 μmol/l, the half-life of $NO^{\bullet}$ is several minutes, and at physiological concentrations in vivo (1–10 nmol/l), its half-life would be considerably longer (hours) (Halliwell and Gutteridge 2007).

Nitric oxide is synthesized in vivo from L-arginine and oxygen. This reaction is catalyzed in animals by the nitric oxide synthase (NOS) which contains four cofactors – FMN, FAD, tetrahydrobiopterin, and heme, and where NADPH serves as an electron donor. There are three types of NOS. Two are constitutive and Ca^{2+}-dependent enzymes (requiring calmodulin). Depending on their location, we recognize *neuronal NOS* (nNOS, NOS1) and *endothelial NOS* (eNOS, NOS3). Neuronal NOS was first identified in nervous tissue, but later also in "working" skeletal muscles, where $NO^{\bullet}$ participates in the regulation of contractions. eNOS in endothelial cells is important for relaxation and vasodilatation processes as well as for regulation of blood pressure. The third type of NOS – *inducible NOS* (iNOS, NOS2) – is Ca^{2+}-independent, and is formed at the sites of inflammation and catalyzes the formation of high concentration of $NO^{\bullet}$ (more than μmol/l) (Ďuračková 1998). Increased concentrations of $NO^{\bullet}$ have been detected in various diseases (for example, see chapters 9, 10, 34, 35, 36, 145, 146).

Nitric oxide reacts with oxygen in aqueous solutions to form nitrite NO_2^-

$$4\ NO^{\bullet} + O_2 + 2H_2O \leftrightarrow 4H^+ + 4\ NO_2^- \tag{1.16}$$

This reaction is used for spectrophotometrical determination of nitric oxide.

$NO^{\bullet}$ generated in the vascular system can be quenched by interaction with hemoglobin, where it forms complex with Fe^{2+} ion of heme. This reaction is called *nitrosylation*. After reaction with oxyhemoglobin, toxic methemoglobin (Hb-Fe^{3+}) and nitrite are formed (Halliwell and Gutteridge 2007). However, this reaction is limited by the rate of $NO^{\bullet}$ entry into erythrocytes (the half-life of $NO^{\bullet}$ in whole blood is about 1.8 ms) (Kim-Shapiro 2004). However, under hypoxic conditions, deoxyhemoglobin can exhibit nitrite reductase activity when it reduces nitrite NO_2^- to $NO^{\bullet}$. By using this pathway, $NO^{\bullet}$ is released as a gas where it can participate in hypoxic vasodilation (Huang et al. 2005).

In recognition of its extensive and important physiological roles, the journal Science named nitric oxide as the "molecule of the year" in 1992. Four years later, the Nobel Prize in Medicine was awarded to R. F. Furchgott, L. J. Ignarro and F. Murad for the unravelling signaling properties of nitric oxide.

The most important reactions of $NO^{\bullet}$ include, for example in the vascular system, activation of guanylate cyclase to form cyclic GMP which reduces intracellular free Ca^{2+} concentrations and smooth muscle relaxation and lowering of blood pressure. Nitric oxide also inhibits platelet aggregation. In the nervous system, $NO^{\bullet}$ plays a role in response to excitatory amino acids (glutamate), neuromodulation, and synaptic plasticity influencing long-term memory. Other roles of $NO^{\bullet}$ are in penile erection bladder control, lung vasodilatation, gastrointestinal function, etc.

$NO^{\bullet}$ molecule can also be regarded *as a free radical scavenger*.

$NO^{\bullet}$ can quickly react with hydroxyl, peroxyl, or thiyl radicals according to the reactions

$$NO^{\bullet} + HO^{\bullet} \rightarrow HNO_2 \tag{1.17}$$

$$NO^{\bullet} + ROO^{\bullet} \rightarrow ROONO \tag{1.18}$$

$$NO^{\bullet} + RS^{\bullet} \rightarrow RSNO \tag{1.19}$$

when nitric acid (Eq. 1.17), nitrosoperoxyl (Eq. 1.18), or nitrosothiol (Eq. 1.19) can be formed and reaction 1.18 can be responsible for inhibition of lipoperoxidation by $NO^{\bullet}$.

However, at high concentrations (>μmol/l), $NO^{\bullet}$ can have deleterious effects. It can be involved in the pathology of septic shock, chronic inflammation, increased cancer risk, transplant rejection, asthma, epilepsy, and in excitotoxity in neurodegenerative diseases (Halliwell and Gutteridge 2007).

An important reaction is the reaction of $NO^{\bullet}$ with superoxide to form the nonradical molecule *peroxynitrite* (*oxoperoxonitrate*):

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^{-} \quad (1.20)$$

This reaction is faster than the reaction of superoxide with SOD or nitric oxide with heme compounds ($NO^{\bullet}$ forms complex with ferrous compounds through nitrosylation, e.g., with deoxyhemoglobin). It indicates that $NO^{\bullet}$ and $O_2^{\bullet -}$ antagonize each other's physiological functions.

Peroxynitrite formation is important from a toxicological point of view. Peroxynitrite itself is relatively little reactive. However, $ONOO^{-}$ can be protonated by glutathione or other antioxidants which could lead to exhaustion of antioxidants. $ONOO^{-}$ may diffuse to more distant places in the cell where lipids, proteins, or DNA can be oxidized and nitrated.

Nitration of tyrosine units in proteins is generally used as a biomarker of peroxynitrite formation, although some other residues of amino acids can be modified by $ONOO^{-}$ too.

Reactive Metabolites

Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a two-electron reductive product of oxygen. It has no unpaired electrons and is not a radical in nature. H_2O_2 is only a weak oxidizing or reducing agent able to participate in signal transduction. For example, protein kinases are involved in cell proliferation, differentiation, and response to stress. The alteration of their activity by RS (H_2O_2, $ONOO^{-}$, HOCl), for example, due to oxidation of –SH groups (more sensitive to oxidation is thiolate – S^{-}) can lead to modulation of redox-dependent transcription factor activities and gene expression. All classes of protein kinases can be activated by RS dependent on cell type, level of RS, and time of action, resulting in proliferation, cytoprotection, cell survival, or apoptosis and thus influencing fate of cells.

H_2O_2 is produced in probably all tissues, for example at steady-state concentrations of 10–100 nmol/l in the liver or 5 nmol/l in mitochondria (Boveris and Cadenas 2000). However, there may be some variations between tissues. In addition to the dismutation reaction of superoxide catalyzed by SOD, hydrogen peroxide can also be produced by other enzymes, such as xanthine, glucose monoamine, and D-amino acid oxidases (Mahns et al. 2003) or during ascorbate and polyphenol oxidation.

Hydrogen peroxide at μmolar levels is toxic to many cells. It can directly damage some enzymes such as glyceraldehyde-3-phosphate dehydrogenase (G3PDH) through oxidation of –SH group (Brodie and Reed 1987).

H_2O_2 is soluble in water and can diffuse within and between cells. It can accumulate there and react with ions of iron or copper at a different oxidative state and can damage biomolecules such as lipids, DNA, and proteins through secondarily formed hydroxyl radical from H_2O_2 and transition metal ions.

Singlet Oxygen

To nonradical oxygen species belongs singlet oxygen, $^{\mathbf{1}}\mathbf{O_2}$, which is a molecule of oxygen where one of two unpaired electrons is changed from the ground state to the exciting state. Singlet oxygen can then transfer the energy to a new molecule and act as a catalyst for free radical formation.$^1O_2$ can exist in two states (Ďuračková 1998). Sigma singlet oxygen ($^1\Sigma g^+O_2$) has two unpaired electrons in two orbitals and is not stable. It rapidly decays to the more stable delta state ($^1\Delta gO_2$), which has both of the originally unpaired electrons in the same orbital. In a biological system, only delta $^1O_2$ can be detected, although both states can be formed. A further discussion of the single oxygen delta species follows below.

The biochemical production of singlet oxygen has been proposed to contribute to the destructive effects seen in a number of biological processes. Several models of biochemical systems have been shown to produce singlet oxygen. These systems include the peroxidase-catalyzed oxidations of halide ions, the peroxidase-catalyzed oxidations of indole-3-acetic acid, the lipoxygenase-catalyzed oxidation of unsaturated long chain fatty acids, and the bleomycin-catalyzed decomposition of hydroperoxides. Results from these model systems should not be uncritically extrapolated to living systems. The human eosinophil was shown to generate detectable amounts of singlet oxygen. This result suggests that singlet oxygen may be a significant biochemical intermediate in a few biological processes. Also singlet oxygen generated from ozone and cysteine, methionine, GSH, albumin, ascorbic acid, and NAD(P)H was recognized (Kanofsky and Sima 1991).

Important reactions leading to singlet oxygen formation are *photosensitization reactions*. After illumination of certain molecules with light of the appropriate wavelength, the light is absorbed and the molecule (called photosensitizer, S) is raised into an excited state (reaction 1.21). Excitation energy can then be transferred into oxygen forming singlet oxygen, forming singlet oxygen, and the photosensitizer returns back to its ground state (reaction 1.22). The list biologically important photosentiizers includes riboflavin, FMN, FAD chlorophylls *a* and *b*, bilirubin, and porphyrins.

$$S \xrightarrow{h\surd} S^* \tag{1.21}$$

$$S^* + O_2 \rightarrow S + {}^1O_2 \tag{1.22}$$

An excited photosensitizer can by itself be a damaging agent (*mechanism of type I*), while a singlet oxygen can cause damage (*mechanism of type II*) to biologically important molecules in vivo, for example, in skin, eye, or chloroplasts. After exposure to light, accumulation of porphyrins in skin (cutaneous porphyrias) causes swelling, blistering, eruptions, and scarring of skin. Also an application of some drugs can form $^1O_2$ after their photosensitizing (some antidepressants, tetracycline, and fluoroquinolone antibiotics and some nonsteroidal anti-inflammatory drugs).

However, in some cases, photosensitizing processes might be medically relevant, for example, PUVA (psoralen ultraviolet A) therapy, where UV light and synthetic psoralens (furocoumarins) are applied through skin absorption to patients

with psoriasis. Psoralens are also produced by plants, for example, by celery or *Heracleum mantegazzianum*. The latter is used also for the treatment of vitiligo.

However, the relation of this method to possible risk of skin cancer is disputable.

$^1O_2$ can react with other molecules containing conjugated double bonds to give unstable endoperoxides. Another mechanism of $^1O_2$ reaction is the transfer of excitation energy to a co-reactant which enters an excited state and the singlet oxygen by the so-called *quenching of* $^1O_2$. The quenchers include, for example, carotenoids–especially lycopene (Di Mascio et al. 1989), thioredoxin (Das and Das 2000), or vasoactive intestinal peptide (VIP), a highly basic 28-amino acid peptide (Misra and Misra 1990).

Hypochlorous Acid

Another reactive species includes hypochlorous acid, HOCl, which is produced by enzyme *myeloperoxidase* (MPO)

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^- \tag{1.23}$$

Hypochlorous acid at acidic pH can react with chloride ions to form the toxic gas Cl_2

$$HOCl + Cl^- + H^+ \rightarrow Cl_2 + H_2O \tag{1.24}$$

HOCl is a strong two-electron oxidizing agent to different biomolecules. It can pass through the membranes, causing damage to membrane proteins and lipids or also intracellular molecules. In extracellular space, HOCl inactivates through the methionine unit of α_1-antitrypsin, an inhibitor of proteolytic enzymes such as *elastase*. Elastase catalyzes the hydrolytical cleavage of elastin, especially in lungs (as in the disease emphysema). The methionine residue of *thrombomodulin*, a glycoprotein from endothelium involved in blood coagulation, is inactivated also by HOCl (Glaser et al. 1992). HOCl also reacts with many other compounds such as thiols, ascorbate, and NAD(P)H. It chlorinates pyrimidine bases in DNA and tyrosine units in proteins, and it causes fragmentation of proteins by different mechanisms (Halliwell and Gutteridge 2007). In addition, HOCl toxicity is used during phagocytosis for bacterial killing. Activated neutrophils generate both superoxide and HOCl. Reaction of these compounds leads to the formation of hydroxyl radical (reaction 1.4). After the reaction of HOCl with H_2O_2, another killing molecule, singlet oxygen is formed (Fig. 1.1).

Free radicals and RM have long been supposed to have only negative functions in the organism, especially when produced in large quantities, at the wrong place, in the presence of transition metal ions catalyzing radical reactions and if protection of the organism against their toxic effect fails (Ďuračková et al. 1999).

FR and RM can be produced in both the exogenous and endogenous environments of the organism.

The *endogenous sources of RS* are particularly phagocytes; mitochondria; peroxisomes; reactions of some enzymes, such as xanthine oxidase; cascade of

arachidonic acid; NADPH oxidase; reactions of ions of transition elements; inflammation; ischemia-reperfusion states; atherogenesis; hemodialysis; or intensive exercise. *Exogenous sources of RS* include cigarette smoke, radiation, chemotherapeutics, ozone, and exhalates.

However, a living organism is a very well-organized and sophisticated system in which free radicals act under strict control at predefined locations. An organism's ability to protect itself against the toxic effects of reactive oxygen metabolites is fascinating. If the free radicals have already been generated and have damaged some tissues, recognition of damaged molecules and the ability to remove damaged products are remarkable. On the other hand, nature is so ingenious that it can use the toxicity of free radicals at some places and in certain processes in the body, in favor of life. However, if one of the protective mechanisms of the organism against free radicals fails, free radicals action becomes uncontrollable, causing damage to molecules, cells, and organs and ultimately death of the organism.

Protective Systems, Antioxidants

To fight excessive production of FR and RM, the organism has built protective systems and mechanisms against their toxic effects. Protection is organized at three levels: (a) systems preventing FR formation, such as inhibitors of enzymes catalyzing FR formation. Included in this category is xanthine oxidase producing superoxide, which can be inhibited by allopurinol, or chelating agents trapping ions of transition metals and eliminating them from their catalytical activity during production of FR. (b) When these primary protective systems are insufficient and FR and RM are already formed, scavengers and trappers of FR come into action and eliminate high reactivity of FR by turning them into nonradical and nontoxic metabolites. These compounds are called *antioxidants* and they prevent oxidation of biologically important molecules by FR or RM. (c) If protection of the organism fails at this level, then repair systems recognize impaired molecules and decompose them, as it is in case of proteinases at oxidatively modified proteins, lipases at oxidatively damaged lipids, or DNA repair systems at modified DNA bases (Ďuračková 1998).

From the biological point of view, antioxidants are compounds which at low concentration prevent oxidative damage to molecules by oxidants (free radicals and reactive metabolites), while products of the reaction between oxidant and antioxidant should not be toxic and should not reinvigorate the radical reaction. For newly defined antioxidants, the source of oxidant and the method for detection of antioxidant ability should always be reported (Halliwell and Whiteman 2004; Gutteridge and Halliwell 2010).

Antioxidants have various structures, and according to the size of their antioxidative molecules, they can be classified into the high-molecular-weight and low-molecular-weight compounds (Table 1.1). *High-molecular-weight antioxidants* include, for example, the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase, or nonenzymatic proteinaceous antioxidants, for example,

Table 1.1 Examples of endogenous and exogenous antioxidants

Endogenous and exogenous antioxidants	
High molecular weight	**Low molecular weight**
Superoxide dismutase	Uric acid
Glutathione peroxidase	Ascorbic acid
Catalase	Lipoic acid
Albumin	Glutathione
Transferrin	Tocopherol (vitamin E)
Metallothionein	Ubiquinol (CoQ)
	Flavonoids/Polyphenols

albumin, transferrin, and various metallothioneins. The *low-molecular-weight antioxidants* include hydrophilic as well as lipophilic compounds produced in organism, for example, uric acid, lipoic acid, glutathione, or ubiquinol. The organism obtains exogenous antioxidants from food, for example, vitamin C and vitamin E and also compounds of polyphenolic structure synthesized in plants, including flavonoids (Williams et al. 2004; Ďuračková 2008).

The content of individual antioxidants is different in various organs and animal species. For example, the human eye lens contains little SOD and considerable amounts of ascorbic acid, while the rat eye is rich in SOD and has very little ascorbate. In rat peripheral nerve tissues, SOD has an activity of 90 U/mg nerve tissue, but in mice only 1 U/mg nerve tissue (Romero 1996).

Plasma antioxidant capacity also varies with age. For example, the concentration of urates increases by 25–30% with age, while ascorbic acid is more abundant in children. In adults, ascorbic acid represents about 15 % of the plasma total antioxidative capacity. Moreover, protein thiols (25 %), albumin (25 %), and vitamin E (5 %) also contribute to antioxidative capacity. In addition to these main antioxidants, other low-molecular-weight as well as enzyme antioxidants also participate in plasma antioxidative capacity to a lesser extent (Sies 1991; Polidori et al. 2001).

A compound is characterized as an effective antioxidant in vivo when it meets the following requirements: (1) it must react with biologically effective reactive metabolites; (2) the product of the reaction of prooxidant + antioxidant must not be more toxic for the organism than the removed prooxidant; (3) the potential antioxidant must be present in the organism at sufficient concentration; (4) the half-life of the antioxidant must be long enough to react with the oxidant (Halliwell and Whiteman 2004).

Generally, an antioxidant in one system and under certain circumstances need not act as antioxidant under other circumstances and in other systems.

An increasing body of evidence from experimental studies as well as clinical practice concerning their effect suggest that antioxidants need not always play a positive role. This fact has to be kept in mind especially during therapeutic administration of these compounds.

Enzyme and Protein Antioxidants

Cu/Zn-Superoxide Dismutase

Enzyme antioxidants have a negligible significance in the extracellular space. In the plasma, extracellular superoxide dismutase (EC-SOD) (Marklund 1984) is present only in a small quantity; there is little of GSH-dependent peroxidase activity and a questionable quantity of catalase. In the intracellular space, enzyme antioxidants play a significant role. Cu/Zn-superoxide dismutase (Cu/Zn SOD), similar to EC-SOD, specifically catalyzes dismutation of the superoxide radical to the nonradical molecules O_2 and H_2O_2. The general mechanism of superoxide dismutation catalyzed by SOD can be expressed by Eqs. (1.25) and (1.26). It is characterized by the redox cycle of copper ion Cu(II)→Cu(I)→Cu(II), etc.

$$Cu^{2+} + O_2^{\bullet -} \rightarrow Cu^{+} + O_2 \tag{1.25}$$

$$Cu^{+} + O_2^{\bullet -} \rightarrow Cu^{2+}\left(O_2^{2-}\right) \xrightarrow{2H^{+}} Cu^{2+} + H_2O_2 \tag{1.26}$$

The redox potential is significantly changed by some SOD inhibitors, for example, CN^- and N_3^- ions. Human and bovine Cu/Zn SOD is inhibited by higher concentrations of H_2O_2 which oxidatively damages histidine unit in the active enzyme center (Bertini et al. 1998).

Three isoforms of SOD are present in humans. They differ in the content of a metal in the active site, in the number of subunits, in the amino acid composition of the apoenzyme, in the sensitivity to inhibitors, etc. In addition to extracellular EC-SOD and intracellular Cu/Zn SOD, there is Mn SOD in mitochondria.

In spite of the fact that SOD is one of the most important intracellular antioxidative systems, its low activity does not always have to lead to pathological conditions. Antioxidants can partially substitute for one another and the lack of one antioxidant can trigger an increased synthesis of another antioxidant. On the other hand, increased enzyme activity need not always be a positive phenomenon as, for example, in Down syndrome. For individuals with Down's syndrome, trisomy of the 21st chromosome is characteristic, where the gene for Cu/Zn SOD is also coded. These patients have a 50% increased activity of Cu/Zn SOD, which is related to increased oxidative stress (Ďuračková et al. 2000; Muchová et al. 2001; Garaiová et al. 2004; Žitňanová et al. 2004).

Catalase

The majority of aerobic organisms, excluding some bacteria and algae, contain catalase. In animals, catalase is located in all important organs, especially in the liver and erythrocytes. The brain, heart, and the skeletal muscles contain only small amounts of catalase. Catalase is bound to *peroxisomes* in cells. Mitochondria,

chloroplasts, and the endoplasmic reticulum contains negligible amounts of catalase. The molecular weight of catalase is 240,000 and it is composed of four subunits containing heme with coordinated Fe^{3+} atom in their active site. Each subunit binds one molecule of NADPH, stabilizing the enzyme molecule.

Catalase catalyzes the decomposition of H_2O_2 to water and oxygen (reaction 1.27).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \tag{1.27}$$

Glutathione Peroxidase

The enzyme glutathione peroxidase (GPx) occurs in two forms: selenium-dependent and selenium-independent GPx, differing in the number of subunits, in the selenium bond in the active center, and in the catalytical mechanism.

Selenium-independent GPx (*glutathione S-transferase*, *GST*) catalyzes detoxification of various xenobiotics. The selenium atom with oxidative number (II) which is present in the enzyme molecule does not participate in the catalytical mechanism (Holovská et al. 1998). *Selenium-dependent glutathione peroxidase* (*GPx*) is composed of four subunits, while each subunit contains one selenium atom in the active center bound in the modified amino acid selenocysteine.

All GPx can reduce peroxides by two electrons producing selenols −Se−OH. In the second stage of the catalytical cycle, selenols are reduced by two GSH back to –SeH.

The significance of these selenoenzymes is based on elimination of peroxides – potential substrates for Fenton-type reaction. In addition, selenols react faster than thiols. During redox reactions, they transfer two electrons preventing the formation of the superoxide from the oxygen molecule. For the formation of superoxide, only one electron is required.

A high GPx activity was detected in the liver; medium activity in the heart, lungs, and brain; and low activity in muscles (Ďuračková 1998).

Glutathione peroxidase cooperates with the tripeptide glutathione (GSH) present in cells at relatively high (millimole) concentration. The substrate for GPx reaction is H_2O_2 or an organic peroxide. Glutathione peroxidase decomposes peroxides to water or alcohol and at the same time it oxidizes GSH (reaction 1.28 and 1.29). It is supposed that GSH reduces selenium in GPx and this reduced form of enzyme catalyzes decomposition of the hydrogen peroxide:

$$2\,GSH + H_2O_2 \rightarrow GSSG + 2H_2O \tag{1.28}$$

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O \tag{1.29}$$

The majority of glutathione present in cells is mostly in the reduced form (GSH) compared to the oxidized form (GSSG). A part of the total glutathione in cells is present in the form of “mixed” glutathione

$$\begin{array}{c} \gamma - \text{glu} - \text{cys} - \text{gly} \\ | \\ \text{S} - \text{S} - \text{R} \end{array}$$

where –R can be the cysteine residue, coenzyme A, or a protein containing –SH groups (Halliwell and Gutteridge 2007). The ratio GSH: GSSG is 10–100:1 (Devlin 2006). In cells, the enzyme *glutathione reductase* (GR) catalyzes GSSG reduction to GSH (Eq. 1.30). Glutathione reductase can reduce not only GSSG but also "mixed" disulfides (GSSR). The cofactor of GR is NADPH, produced in the pentose cycle by *glucose-6-phosphate dehydrogenase*:

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \tag{1.30}$$

Glutathione peroxidase activity depends on the concentration of GSH. Therefore, its physiological activity requires sufficient concentration of glutathione.

Paraoxonases

Paraoxonases (PONs 1, 2, and 3) are additional examples of antioxidants (Mackness and Mackness 2004; Ďuračková 2009). Mammalian paraoxonases (PON1, PON2, and PON3) are a unique family of calcium-dependent hydrolases, with enzymatic activity towards a broad range of substrates. PON1 and PON3 are associated with the HDL (high density lipoprotein) fraction and PON2 is an intracellular enzyme. Although PONs' physiological substrates have not been identified yet, some studies suggest some lactones, or some specific oxidized phospholipids, or products of oxidation of arachidonic and docosahexaenoic acid as well as N-acyl homoserine lactones to be the suitable substrates for the enzyme. All three members of the PON family were shown to protect from atherosclerosis development, because oxidatively modified lipoproteins are the main risk factors that contribute to the pathology of cardiovascular diseases. Their biological activities are determined towards three main substrates – organophosphate paraoxon, aromatic carboxylic acid ester (phenylacetate), and different lactones. Arylesterase activity is a more decisive indicator of antiatherogenic activity than paraoxonase activity. However, structure-reactivity studies indicate that lactonase activity of PON1 is its native activity (James 2006; Aviram and Rosenblat 2008).

Transferrin

Transferrin is a protein in blood plasma that binds Fe^{3+} atoms. Its binding capacity represents only 20–30 %. Iron ions bound strongly to transferrin do not catalyze lipid peroxidation. Apotransferrin (transferrin without Fe^{3+} atoms) has a great

ability to bind Fe^{3+} atoms released from ferritin or other sources. Thus, transferrin acts as an antioxidant because it inhibits formation of $^{\bullet}OH$ radical and lipid peroxidation through Fenton-type reactions.

An important protein of iron metabolism is **ferritin** present in plasma and cells as a stock protein. Unlike transferrin, ferritin binds Fe^{3+} by a weak bond, releasing Fe^{3+} very easily. Such a release of Fe^{3+} from ferritin requires the presence of the appropriate reductant, for example, $O_2^{\bullet -}$. Released and reduced Fe^{3+} atoms can exert their catalytical properties at hydroxyl radical formation. Therefore, *ferritin cannot be considered an antioxidant*; on the contrary, it can be a source of pro-oxidatively acting Fe^{2+} atoms (Rieth et al. 1992).

Ceruloplasmin

Ceruloplasmin is a protein transporting Cu^{2+} atoms. It binds almost all Cu^{2+} atoms present in plasma. Its antioxidative effect is based on the following:

1. Ceruloplasmin is able to oxidize Fe^{2+} to Fe^{3+} atoms (it is called also *ferroxidase I*) at the simultaneous reduction of O_2 to H_2O (reactions 1.31 and 1.32). Fe^{3+} atoms can bind to transferrin. During oxidation of Fe^{2+} to Fe^{3+} by ceruloplasmin, superoxide is not formed as it is during nonenzymatic oxidation of Fe^{2+} atoms. By changing the oxidative number of iron, ceruloplasmin expels Fe^{2+} from participation in Fenton reaction (Halliwell and Gutteridge 1990):

$$CP - \left[Cu^{2+}\right] + 4Fe^{2+} \rightarrow CP - \left[Cu^{+}\right] + 4Fe^{3+} \tag{1.31}$$

$$CP - \left[Cu^{+}\right] + O_2 + 4H^{+} \rightarrow CP - \left[Cu^{2+}\right] + 2H_2O \tag{1.32}$$

2. Cu^{2+} or Cu^{+} atoms bound to ceruloplasmin do not produce the hydroxyl radical in the presence of H_2O_2.
3. Ceruloplasmin can react directly with superoxide. However, the mechanism of the reaction is not dismutative as it is in the reaction with superoxide dismutase (Bannister et al. 1980).

Thanks to these properties, ceruloplasmin belongs to the most important extracellular antioxidants in the human body.

Albumin

Its antioxidative ability is based on the bond of Cu^{2+} atoms and heme, thus preventing oxidative damage of other molecules in the extracellular space. Albumin is also termed a "self-sacrificing" antioxidant. In the case of Cu^{2+} ions bound to albumin, radical reactions can be accelerated. Cu^{2+} atom can be oxidized by the hydrogen peroxide to the highly toxic atom Cu^{3+}. At these redox reactions, copper from albumin is not liberated and the protein can be damaged

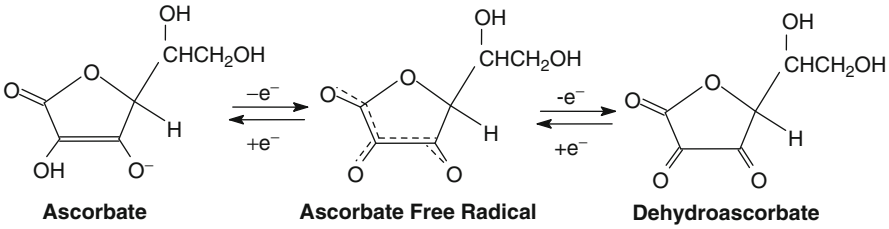
at the site where Cu^{2+} binds to albumin. However, in plasma, albumin is in such a high concentration (600 μmol.l^{-1}) and its overall change is so rapid that possible biological consequences of such damages are nonsignificant. Albumin thus significantly contributes to the antioxidative activity of ceruloplasmin in the extracellular space. One of the important functions of serum albumin is also its protection against *the oxidative damage by hypochlorous acid* (HOCl) produced by *myeloperoxidase* (Halliwell 1988), when HOCl binds to albumin thiol groups.

Low-Molecular-Weight Antioxidants

In addition to high-molecular-weight enzymatic as well as nonenzymatic antioxidative systems, *low-molecular-weight antioxidants* also participate significantly in the metabolism of free radicals. According to their solubility, they are classified to hydrophilic and lipophilic antioxidants. They occur in cells as well as in the extracellular space.

Ascorbic Acid

Possible redox reactions of ascorbic acid are shown in reaction scheme (1.33).



(1.33)

Ascorbic acid is also able to recycle other important antioxidants from their radical forms, as, for example, tocopherol and glutathione.

The antioxidative effect of ascorbic acid is strictly confined by the presence of ions or chelatory bound atoms of transition metals. In their presence, ascorbate behaves pro-oxidatively through the "Fenton-type" reaction (Fig. 1.2). In the presence of ascorbic acid, metal ions in so-called catalytically effective form can catalyze the formation of toxic reactive metabolites, such as $^{\bullet}OH$ radical. Moreover, the pro-oxidative effect of ascorbic acid in vivo is associated with autoxidation of ascorbate to dehydroascorbate, thus changing the redox state of cells leading to changed expression of some genes, for example, transcription factors (NF-kappaB and AP-1) and signal molecules influencing regulation of signaling transduction pathways (Fig. 1.3).

Intracellular semidehydroascorbate (ascorbate free radical, Eq. 1.33) and dehydroascorbate (DHA) can be enzymatically reduced back to ascorbate,

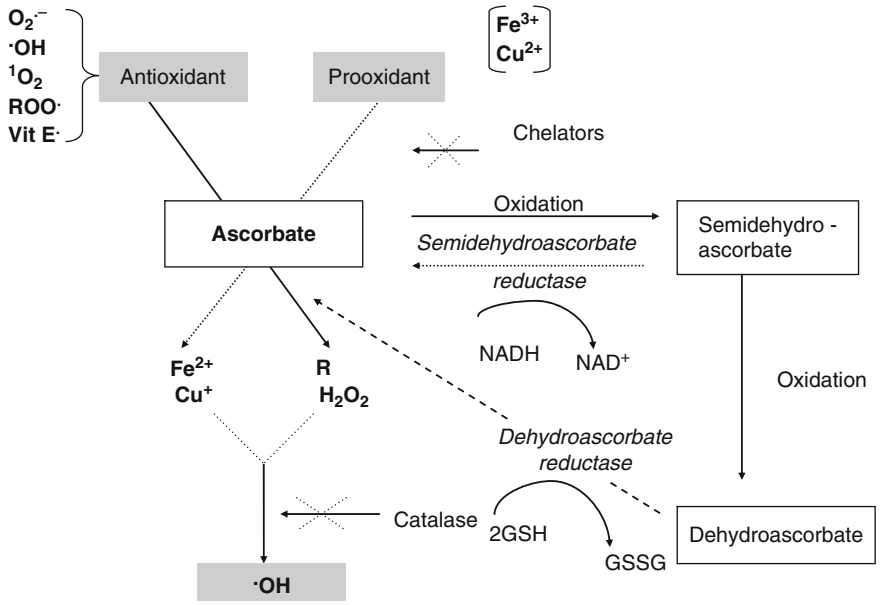


Fig. 1.2 Antioxidant and possible prooxidant properties of ascorbic acid

producing H_2O_2, which can oxidatively modulate surrounding molecules and thus changing also the cell redox state (Duarte and Lunec 2005; Ďuračková 2010).

Vitamin E

Vitamin E is a term used for a group of compounds composed of the mixture of eight derivatives exerting the activity of α-tocopherol (Pryor 1997; Singh et al. 2005) (Fig. 1.4).

The main function of vitamin E is its antioxidative ability. It is able to disrupt radical chain reactions (e.g., in the reaction with peroxyl radical $LOO^{\bullet}$) or to trap directly oxygen radicals (e.g., hydroxyl radical $HO^{\bullet}$). During these reactions, tocopheryl radical (vitamin $E^{\bullet}$) is formed. This radical can be generated by the extraction of hydrogen atom from various places of the chroman nucleus (from position 5,7,9) or more often from the – OH group at position 6. Tocopheryl radical can be reduced by ascorbate, glutathione, or ubiquinol to the active α-tocopherol. When vitamin E is located in the lipid part of membrane, where the process of lipoperoxidation is carried out, tocopheryl radical can be reduced by lipoperoxides, thus branching the radical reaction (Ďuračková 1998).

Vitamin E acts synergically with vitamin C. This is possible due to the location of vitamin E in the membrane where its chroman ring with the hydroxyl group is oriented into the hydrophilic part of the membrane where at the borderline of the

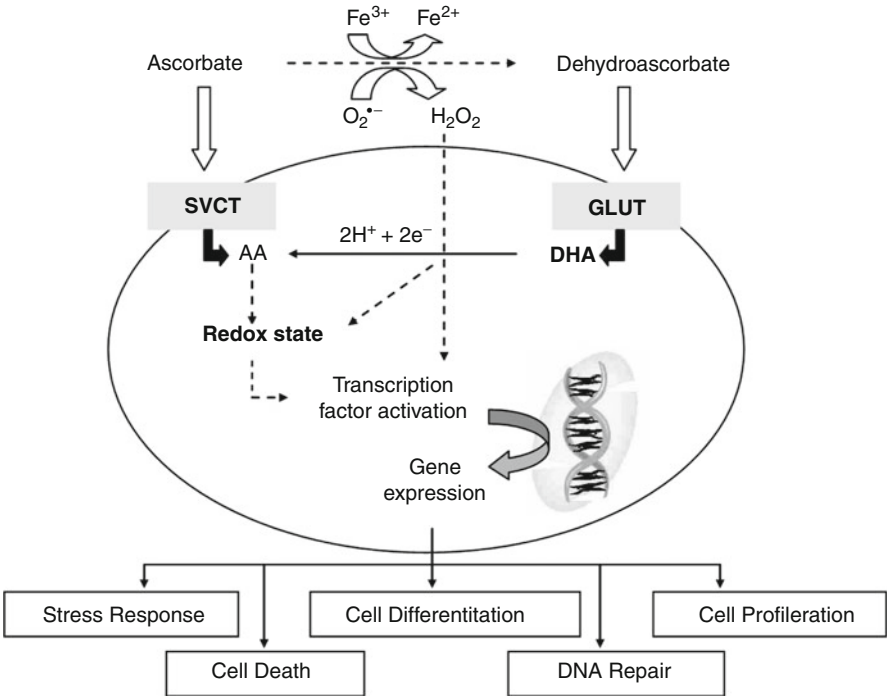


Fig. 1.3 Putative mechanism of ascorbic acid action in vivo (According to Duarte and Lunec 2005). *AA* ascorbate, *DHA* dehydroascorbate, *SVCT* sodium-dependent vitamin C (ascorbate) transporter, *GLUT* glucose transporter

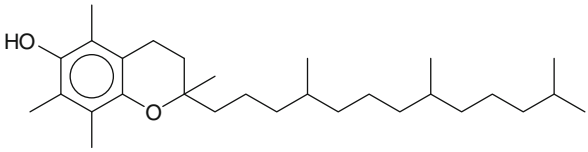


Fig. 1.4 Structure of α-tocopherol

two phases, it can come into contact with ascorbic acid which can regenerate vitamin E (Niki 1991) (Fig. 1.5). However, there are studies refusing cooperation of vitamin E with C in vivo (Burton et al. 1990).

Carotenoids

Carotenoids are pigments of plant or microbial origin. At present, around 600 derivatives of carotenoids are known. Of these, 10 % possess the activity of provitamin A and can be metabolized to retinol. Only a small number of carotenoids are present in plasma and tissue in sufficient amount. Carotenoids can react with singlet oxygen and

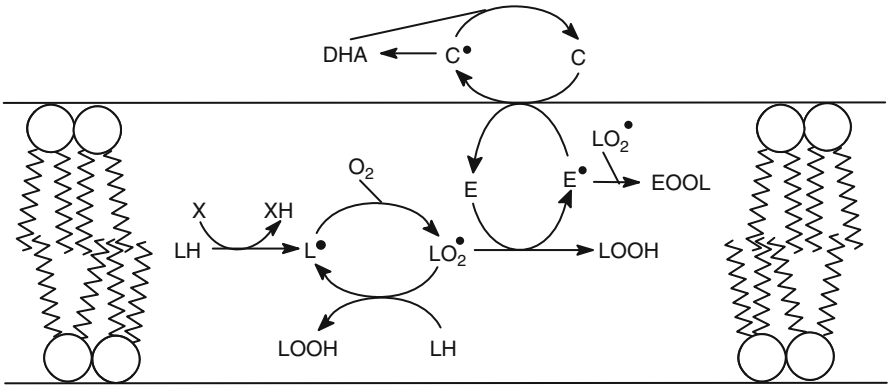


Fig. 1.5 Regeneration of tocopheryl radical by ascorbate. *E* tocopherol, *E*• tocopheryl radical, *C* ascorbate; *C*• ascorbate radical, *DHA* dehydroascorbate, *LH* lipid, *LOOH* lipoperoxide, *X* oxidant, $LO_2^{\bullet}$ lipoperoxyl radical

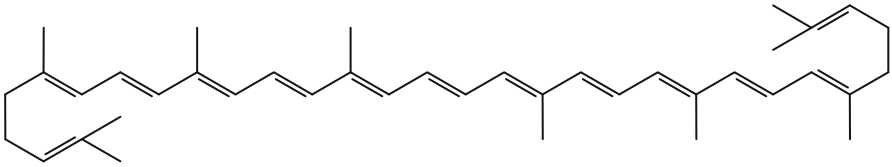


Fig. 1.6 Structures of lycopene

return the molecule of the excited oxygen into the basic energetic state. Due to this property, β-carotene is an important in vitro antioxidant that is part of a group referred to as *quenchers*. Carotenoids can also directly trap free radicals (Krinsky 1993; Stahl and Sies 1993). Of the biologically important natural carotenoids, the most efficient quencher is lycopene (Devasagayam et al. 1992) (Fig. 1.6). Vitamin A exerts only a negligible antioxidative ability (Sundquist et al. 1994).

Coenzyme Q

Coenzyme Q (CoQ) is a widely used name for 2,3-dimetoxy-5-methyl-6-multiprenyl-benzoquinone. The synonym *ubiquinone* is derived from the English word *ubiquitous*, present everywhere. Coenzyme Q has isoprene units in its side chain, whose number differs in various species (in microorganisms 6, in rats 9, in man 10).

Coenzyme Q was discovered by Crane et al. (Crane 1965) as a constant component of the mitochondrial succinate dehydrogenase system. In addition, coenzyme Q is associated with metabolism of free radicals: with their formation, for example, in mitochondria (pro-oxidative properties) (Fig. 1.7), as well as with their elimination (antioxidative properties).

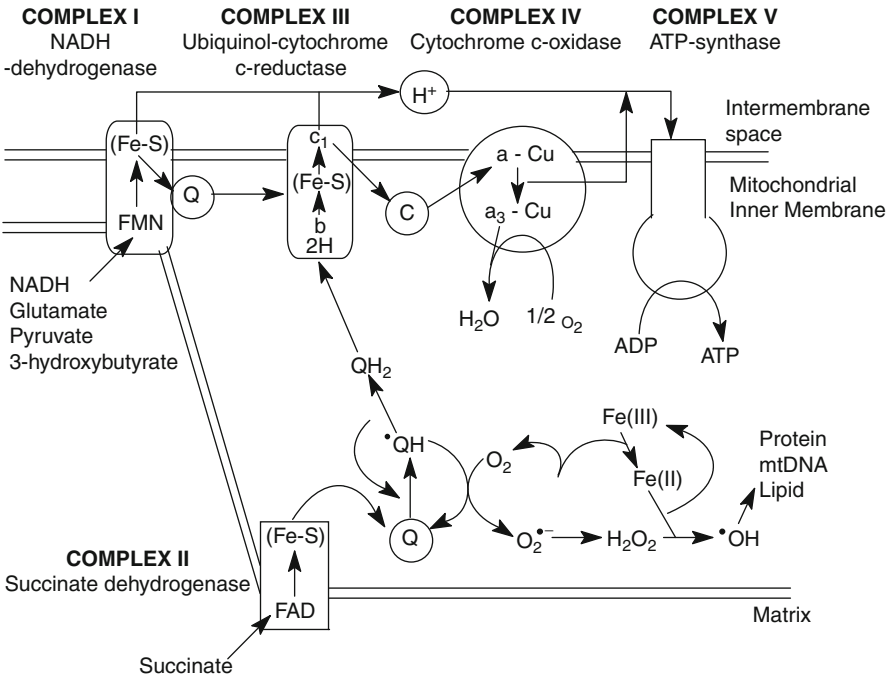


Fig. 1.7 Expected sites of RM formation in mitochondria (Modified according to Gabbita et al. 1997 with his kind permission) (Gabbita et al. 1997). *Q* ubiquinone, QH_2 ubiquinol, $^{\bullet}QH$ semiquinone radical

Coenzyme Q in its reduced form ($CoQH_2$, ubiquinol) is a more effective antioxidant than ubiquinone (CoQ). Coenzyme QH_2 can react directly with superoxide to semiquinone (reaction 1.34), lipid radical (reaction 1.35), as well as lipid peroxyl radical (reaction 1.36) (Beyer 1990).

$$CoQH_2 + O_2^{\bullet -} \rightarrow H_2O_2 + CoQ^{\bullet -} \tag{1.34}$$

$$CoQH_2 + 2L^{\bullet} \rightarrow 2LH + CoQ \tag{1.35}$$

$$CoQH_2 + 2LOO^{\bullet} \rightarrow 2LOOH + CoQ \tag{1.36}$$

The reactions shown in Fig. 1.7 can also proceed in one stage. Formed semiquinone (reaction 1.37) can be reduced in the respiratory chain in mitochondria (by cytochrome b_{562}), or it can be oxidized to quinone with the potential danger of the superoxide formation (Fig. 1.8):

$$CoQH_2 + ROO^{\bullet} \rightarrow CoQ^{\bullet -} + ROOH + H^{+} \tag{1.37}$$

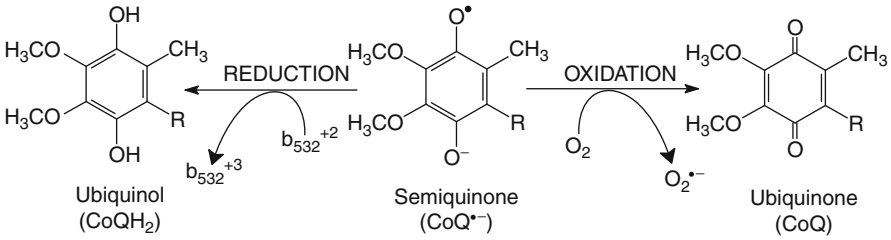


Fig. 1.8 Two possible directions of the semiquinone change. R (in human mitochondria) = $(-CH_2-CH=C(CH_3)-CH_2-)_{10}H$

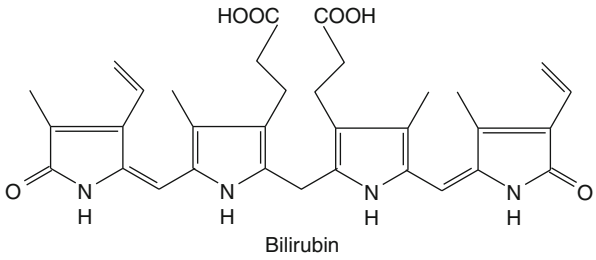


Fig. 1.9 Structure of bilirubin

The antioxidative ability of coenzyme Q can be exerted also through regeneration of vitamin E due to the neighboring location of both compounds in the membrane lipid bilayer.

Although α-tocopherol is ten times more effective as an antioxidant than $CoQH_2$, in case of vitamin E insufficiency in membranes, coenzyme Q becomes an irreplaceable antioxidant. In case of sufficient concentration of α-tocopherol in membranes, $CoQH_2$ has a regenerative function (Kagan et al. 1994).

Bilirubin

Bilirubin is a linear tetrapyrrole formed in vivo by the oxidative splitting of the heme (Fig. 1.9). Bilirubin inhibits oxidation of linoleic acid which is bound to albumin, initiated by heme proteins or by peroxyl radical. It quenches $^1O_2$ and inhibits hyperoxia (>95 % O_2) evoked by lipoperoxidation and production of carbonyl proteins (Dennery et al. 1995). Bilirubin bound to albumin (similarly as uric acid) reacts slowly with the oxidatively effective hypochlorous acid, and the rate of the reaction increases after conjugation of bilirubin with glucuronic acid. Similar to vitamin C, bilirubin acts synergically with vitamin E (Stocker and Frei 1991).

In healthy adults bilirubin occurs in plasma only at micromolar concentrations, and its competition with other antioxidants present in plasma at higher concentrations is therefore debatable. On the other hand, several in vivo studies suggest that

bilirubin has a potent scavenging ability of peroxyl radicals. Mildly increased circulatory bilirubin provides a physiological function to protect the organism against disease processes that involve oxygen and peroxyl radicals, such as atherosclerosis, coronary artery disease, and inflammation (Mayer 2000).

Uric Acid

Uric acid was originally considered a catabolic product of degradation of purine metabolites. Uric acid is present in human plasma at high concentration (0.12–0.45 mmol.l^{-1}). It is a significant quencher of $^1O_2$ and a trapper of the hydroxyl radical. Uric acid exerts its antioxidative ability by two mechanisms: (1) by direct reaction with some free radicals and (2) by the chelatory ability binding ions of transition metals from the environment, eliminating them from their catalytical action in Fenton-type reactions (Glantzounis et al. 2005).

At physiological conditions, uric acid stabilizes ascorbate in human serum which is ascribed to its chelatory ability (Davies et al. 1986). Uric acid, by formation of stabile complexes with Fe^{3+} atoms, inhibits oxidation of ascorbate and suppresses peroxidation of lipids. If uric acid chelates Fe^{2+} atoms, it inhibits the Fenton reaction and formation of $OH^{\bullet -}$ radical.

Since humans have no *uricase*, allantoin, a product of uricase activity, should not be present in plasma under physiological conditions. However, increased concentrations of allantoin were found in plasma of, for example, patients with rheumatoid arthritis which is associated with increased oxidative stress (Halliwell 1990). Similarly in patients with Down's syndrome who are assumed to have increased oxidative stress, allantoin was detected in their plasma which together with increased levels of uric acid is an indicator of the antioxidative role of uric acid in these patients (Žitňanová et al. 2004).

Glutathione

Tripeptide glutathione (GSH) plays a significant role in protection of the organism against oxidative damage for several reasons: (1) It is a cofactor of some enzymes participating in detoxification mechanisms of oxidative stress, as, for example, *glutathione peroxidase*, *glutathione transferase*, *and dehydroascorbate reductase*. (2) GSH is a direct trapper of $^{\bullet}OH$ radical and $^1O_2$, and it detoxifies H_2O_2 and lipoperoxides during catalytical action of glutathione peroxidase. (3) Glutathione can reduce the tocopheryl radical directly or indirectly during the reduction of semidehydroascorbate to ascorbate regenerating these important antioxidants back to their active form (Fig. 1.10). Glutathione plays an important role as an antioxidant in intracellular space and indirectly affects the antioxidative status also in extracellular space and membranes where in cooperation with α-tocopherol, it can inhibit lipoperoxidation (Maxwell and Lip 1997; Ďuračková 1998; Bánhegyi et al. 2003).

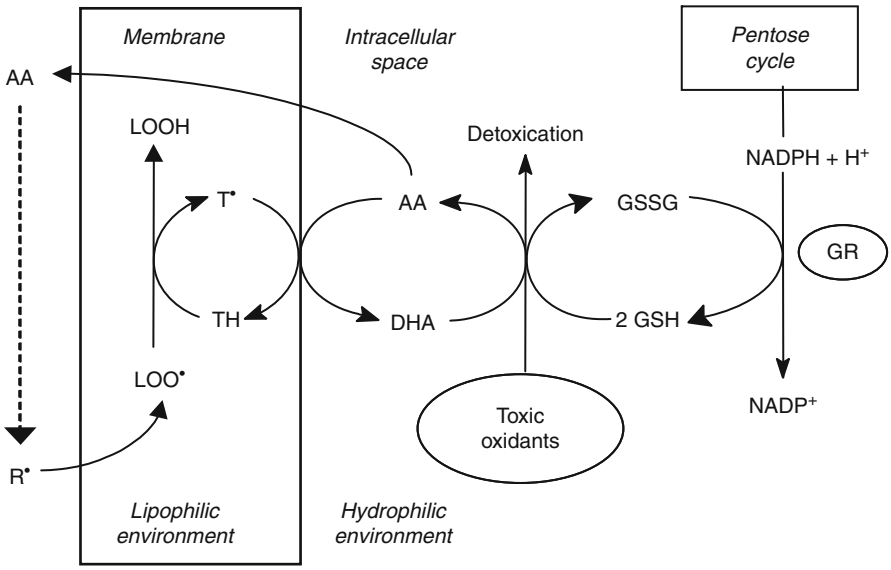


Fig. 1.10 Cooperation of glutathione with vitamins C and E. $R^{\bullet}$ initiator of a radical reaction, $LOO^{\bullet}$ lipoperoxide, *LOOH* hydroperoxide, *TH* tocopherol, $T^{\bullet}$ tocopherol radical, *AA* ascorbate, *DHA* dehydroascorbate, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *GR* glutathione reductase

Oxidized glutathione is regenerated by *glutathione reductase* (GR) cooperating with NADPH which is produced in the pentose cycle of glucose degradation. The most important source of GSH is the liver where 90 % of the circulating GSH is synthesized de novo. The intracellular concentration of GSH is 500 times higher than its extracellular concentration; thus, GSH has an important function in detoxification processes in the cell and also has the function of "redox buffer." Moreover, the correct ratio of GSH/GSSG significantly contributes to the total redox state of the cell. Since many proteins participating in the signaling pathways such as transcription factors and receptors have thiols groups in the active sites, their function can be influenced by the redox state of the cell. At increased levels of GSSG, thiol groups of proteins can form mixed disulfides, thus changing their physiological function. In this regard, GSSG appears to act as a nonspecific signaling molecule (Valko et al. 2006).

Natural Antioxidants, Flavonoids, and Polyphenols

Flavonoids

Flavonoids are phenolic compounds that are widely distributed in the plant kingdom. They include more than 4,000 different derivatives and their list

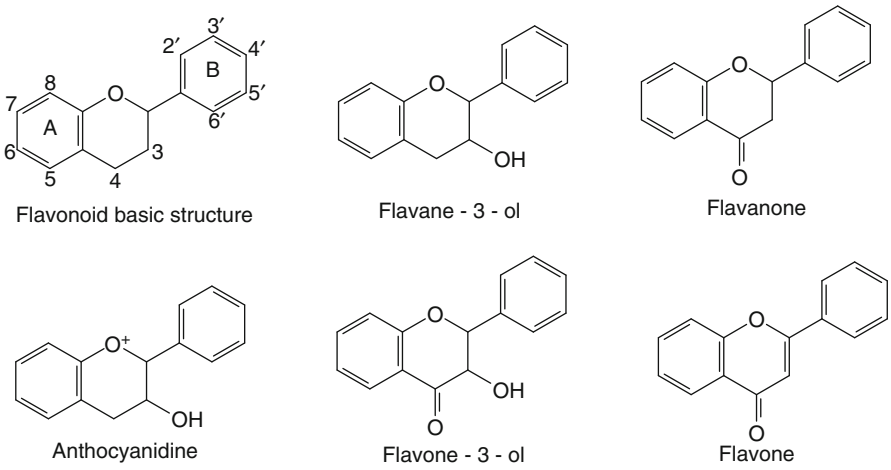


Fig. 1.11 Basic flavonoid structures

constantly increases. Formation of so many derivatives is possible due to the substitution of hydrogen atoms at different sites of the basic structures by hydroxyl, methoxyl, and other groups. The basic flavonoid structures include the following: *flavan-3-ols* (epicatechin, gallocatechin), *flavanones* (naringenin, hesperidin), *flavones* (apigenin, luteolin), *flavone-3-ol* (quercetin, myricetin), *anthocyanidins* (cyanidin, pelargonidin), and *isoflavones* (genistein, daidzein) (Ross and Kasum 2002) (Fig. 1.11).

Flavonoids occur in food either as free monomers (quercetin, catechin) or oligomers (procyanidins). They are bound to saccharides as glycosides or occasionally they are found as aglycones. After ingestion, flavonoids can undergo biotransformation to their metabolites which can be detected in plasma reaching concentration of about 1 μmol.l^{-1} (Manach and Donovan 2004; Grimm et al. 2006).

Consumption of flavonoid-rich food is associated with a lower incidence of coronary heart disease, myocardial infarction (Curin et al. 2006), cancer (Valko et al. 2006), neurodegenerative diseases (Ramassamy 2006), psychical diseases (Trebatická et al. 2006), and other chronic diseases (Knekt et al. 2002). Since in the pathology of these diseases, in addition to other factors, oxidative stress has been assumed to play a role, dietary flavonoids have been suggested to exert health benefits through antioxidant mechanisms. In experiments in vitro, flavonoids exert a significant antioxidative ability, especially due to hydroxyl groups present in the B ring donating hydrogen atoms to radical reactions (Williams et al. 2004). The presence of the double bond at position 2,3 in conjugation with 4-oxo-group in the C ring and the presence of hydroxyl groups capable of binding transition metal ions, such as iron and copper, contribute to the chelating ability of flavonoids. In addition, flavonoids have a relatively low redox potential, enabling them to easily

oxidate and reduce free radicals and also to regenerate other antioxidants, for example, which has a higher redox potential (Lotito and Frei 2006).

However, the antioxidative ability of flavonoids cannot be exerted in vivo because flavonoid absorption from food is low. The plasma level of flavonoids is an order of magnitude lower than the level of other antioxidants in plasma, such as vitamins C and E and uric acid (Chovanová et al. 2006). Moreover, the half-life of flavonoids in plasma is short because after ingestion, they are metabolized to other derivatives (Halliwell et al. 2005).

Low concentrations of flavonoids exert other effects in vivo that are separate from their antioxidative effects. Flavonoids can participate in signaling pathways in cells, thus influencing the fate of a cell (Ďuračková 2010). Flavonoids may interact selectively within the MAP kinase signaling pathway which is involved in signaling to neuronal survival, regeneration, development, and death (Spencer 2005).

The positive effect of flavonoids on the organism is manifested in several ways. In addition to the secondary effect of the antioxidative ability, for example, through stimulation of the activity of antioxidant enzymes (Koláček et al. 2010), a vasodilating effect (Ďuračková et al. 2003), antithrombic effect (Golański et al. 2006), anti-inflammatory effect (Schäfer et al. 2006), and anti-apoptotic effect. Flavonoids also possess antimutagenic ability (Križková et al. 2008) and can inhibit the binding of carcinogenic compounds to DNA.

Oxidative Stress

Results of many epidemiological studies indicate an exceptional place of flavonoids among biomodulating compounds, of which "the French paradox" is much discussed. Based on the living style of French people, it could be assumed that their life includes several risk factors for the occurrence of coronary heart disease. However, in one region of France, a markedly low incidence of this disease has been detected in comparison with other regions. This fact has been attributed to the consumption of red wine with a high content of flavonoids and polyphenolic compounds (Renaud and De Lorgeril 1992).

On the other hand, flavonoids also have pro-oxidative properties, for example, in the presence of high concentration of Cu^{2+} ions (25–100 $mol.l^{-1}$) and oxygen. The issue whether pro-oxidative effects of flavonoids can also be exerted in vivo requires further studies (Labuda et al. 2003; Procházková et al. 2011).

Oxidative stress occurs when the balance between the production of FR and RM on the one hand and the antioxidative defence mechanisms is disturbed and cellular damage results (Štípek et al. 2000; Halliwell and Whiteman 2004; Ďuračková 2007) (Fig. 1.12). In spite of the fact that this term has been used commonly, it is important to realize that the term is not completely correct. Oxidation never proceeds alone, but reduction must be its chemical partner. Therefore, the more precise term for this phenomenon should be the "**redox stress**" (Ďuračková 2007).

Redox stress is a very complicated and complex process. Its impact depends on the type of oxidant, on the place and intensity of its production, on composition

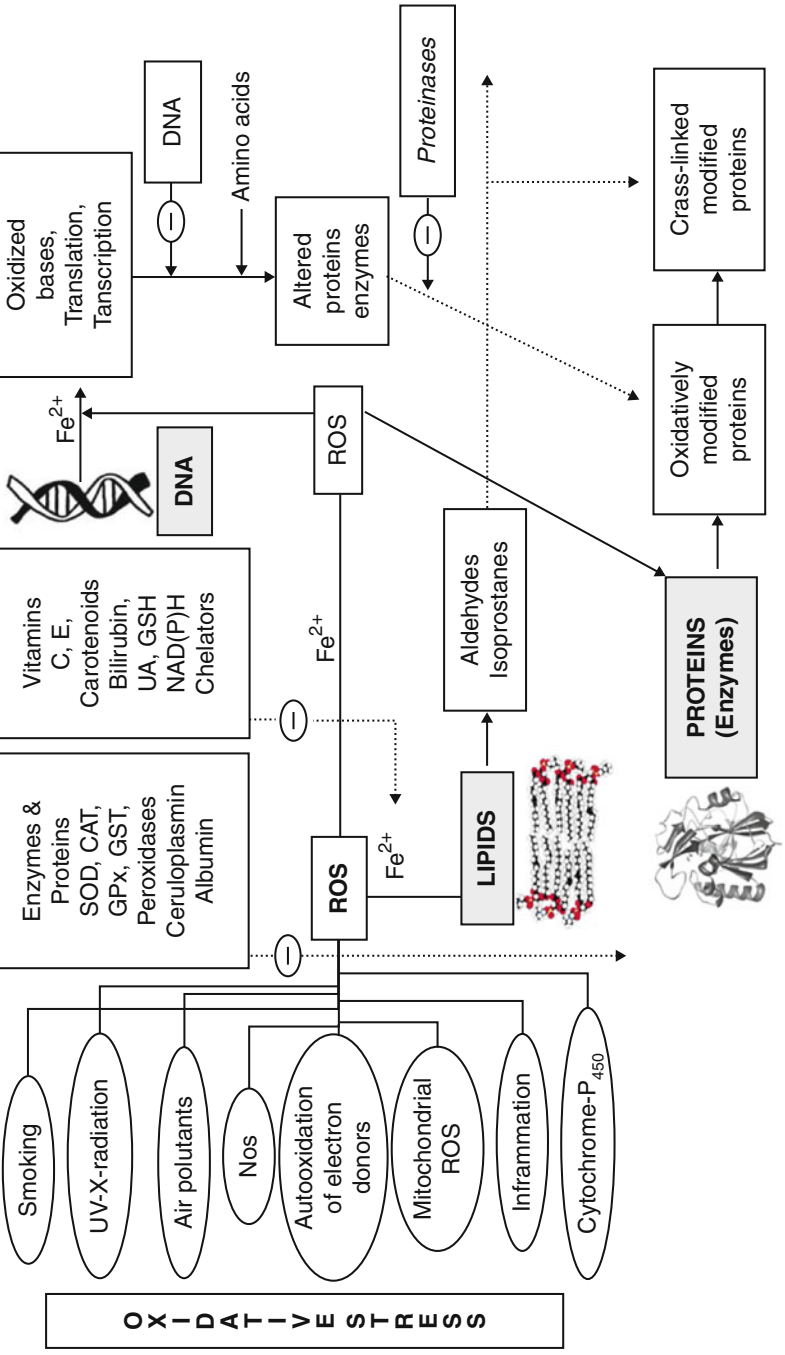


Fig. 1.12 Mutual association between oxidants and antioxidants. *NOS* NO synthase, *ROS* reactive oxygen species, *CAT* catalase, *SOD* superoxide dismutase, *GPx* glutathione peroxidase, *GST* glutathione S-transferase, *UA* uric acid, *GSH* glutathione reduced

and activities of various antioxidants, and on the ability of repair systems (Ďuračková 2007).

Oxidative stress has been associated with several diseases, such as atherosclerosis and cardiovascular diseases (Racek et al. 1995; Pechan et al. 2003; Ballinger 2005; Cherubini et al. 2005); neurodegenerative diseases (Aruoma et al. 2007), such as Alzheimer and Parkinson diseases; diabetes mellitus (Muchová 1999; Muchová et al. 1999) and metabolic syndrome; skin and tumor diseases (Klaunig and Kamendulis 2004; Valko et al. 2006; Ghaffari 2008); and also psychological impairments such as schizophrenia or ADHD (attention deficit hyperactivity disorder) (Chovanová et al. 2006; Trebatická et al. 2006; Dvořáková et al. 2007). Even physiological process such as aging has been associated with FR, RM, and antioxidants actions.

As discussed earlier, production of FR and RM are necessary for some cell functions, but if produced in excess, these FR and RM can have deleterious effects. This FR and RM, and antioxidants under some conditions, have dual roles in the organism, some of which are beneficial and others harmful. Complete suppression of FR formation would not be beneficial; therefore, under physiological condition, production of modest amounts of FR and RM are required (Ďuračková et al. 1999). The positive function is associated with, for example, reproduction processes. Free radicals play irreplaceable role in phagocytosis as a significant microbiocide; FR also have important roles in several biochemical reactions, for example, hydroxylating, carboxylating, or peroxidating reactions or in reduction of ribonucleotides.

At present FR and RM are assumed to have important biomodulating activity and regulatory ability in signal transduction processes during transduction of intercellular information.

Depending on the type and concentration of RM, exposure time antioxidant status, and activation of cellular repair systems, cells exposed to RM can respond differently – increased proliferation, halted cell cycle, senescence, apoptosis, or necrosis (Halliwell 2007). The response of cells to RM is dependent on the type of cells. The "fate" of cells is dependent on the activities of different signal transduction pathways (Ďuračková 2010).

The necessity to maintain the natural redox homeostasis of the organism is important under physiological conditions. A special challenge arises when maintaining redox homeostasis in illness. While it is certain that endogenous antioxidants may be considered biological response modifiers to redox homeostasis, it remains to be clarified whether exogenous natural and synthetic antioxidants are beneficial (Augustyniak et al. 2010). However, positive effects of exogenous antioxidants were observed in some rodent and murine models of disease. This gives us hope that antioxidants could be beneficial, if we could find ones that actually work in humans, getting to the correct sites of action and diminishing oxidative damage at those sites (Halliwell 2009).

At present, information on OS could be summarized as follows: generated FR and RM, and antioxidants significantly interfere with oxidative-reduction processes in cells and organisms, changing the redox state of the cell. Changed redox state stimulates or inhibits activities of various signal proteins, leading to the changed ability of signal pathways to influence the fate of cells. An oxidative milieu is

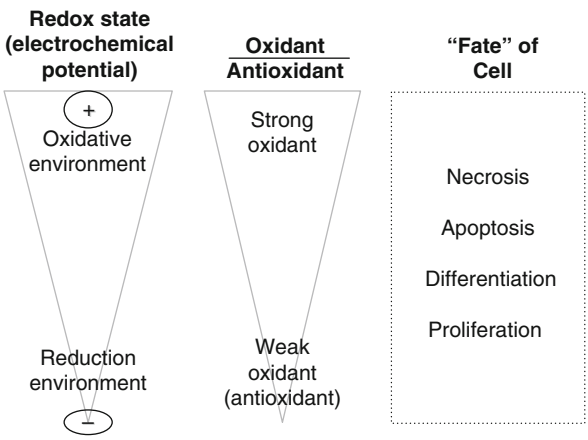


Fig. 1.13 Oxidative and reductive environment and "fate" of cell

suitable for cell destruction by apoptosis or necrosis and vice versa, reducing milieu for cell survival (Fig. 1.13) (Ďuračková 2007).

In conclusion, FR and RM are not "only bad." Antioxidants are not "only good." They both have regulatory functions in many physiological and pathological processes especially through the modulation of the redox state of the organism. However, at present, we do not know exactly where a border between their physiological and pathological effect is, what that effect depends on, and how we can benefit the wellbeing of organisms by modulating the effects of FR, RM and antioxidants by influencing the balance of these determinants of the redox state of cells.

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