Chapter 1

Oxidative Stress: Noxious but Also Vital

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73394

Abstract

The imbalance between reactive oxygen species (ROS) production and antioxidant defenses determines the condition called oxidative stress. When there is an increase in ROS production or a decrease in the antioxidant defenses, this systemic antioxidant/pro-oxidant imbalance may lead to the accumulation of oxidative damage, which, in turn, may lead to a modification of biomolecules. These consist of reactions resulting in protein adducts, DNA oxidation, and formation of lipid peroxides, which, in turn, reduce the cellular functional capacity and increase the risk of disease development. The body has natural scavenging systems against free radicals and other reactive species. However, sometimes the endogenous antioxidant capacity is exceeded by the production of ROS. When this occurs, exogenous antioxidants exert important function for the human health. These bioactive compounds act preventing and neutralizing the formation of new reactive species and free radicals. In some cases, an increase of ROS can help the host to resolve an infection or even to control the tumor growth. Finally, the levels of ROS can be perceived by signal transduction pathways involving known targets (i.e., p53, Ras, and NF-κB) and regulate physiopathological events such as the cellular cycle, apoptosis, and inflammation.

Keywords: reactive species, cellular oxidation, antioxidant system, health, disease

1. Cellular respiration and generation of reactive species in the mitochondria: implications in cell viability and aging

Oxidative phosphorylation is the center of energy metabolism in plants, animals and several microbial life forms [1]. In eukaryotes, this process occurs in mitochondria. The mitochondria is a cytoplasmic organelle surrounded by two membranes, outer and inner membrane, which main function is the production of most of the phosphate compounds necessary for the energetic balance of the cell. In addition, other functions such as the regulation of the body's heat generation [2–4] programmed cell death [5–7], reactive oxygen species (ROS) generation and cell signaling [8] is also associated with mitochondria. Cellular vitality is directly related to mitochondria, and mitochondrial dysfunctions are frequent causes of accidental cell death [5, 9–11], cancer [12, 13], diabetes [14–16] and neurodegenerative diseases [17–19], among others.

The characterization of the respiratory electron chain could be performed in studies using the fractionation of its components by certain detergents that at low concentrations break the interactions between proteins and lipids in the membranes, leaving associations between proteins intact [20]. In electron transport chain, through this process, four protein complexes were found. They were named complex I (or NADH-Ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (Ubiquinol -cytochrome c oxidoreductase, or complex bc_1) e complex IV (cytochrome c oxidase). The complex V is also known as ATP synthase. Despite glycerophosphate dehydrogenase (glycerol-3-phosphate dehydrogenase) and ETF–ubiquinone oxidoreductase have not complex nomenclature, they are connect to the electron transport chain, as complex I and complex II, i.e., delivering electron to ubiquinone [21].

The redox carriers within the respiratory chain consists of flavoprotein containing tightly bound FAD or FMN as prosthetic groups, protein-bound couper, ironsulphur (nonhaem iron) proteins and cytochromes, with haem prosthetic groups. The ubiquinone also participated in electron transport chain as a free and diffusible cofactor [20]. While electron transport occurs through the mitochondrial complexes, complexes I, II, and III pump protons from mitochondrial matrix to the intermembrane space. The energy associated to this process is used to the production of ATP by ATP synthase (**Figure 1**) [22].

1.1. Reactive species in mitochondria

The ROS comprise a variety of molecules derived from molecular oxygen, including oxygen radicals and non-radical oxygen derivate. The major intracellular site of ROS formation in most tissues is mitochondria [23, 24]. Within mitochondria, the electron transport chain continuously generates water from O_2 through the electronic reduction at the cytochrome c oxidase level (**Figure 1**). These electrons reach cytochrome c oxidase by sequential transfer from the reduction of other components, and are initially removed from NADH and $FADH_2$. During this transfer, a small amount of electrons are lost at intermediate stages in the electron transport chain, mainly in the complex I and complex III [25–27] in mammals, leading to a monoelectronic reduction of O_2 [28].

This monoelectronic reduction of O_2 results in the formation of anion superoxide radical. While complex I releases superoxide only in the mitochondrial matrix, complex III releases

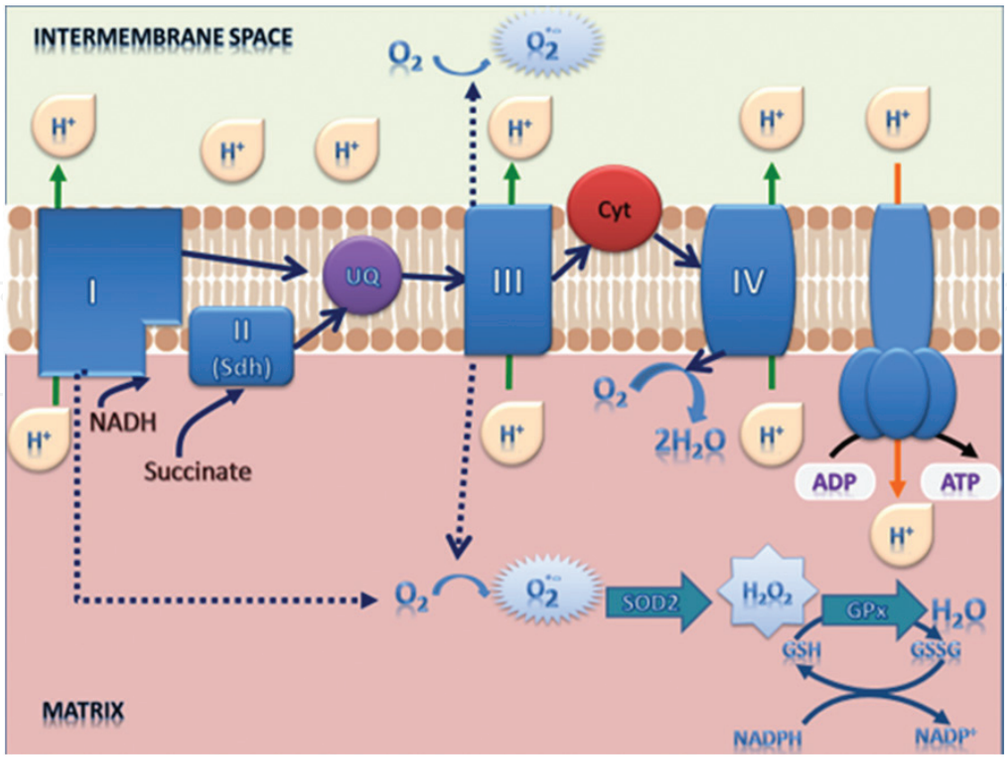

Figure 1. Electron transport chain, ROS, and antioxidant defense. The electron transport chain receives electrons from reduced compounds, as NADH in complex I (also called NADH coenzyme Q reductase) and succinate or $FADH_2$ in complex II (succinate dehydrogenase) and transfers them successively to coenzyme Q or ubiquinone, complex III, complex IV and finally to molecular oxygen with the formation of water. Concomitant with electron transport, protons are transferred from the mitochondrial matrix to the intermembrane space by complexes I (in mammals, but not in yeasts), complex III and IV. The difference in electrochemical potential between intermembrane space and matrix is used by ATP synthase to produce ATP from ADP and inorganic phosphate. During the passage of electrons through the complexes, a small fraction is leaked to oxygen at intermediate points, producing the superoxide radical anion, which in the mitochondrial matrix can be removed by SOD2 forming H_2O_2. Hydrogen peroxide can be converted to water in the mitochondrial matrix by glutathione peroxidase (mammals). Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; UQ, ubiquinone; Cyt, cytochrome c; SDH, succinate dehydrogenase; I, complex I or NADH coenzyme Q reductase; II, complex II or succinate dehydrogenase; III, complex III; IV, complex IV or cytochrome c oxidase; $O_2^{\bullet-}$, superoxide radical anion; SOD2, superoxide dismutase 2 or mitochondrial MnSOD; GPx, glutathione peroxidase. Modified from Ref. [22].

superoxide in both sides of inner mitochondrial membrane [29]. Complex II could theoretically generate superoxide, due presence of flavoprotein in its structure. However, the redox centers are arranged in a manner that aids the prevention of ROS by avoiding the access of O_2 to the flavoprotein. This may explain the reason why this complex does not show a ROS formation by itself [30], but only due reverse electron transfer, i.e., when electrons flow from succinate to ubiquinone and back to complex I [31].

In addition to the electron transport chain, recent studies in mammalian tissues have shown that proteins belonging to the α-ketoglutarate dehydrogenase complex located in the mitochondrial matrix are also a source of ROS in a mechanism stimulated by the low concentration of NAD^+ [32, 33]. In *Saccharomyces cerevisiae*, the deletion of the LPD1 gene, which leads to the inactivation of the enzyme dihydrolipoyl dehydrogenase, E3 subunit of the pyruvate dehydrogenase complex, also leads to a decrease in ROS production. This finding shows the importance of other mitochondrial proteins, other than those associated with the electron transport chain, in the regulation of redox balance [34].

The term reactive specie is not restricted to oxygen, but is also include others, as reactive nitrogen (RNS). Nitric oxide is a membrane permeable free radical that participates in a multiple process in the cells as signaling molecule, but also can contribute in cell oxidative damage. Its effect depends on NO levels and localization in the cell microenvironment [35, 36]. When nitric oxide is present in environment, as in mitochondrial matrix, the reaction of this free radical with superoxide can form others RNS, as peroxynitrite.

Besides mitochondria electron chain and enzyme linked to mitochondrial dehydrogenase complexes, other sources of ROS in cells include enzymes, as NADPH oxidases, cytochrome P450, cyclooxygenases, and the system xanthine/xanthine oxidase. Autoxidation is another example of source of ROS that in cells occurs when a biochemical compound is exposure to O_2, as it occurs in $FADH_2$, L-DOPA and in nitric oxide synthase with generation of superoxide. The auto oxidation can be catalyzed by metallic ions, finally, harm proteins, in which O_2 bind Fe^{2+} could lead to superoxide, as in hemoglobin [37].

1.2. Mitochondria and reactive species: physiological level, oxidative stress, and its implications

ROS and RNS are normally produced in metabolism and have an important role as signaling molecules regulating diverse physiological cell events, as cell signaling, metabolism and regulation of transcription factors [35, 38–42].

The steady state of reactive species will depend on their generation, reactivity and removal by antioxidant defenses. When the level of reactive species generation is much larger than their removal it is said that there is a condition called oxidative stress, i.e., an imbalance between reactive species and antioxidants in favor of reactive species. The maintenance of cell redox state is important to cell viability [43]. The increased level of reactive species can lead to oxidative damage to a vast number of biological molecules, as DNA [44–46], proteins [47], lipids [48], including membranes [3] leading to a range of pathologies, as cancer [36], neurological disease [49], cardiac disease [50, 51], inflammation process [52] and aging.

There is a grand amount of theories about aging process, at least 300 theories according Medvedev [53]. In 1956, Harman proposed in his "free radical theory of aging" that the damage of biomolecules that occurs during aging is due oxidative stress, ROS increments [54]. Mitochondria, as the major site of ROS production, have been associated with aging process [55, 56]. Moreover, studies with caloric restriction in yeast and mammals have shown that the mitochondria, ROS, and RNS have an important role in the aging process [34, 56–61].

2. Protein adducts, DNA oxidation and epigenetic regulation, and effects on biological membranes

During oxidative stress, ROS can attack molecules at electron-dense sites or abstract protons, producing secondary radical species, which undergo conformational change generating more stable products. The molecules that are vulnerable to these deleterious modifications include

the lipids, proteins and nucleic acids. In other words, when the generation of reactive species exceeds antioxidant capacity, the cellular macromolecules also become targets of oxidation by these species. The possible consequences originated from this extensive oxidation, including an increased risk for cardiovascular disease, cancer and neurodegenerative disease (as detailed in Section 4).

2.1. Protein adducts

Under oxidative stress conditions, proteins suffer extensive modification [62–65]. Basically, ROS can oxidize amino acids cysteine and methionine, resulting in the production of dithiol and methionine sulfoxide crosslinks, respectively [66]. Moreover, reactive species also can cause protein modification by nitration of tyrosine and by nitrosation of amino acids with thiol group. These changes often result in the alteration of function or inhibition of enzyme activities. The protein adducts have been observed in several pathologic conditions [67, 68], suggesting their deleterious effects. However, whether these endogenous modifications are produced in a controlled manner, they may also control physiological responses [69, 70].

It is important to stress that the presence of proteins containing nitrotyrosine residues, for example, has been a biomarker of damage by reactive species [67, 68]. The tyrosine nitration occurs by addition of NO_2 to the ortho position of the phenolic ring of this amino acid. In fact, this NO_2 group is obtained from peroxynitrite ($ONOO^-$), a very strong oxidant [71]. During oxidative stress conditions, especially in inflammatory processes, a proportion of $O_2^{\bullet -}$ reacts with NO to form $ONOO^-$. This last is a much more powerful oxidant than $O_2^{\bullet -}$ and, beyond the tyrosine residues, can damage several classes of molecules. $ONOO^-$, its protonated form peroxynitrous acid (ONOOH), and its secondary radical product, react with electron-rich groups, such as sulfhydryls, ironsulphur centers, zinc-thiolates and active site sulfhydryl in tyrosine phosphatases [67, 68, 72, 73].

The thiol group (-SH) of cysteine, for example, it is another relevant protein targets of ROS. Disulfide bond is important in protein structure and function [74], and recently its role in redox signaling has also been evidenced [75]. The reaction of H_2O_2 with the deprotonated thiol group of cysteine produces a sulfenic acid (R-SOH). This last may be oxidized again producing a sulfinic acid ($R\text{-}SO_2H$). With high levels of stress oxidative, cysteines can further be oxidized to a sulfonic acid ($R\text{-}SO_3H$) [70, 76]. While sulfenic and sulfinic acids can be enzymatically reversible by the glutathione and thioredoxin enzyme systems [77] (Details about antioxidant mechanisms in next section), the sulfonic acid in cysteine residues seems to represent an irreversible protein damage.

2.2. DNA oxidation

The reactive species react directly with nucleic acids producing oxidative damage. Since oxidative DNA damage is a major threat to genetic integrity, causing mutations and modifications in gene expression pattern, it has been implicated in a wide variety of diseases, including cancer, cardiovascular and neurodegeneration disease, as well as aging process [46, 73].

The nitrogenous bases as well as the sugar suffer radical attacks, causing several base alterations and strand breaks [78]. In fact, around 80 different bases have been observed in DNA exposed to oxidants [79]. In this context, •OH is the most important reactive species that attacks DNA, since it reacts with the four bases and sugar moiety of the DNA backbone [78, 80] with a reaction rate limited by diffusion (4.5×10^9 to 9×10^9 $M^{-1}s^{-1}$) [79]. •OH attacks carbo-carbon double bonds of bases due to the high electron density. These attacks produce the hydroxylation at C5 and C6 of pyrimidines and C4, C5 and C8 of purines [78, 80]. These secondary radicals are subjected to other oxidation and reduction reactions, producing a wide DNA lesions, including the well characterized derivatives, 7,8-dihydro-8-oxodeoxyguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (FapyGua) [71]. 8-oxo-G is the most stable of these altered bases and can give rise to mutations due to insert Adenine (A) opposite 8-oxo-G during DNA replication, instead of the Cytosine (C) [46, 71].

Another mutation produced by oxidative damage is C to thymine (T) transition, mainly due to the cytosine-derived products uracil glycol and 5-hydroxyuracil mispairing with A, instead of the G [71]. Although other pathways also induce this mutation, it is important to stress that C to T transition is the most frequent mutations found in cancers and in the tumor suppressor gene p53 [81, 82].

2.3. Effects on biological membranes

Under conditions of oxidative stress occur an oxidative process termed lipid peroxidation that affects lipids containing multiple double bonds, such as fatty acids, phospholipids, glycolipids and cholesterol, modifying properties of cellular membranes [73, 83]. This degenerative process is believed to contribute to aging and several diseases, such as atherosclerosis, Alzheimer's disease, peptic ulcer disease, and cancer [84, 85].

Cellular membranes are especially vulnerable to lipid peroxidation not only because of their high levels of unsaturated fatty acids, but also because of their connection with molecules capable of producing reactive species. They attack mainly the unsaturated fatty acids which contain carbon-carbon double bonds and CH_2 groups with particularly reactive hydrogen, and start radical peroxidation chain reactions [86]. These chain reactions are going to terminate when primary or secondary radicals directly react. Lipid peroxidation is accelerated by the presence of Fe^{2+} and Cu^{2+} ions [87, 88]. It is important to stress that lipid peroxides are unstable derivatives from the oxidation of unsaturated fatty acids and decompose to form reactive carbonyl molecules, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [85, 89]. These two products are abundant biomarkers of lipid peroxidation [85, 90].

Membrane-bound proteins are also involved in the process of lipid peroxidation. Aldehyde products, such as MDA and 4-HNE, react with amine and thiol groups of membrane protein, causing several damages, including inactivation of enzymes. Conformational changes of membrane molecules also include lipid–lipid cross-links and lipid–protein cross-links [91, 92].

Moreover, lipid peroxidation modifies the global biophysical properties of the membranes. This process affects the packing of lipids and the permeability to solutes, which in turn, changes its function, including the membrane potential. Furthermore, the process

of peroxidation can inhibit the activity of protein transporters and ion channels [89, 91]. The increase of the permeability also seems to occur in internal mitochondrial membrane, uncoupling respiratory-chain phosphorylation [93]. Finally, the lipid peroxidation leads the severe damages: modification of membrane permeability, enzymatic inhibitions, inactivation of transporters [37, 92].

3. Endogenous/exogenous defense mechanisms

The exposure cells and tissues to the harmful effects of free radicals cause a cascade of reactions and induces activation of some strategies to damage prevent, repair mechanism to alleviate the oxidative damages, physical protection mechanism against damage, and the final most important is the antioxidant defense mechanisms [94, 95].

The antioxidant defenses are the first line of choice to take care of the stress. Endogenous antioxidant defenses include antioxidant enzymes and non-enzymatic molecules that are usually distributed within the cytoplasm and various cell organelles [94]. The exogenous antioxidants are present in consumed fruits, vegetables, juice, tea, coffee, nuts and cereal products [95].

The concept of biological antioxidant refers to any compound present at a lower concentration which is able to either delay or prevent the oxidation of the substrate. Antioxidant functions imply lowering oxidative stress, DNA mutations, malignant transformations, as well as other parameters of cell damage [96]. Antioxidants reactions can deplete molecular oxygen or decreasing its local concentration, removing pro oxidative metal ions, trapping aggressive ROS such as superoxide anion radical or hydrogen peroxide, scavenging chain initiating radicals like hydroxyl OH·, alkoxyl RO· or peroxyl ROO·, breaking the chain of a radical sequence or quenching singlet oxygen ($^1O_2$) [97].

The antioxidants include some high molecular weight (SOD, GPx, catalase, albumin, transferrin, and metallothionein) and some low molecular weight substances (uric acid, ascorbic acid, lipoic acid, glutathione, ubiquinol, tocopherol/vitamin E, flavonoids). Natural food-derived components have received great attention in the last 2 decades, and several biological activities showing promising anti-inflammatory, antioxidant, and anti-apoptotic-modulatory potential have been identified. These enzymatic and nonenzymatic antioxidant systems are necessary for sustaining life by maintaining a delicate intracellular redox balance and minimizing undesirable cellular damage caused by ROS [94, 97, 98].

3.1. Enzymatic antioxidant system

Antioxidant enzymes catalyze ROS conversion directly via an active-site metal ion or through pathways involving the donation of an electron from the moiety-conserved redox couples thioredoxin and glutathione, which require continuous regeneration of the reduced species [99]. Superoxide and H_2O_2 metabolizing enzymes are generally considered to be the primary antioxidant enzyme defense system in the body [98].

The SOD is a family of enzymes catalyzing dismutation of superoxide into oxygen and H_2O_2. Three types of superoxide dismutases can be encountered in mammalian tissues: copper-zinc containing superoxide dismutase (SOD1) present in the cytosol, manganese containing superoxide dismutase (SOD2) found in the mitochondrial matrix and extracellular superoxide dismutase (SOD3). All three are highly expressed, mainly in the renal tubules of healthy kidneys [15, 98, 100]. The final product of the SOD activity - H_2O_2, is then converted into water and oxygen by the catalase (CAT). This enzyme is a homotetrameric protein containing four iron heme and largely located in the peroxisomes [15, 100].

Other important enzymatic antioxidants in the first line of defense include glutathione peroxidase (GPX) and myeloperoxidase (MPO) enzymes. The GPX is a selenium-containing enzyme, catalyzes both the reduction of H_2O_2, and organic hydroperoxides to water or corresponding alcohols. Reduced glutathione functions as effective electron donor in the process, as free thiol groups are oxidized to disulfide bonds: $H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O$ [97]. The MPO, a heme peroxidase, abundant in granules of human inflammatory cells, catalyzes the conversion of H_2O_2 to HClO with the production of ROS. The ROS production is associated with cardiovascular disease, chronic obstructive pulmonary disease, and Alzheimer's disease. Oxidant species derived from MPO lead to the production of specific oxidation products, such as 3-Cl-Tyr. This can be used as biomarker in several diseases, as above described, and its levels correlate with MPO [100].

Other enzymes could be cited by our antioxidant activity, such as Peroxiredoxin Family (PRX). These enzymes are a family of abundantly present 20–30 kDa peroxidases that are excessively reactive with H_2O_2. So, they are likely to be critical for both oxidative stress protection as well as redox signaling [98]. The antioxidant enzymes may possibly offer novel treatment options for redox-related diseases, provided that the molecular mechanisms are known and can be specifically targeted. Besides that, inhibiting a given antioxidant enzyme or specifically silencing its gene expression may help treat disorders related to a gain of enzymatic function [98] and this fact can will help the researchers to explore future options in enzymatic antioxidant system and diseases.

3.2. Nonenzymatic antioxidant systems

Among the nonenzymatic antioxidant compounds, the principals are obtained from dietary as the class of phenolic compounds, vitamins C and E, and carotenoids [101]. Phenolic compounds represent a large group of secondary metabolites [102], among them flavonoids, phenolic acids, tannins and tocopherols as the most common natural source phenolic antioxidants [103].

The phenolic compounds are composed of one or more aromatic rings with varying degrees of hydroxylation, methoxylation and glycosylation, and various studies have associated the structure of phenolic compounds with their antioxidant properties [102, 104]. The antioxidant activity generally increases with the degree of hydroxylation in aromatic rings and decreases with C-3 methoxylation [105, 106]. The antioxidant activity is based on the availability of electrons to neutralize the free radicals; in addition, it is related to the number and nature of the hydroxylation pattern in the aromatic ring and the ability to act as a hydrogen donor [106].

The flavonoid group is the most diverse within phenolic compounds, with two aromatic rings associated via C-C bonds by a 3C oxygenated heterocycle. Flavonoids have antioxidant and chelating properties, inactivate ROS, acting against the oxidation of low density lipoproteins (LDL) and improving inflammation of the blood vessels. They also reduce the activity of the xanthine oxidase enzymes and the nicotinamide adenine dinucleotide phosphate oxidase, enzymes that stimulate the production of ROS [107].

In cellular compartments, flavonoids function as antioxidants inactivating free radicals both in hydrophilic and lipophilic compartments. For example, the antioxidant activity of phenolic compounds present in spices (cinnamon, sweet weed and mustard) differs between aqueous and lipid systems [108].

Vitamins C and E act together to inhibit lipid peroxidation and protect the cell against oxidative damage, as DNA damage. The antioxidant activity of vitamin C involves the transfer of an electron to the free radical and the consequent formation of the radical ascorbate [109]. In addition, vitamin C acts synergistically with vitamin E, which regenerate the vitamin C has better antioxidant activity in hydrophilic media, and in aqueous phase of extracellular fluids, it is able to neutralize ROS in the aqueous phase before they can attack lipids. Vitamin E is an important fat soluble antioxidant, acting as the chain breaking antioxidant within the cell membrane and playing an important role in the protection of membrane fatty acids against lipid peroxidation [110].

Vitamins C and E inhibit lipid peroxidation and protect against oxidative damage by their scavenging actions of ROS, as well as by modulateing numerous enzymatic complexes involved in the production of ROS, endothelial function and aggregation of platelets. These vitamins can also regulate NADPH oxidase, the most important source of $O_2^{\bullet -}$ in the cardiovascular system. It has been reported that ascorbic acid and α-tocopherol, derivated from vitamin C and E respectively, may involved in the transcriptional modulation of NADPH oxidase [111].

The most common carotenoids are xanthophylls and carotenes. Carotenoids can neutralize singlet oxygen by quenching it or can break the chain reaction of free radicals, or scavenging it, not so effective action (scavenging). The structure of the free radical is the main factor that determines if the carotenoid will have quenching or scavenging action. It also depends on the region where the radical is in heterogeneous biological tissue, aqueous or lipid region (plasma, blood, heart, liver, brain etc.), and the structure of the carotenoids (number of conjugated, cyclic or acyclic double bonds), polar or nonpolar groups, redox properties [112–114].

The physical quenching is the transfer of excitation energy from the singlet oxygen to the carotenoid. The oxygen returns to ground state and the carotenoid is in the excited triplet state, the energy is dissipated producing stable carotenoid and thermal energy and the carotenoid can undergo other cycles of singlet oxygen quenching [112, 115].

The chemical quenching the carotenoid combines with oxygen or is oxidized, leading to its destruction and producing a variety of oxidized products. Carotenoids can also extinguish the triplet-excited state of chlorophyll or other excited sensitizers, thus preventing the formation of singlet oxygen [112]. The free radical scavenging can occur in three ways, by electron transfer, by hydrogen abstraction, and by addition [112, 116].

4. Interaction between reactive species, enzymes, and antioxidant molecules in health and disease

All living cells have molecular tools to perceive and respond properly to environmental cues. All the cascades of intracellular reactions involved in promoting a biochemical response are denoted as signal transduction. There are well known receptor types or systems of signal transduction such as the G protein-coupled receptors (GPCR), tyrosine kinase receptors (TKR), ion channels, cell adhesion receptors, nuclear receptors and guanylyl-cyclases. Since cells often need to deal with many signals at the same time, the final biochemical response is a result of the integrations of many simultaneous cascades produced by one or more systems.

Before we move on exploring the targets of ROS in health and disease, an important question is raised: "Which are the main sources of cellular ROS?" Enzymes such as NADPH oxidases (Nox), xanthine oxidase (XO), lipoxygenase, MPO and uncoupled nitric oxide synthase are involved in the production of the anion radical superoxide ($O_2^{\cdot -}$). Furthermore, the mitochondrial aerobic respiration contributes with a huge amount of $O_2^{\cdot -}$. Peroxynitrite ($ONOO^-$) is formed by the reaction of nitric oxide and superoxide and is thought to contribute to eNOS uncoupling [69]. The majority of $O_2^{\cdot -}$ generated within the mitochondrial matrix or the cytosol is dismutated to H_2O_2 by the SOD antioxidant enzyme. Moreover, metal exposure can mediate the generation of H_2O_2, $O_2^{\cdot -}$, and even the hydroxyl radical ($OH^{\cdot}$), mainly via the Fenton or the Haber-Weiss reactions [117].

Some ROS such as $O_2^{\cdot -}$ and $HO^{\cdot}$ are highly reactive and have a brief half life. For this reason they are not considered signaling molecules, but intermediates of nonselective nature. On the other hand, H_2O_2 is relatively stable and can both mediate intracellular signaling and also serve to paracrine signaling (i.e., cell-to-cell communication involving nearby cells), since it can cross biological membranes [118].

Up to date, several proteins have been recognized as downstream targets of ROS, such as kinases, phosphatases, mitogen-activated protein kinases (MAPK), small G proteins, transcription factors, microRNAs, and phospholipases. In this section, we do not intend to deeply review the literature, but to show an overview of important targets and exemplify their involvement in the signal transduction by ROS in health and disease.

ROS can induce alterations in the intracellular and extracellular processes, for example, in the PI3K/AKT signaling. The lipid phosphatidylinositol 3,4,5-triphosphate (PIP3) has a function as a second messenger and is not present in the quiescent cells, but it rises within seconds to minutes when there is a stimuli. PIP3 is produced by the phosphorylation of the phosphatidylinositol 4,5-bisphosphate (PIP2) catalyzed by the phosphatidylinositol 3-kinase (PI3K). This enzyme is activated by ROS through two different pathways, or directly, throught amplications of downstream PI3K pathway, or indirectly by inhibition of the phosphatase and tensing homolog deleted on chromosome 10 (PTEN). PTEN is responsible for the degradation of PIP3 signaling, since it catalyzes the hydrolysis of phosphate in the 3′ position on PIP3 to produce PIP2 [119]. ROS, mainly, H_2O_2, can oxidize and inhibit PTEN, which culminates in an increase in the PIP3 production, that acts in cell signaling, through activation of proteins, as serine/threonine protein kinase, AKT/PKB, among others [120, 121]. The AKT activation provides the transcription of

several targets, such as GSK3, BAD, FOXO, p53, NF-kB, mTOR/p70S6K1 and HIF-1 [122, 123]. In this way, ROS increase the final cascade response in cell, i.e., cell cycle progression, proliferation, anti-apoptosis, invasion, autophagy and angiogenesis [124]. The PI3K/AKT pathway hyper activated by ROS might favor carcinogenesis in the end of the process.

An important class of redox regulated proteins is the Src family of nonreceptor tyrosine kinases (SFKs), a group of structurally related kinases that catalyze the phosphorylation of tyrosine in downstream targets to regulate cellular functions coupling receptors such as the TKR, the cell adhesion molecules (CAMs), and the GPCR to the cellular signaling machinery [125]. For example, during focal adhesion while the extracellular matrix (ECM) contact triggers a slight or partial activation of SFKs, the ROS production is associated with a strong oxidative-dependent activation and recruitment of Src kinases to cell membranes. The redox-activation of SFKs can induce sustained PI3K, protein kinase C (PKC), and extracellular regulated kinase (ERK) activation and, thereby, create conditions for tumor cell growth, invasion, angiogenesis, and resistance to apoptosis [126]. In a variety of human cancers an increased activity of Src kinases have been described, as well as activation of important Src downstream targets such as PI3K/Akt, focal adhesion kinase (FAK), paxillin, p130Cas, signal transducer and activator of transcription 3 (STAT3) and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) [127–130].

Carcinogenesis is also related with activator protein-1 (AP-1) transcription factor activation. Among other systems, ROS are recognized as activators of AP-1; however, the signaling transduction events involved are not totally understood. Chromium, cobalt, cadmium and vanadium are metals involved in the activation of AP-1 through signaling cascades involving the production of ROS and comprised of proteins and enzymes such as thioredoxin (Trx), redox factor-1 (Ref-1), ERK/MAPK, NADPH oxidase, I kappa B kinase (IKK), p38, JNK/c-jun [117, 131, 132].

ROS are important for the regulation of vascular tone, however an excess of reactive species might be associated with pathological dysfunction. Endothelial nitric oxide synthase (eNOS) regulates smooth muscle cells (SMC) relaxation through the production of the second messenger nitric oxide (NO) from L-arginine, which activates guanylyl cyclases to initiate the conversion of GTP to cyclic guanosine monophosphate (cGMP), which allosterically activates the cGMP-dependent protein kinases (PKGs). The enzyme eNOS can be uncoupled and, consequently, change its profile from NO synthesis to $O_2^{\cdot -}$ production instead. Two major events are involved in eNOS uncoupling. First, an increase of ROS might generate the peroxynitrite ($ONOO^-$) through the reaction of NO and $O_2^{\cdot -}$. The anion $ONOO^-$ reacts with and oxidizes tetrahydrobiopterin (THB/BH_4), a cofactor of eNOS [133]. Second, an increased ratio of oxidized glutathione (GSSG)/reduced glutathione (GSH) cause reversible S-glutathionylation and uncoupling of eNOS [134]. Paradoxically, H_2O_2 produced by NADPH oxidase increases eNOS expression and NO production, but this effect is not believed to counteract the effects of oxidative stress [135].

Interestingly, in a scenario of reduced NO levels, in which it would be expected a lack of input signals to PKG activation (e.g., cGMP), the H_2O_2 can cause vasodilation through PKG oxidation [136]. Another target of ROS is the small GTPase RhoA, which when oxidized activates its downstream partner Rho kinase (ROCK), leading to inhibitory phosphorylation of myosin light chain (MLC) phosphatase and, ultimately, to SMC contraction [137, 138]. For a more

explored involvement of ROS in the regulation of signal transduction in the cardiovascular system, check the review of Brown and Griendling [118].

The activating or deactivating switch, in which a group of kinases is active or a group of phosphatases is active, provokes different downstream cascades with consequences in the cellular response. As we described above, several kinases are susceptible to ROS reactions, but also phosphatases are vulnerable to ROS, since they react with a group of amino acids presents in different enzymes. The reaction between ROS and phosphatases causes the oxidation and inhibition of those enzymes, increasing the kinases signaling [139]. Another phosphatase inhibited by ROS is PTEN, which increases the PIP3 signaling, as described above.

A vascular injury promotes an increase in the expression of platelet derived growth factor (PDGF) and PDGF receptor, which in turn cause stimulation for the vascular smooth muscle cells to migrate [140]. The activation of the PDGF receptor is controlled by the action of low molecular weight protein tyrosine phosphatase (LMW-PTP). The Cys12 and Cys17 in LMW-PTP is susceptible to a reaction with ROS resulting in a disulfide bond, and so its inactivation [141]. Therefore, without the LMW-PTP deactivation upon PDGF receptor, its signal is amplified, which generates migration. Oxidized LMW-PTP also increases the Rho family signal, since PDGF receptor is stimulated, and it binds to phospholipase C, Src, and PI3K. As described before, PI3K catalyzes the reaction and formation of PIP3. The Rho-guanine nucleotide exchange factors are activated by PIP3, which triggers Rho-GTPase family members' activation (Rho, Rac, and cdc42). As Nox family is activated by Rac, it produces ROS. Therefore, this process is kept by a positive feedback: generated ROS oxide Rho in a redox sensitive motif and restrain the LMW-PTP action [118, 138].

Phospholipases are enzymes that hydrolyze phospholipids and generate second messengers involved in the regulation of many physiological functions. Phospholipase A2 (PLA2) cleaves the fatty acyl group at the sn-2 position of the glycerol backbone, releasing arachidonic acid (AA) and lysophospholipid. It was attributed a role for the Ca^{2+}-independent PLA2 (iPLA2) isoform in the excessive production of $O_2^{\cdot-}$ by primed neutrophils of patients with poorly controlled diabetes. This study suggested that hyperglycemia is related to the activation of iPLA2 and AA formation which, in part, regulate NADPH oxidase activity (i.e., generation of $O_2^{\cdot-}$) [142].

PLA2 activation has also been related to alterations implicated in the pathogenesis of neurodegenerative diseases, such as neuronal excitation, cognitive and behavioral function, oxidative and nitrosative stress [143]. Phospholipase C (PLC) is a well-known enzyme especially involved in the signaling transduction of GPCR coupled to $G_{q/11}$ protein and some G protein $\beta\gamma$ subunits (PLC-β), but also in RTK (PLC-γ and PLC-ε), Ras and Rho small GTPases (PLC-ε) and Ca^{2+} (PLC-δ) signaling pathways, which involves the generation of the phosphate-containing head group inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) through the hydrolysis of the membrane phospholipid PIP2 [144]. The activation of PLC-γ1 was shown to have an important protective function during mouse embryonic fibroblasts (MEF) response to oxidative stress (H_2O_2) treatment [145]. A further study suggested that this function of PLC-γ1 involved the PKC-dependent phosphorylation of Bcl-2 and inhibition of caspase-3 [146]. Phospholipase D (PLD) cleaves a phosphodiester bold in membrane-bound lipids, similarly to PLC. However, its activity generates phosphatidic acid (PA) and an alcohol, usually choline or

ethanolamine [147]. A link between oxidative stress and PLD has been proposed by Kim et al. [148], in a study that suggests that H_2O_2 induces rat vascular smooth muscle cells tyrosine kinase activity, and PLD1-dependent PKC-α activation.

In the innate immune system, mononuclear monocytes/macrophages eliminate pathogen, antigen and cellular components through generation of ROS/RNS [149]. When there is an imbalance in the equilibrium between oxidative/nitrosative stress and cellular requirements, the stress can generates pathological complications. Among others, rheumatoid arthritis is an autoimmune disease that has oxidative/nitrosative stress as one of the causes. The cellular immune system is vulnerable to reactions caused by ROS, which in turn can affect the regular physiological process and activates inflammatory signaling pathways that produce pro-inflammatory cytokines, chemokines and prostaglandins. The inflammatory mechanism involves synovial cellular infiltrate and peripheral blood inflammatory cells following by polymorphonuclear neutrophils and lymphocytes culminating in the joint damage [150, 151]. The signaling cascade occurs via activation of NFkB for synthesizing pro-inflammatory cytokines and chemokines [149]. The Th1 cytokines are one of the most important because can provide the development of autoimmune disorders. These cytokines can directly or indirectly promote oxidative stress in the cells, intensifying the rheumatoid arthritis.

Prostaglandins have a pivotal role in the formation of the inflammatory response, since they mediate pathogenic mechanisms and provide the development of the cardinal signs of acute inflammation. Their biosynthesis involves the initial enzyme, phospholipase A2 (PLA2). PLA2 catalyzes the conversion of membrane phospholipids in AA. Then, cyclooxygenases convert AA into prostaglandins. Prostaglandin E2, in particular, rises vasoactive components (histamine, bradykinin, and nitric oxide), hence generating edema, pain and hyperalgesia at the local inflammatory sites, and so the inflammation [152]. ROS stimulate this process through the activation of cyclooxygenases. Prostaglandins, also, activate NADPH oxidase, which produces superoxide anion radical [153]. Therefore, this system becomes cyclic, ROS activate cyclooxygenases and so the prostaglandins biosynthesis, further prostaglandins trigger NAPH oxidases, increasing ROS.

The microRNA (miRNA) is a small noncoding endogenous RNA, that has an important role, since it regulates gene expression. Its function can be modified depending on epigenetic changes, chromosomal abnormalities and oxidative stress. It has been found that miRNA can respond to ROS, implying in its ability to activate certain genes transcription during stress, and this is prominent in cancer cells, which was correlated to the adaptation of these cells to unfavorable and/or hypoxic environment [130, 154, 155]. However, studies showed that some types of miRNAs can regulate gene expression of protective proteins and antioxidant enzymes [156, 157]. Some ROS dependent miRNAs play a role as oncogenic (miR21 and miR155), but interesting miR21 also targets SOD, which can be interpreted that this miRNA regulate the ROS levels in the cell. When miR21 is stimulated, it also affects the immune system through the chemokine CXCL10. CXCL10 adjusts innate and adaptive immune response by activating T lymphocytes, macrophages and inflammatory dendritic cells. The miR155 also has opposite actions, it can be oncogenic (the targets are BCL2, FOXO3a, RhoA) or tumor suppressor (the targets are TGF-beta/SMAD) [158]. The literature about miR155 is vast, and we suggest the articles by Higgs and Slack [158] and Mattiske et al. [159] for a deep reading. Besides these two

miRNAs cited above, others miRNAs are upregulated by ROS, such as miR23, miR200, miR210, etc., affecting migration, invasion; tumor growth, angiogenesis; cell cycle, DNA damage (among others), respectively [126].

In addition to the miRNAs that are ROS upregulated as cited above, there are ROS downregulated miRNAs important in the carcinogenic process, such as miR34 family. Some miR34 members regulate p53 causing a cell cycle arrest in G1 and apoptosis when DNA is impaired. The miR34a, for example, induce tumor suppression and metastasis inhibition. Another miRNA, miR124, has been shown to be affected by H_2O_2 [160]. This miRNA is correlated to the regulation of tumor cell proliferation, migration and drug resistance through its action upon R-Ras, PI3-KCA, AKT2, ROCK1, Src, DNA methyltransferases and others. The miR199a is also downregulated by ROS, some of its targets are ERBB2, ERBB3, IKKB, HIF-1alfa, ApoE, CCR7, having an effect upon cell proliferation, invasion, metabolism and metastasis [126, 161]. This is just a

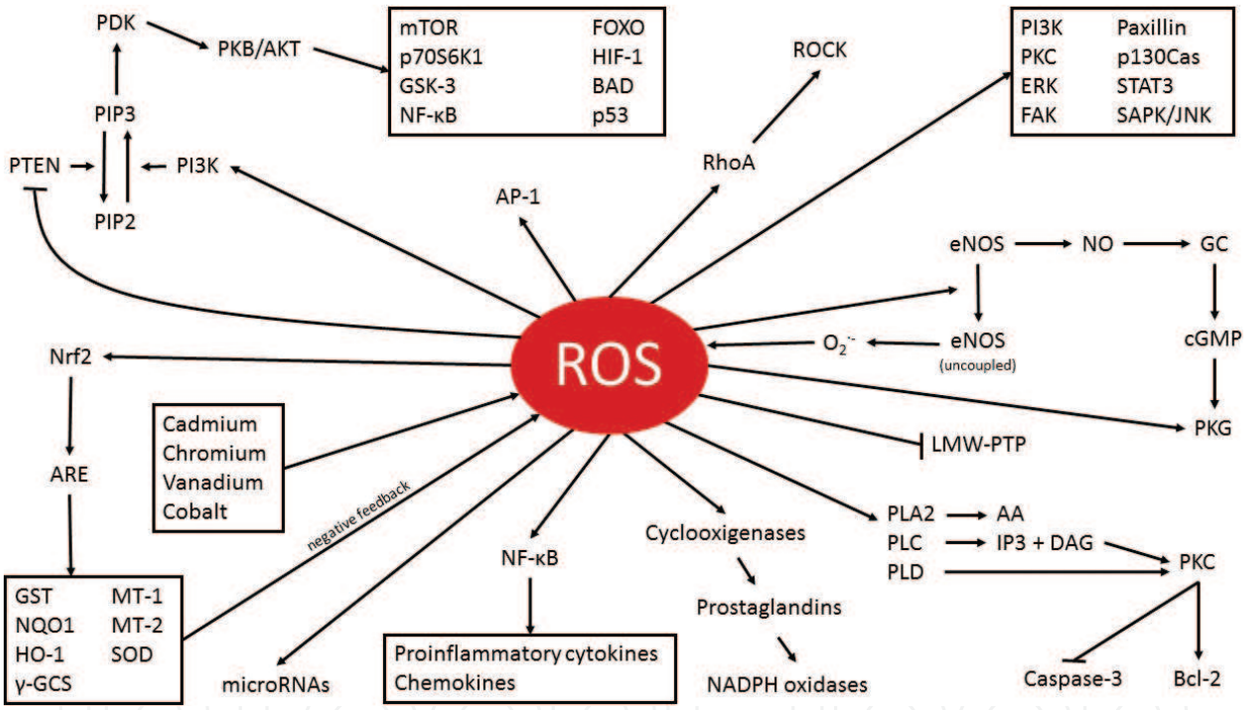

Figure 2. Examples of molecular targets involved in the signal transduction mediated by reactive oxygen species. Abbreviations: AA, arachidonic acid; AP1, activator protein 1; ARE, antioxidant-responsive element; BAD, Bcl-2-associated death promoter; Bcl-2, B-cell lymphoma 2 protein; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; ERK, extracellular-signal regulated kinase; FAK, focal adhesion kinase; FOXO, Forkhead box protein O; GC, guanylyl cyclase; GSK-3, glycogen synthase kinase 3; GST, glutathione S-transferases; HIF-1, hypoxia-inducible factor 1; HO-1, heme oxygenase 1; IP3, inositol 1,4,5-triphosphate; LMW-PTP, low molecular weight phosphotyrosine protein phosphatase; MT-1, metallothionein-1; MT-2, metallothionein-2; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, nuclear-factor erythroid-2 related factor; $O_2^{\cdot-}$, superoxide anion radical; p130Cas, p130 Crk-associated substrate; p53, p53 tumor suppressor protein; p70S6K1, p70S6 kinase 1; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate; PKB/AKT, protein kinase B; PKC, protein kinase C; PKC, protein kinase C; PKG, cGMP-dependent protein kinases; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RhoA, Ras homolog family member A; ROCK, Rho-associated protein kinase; SAPK/JNK, stress-activated protein kinase or c-Jun N-terminal kinase; SOD, superoxide dismutase; STAT3, signal transducer and activator of transcription 3; γ-GCS, gamma-glutamylcysteine synthetase.

summary of some important miRNAs and their responses in carcinogenesis, for more information check the review Mu and Liu [126].

As previously discussed in this chapter, cells have a repertoire of antioxidant molecules and enzymes as a defense mechanism to an increase in ROS production. However, oxidative stress takes place when the antioxidant capacity is overwhelmed by reactive species production. In this scenario, to maintain cell homeostasis and/or terminate the ROS signal transduction there are some stress sensors that regulate the translation of antioxidant proteins. The antioxidant responsive element (ARE) is a region of non-coding DNA (short consensus sequence) which is localized upstream and regulates the transcription of many antioxidant neighboring genes such as glutathione S-transferases (GST), NAD(P)H:quinone oxidoreductase (NQO1) [162], heme oxygenase 1 (HO-1), γ-glutamylcysteine synthetase (γ-GCS) [163], metallothionein-1 and -2 (MT-1 and MT-2) [164], and SOD [165].

It was shown that ARE induction protected against oxidative stress mediated by 6- hydroxydopamine in vitro, a mitochondrial inhibitor used to model Parkinson's disease [166]. The nuclear-factor erythroid-2 related factor (Nrf2) is a central transcription factor involved in the upregulation of ARE-containing genes and, consequently, synthesis of proteins with antioxidant function. However, there are also nuclear factors that negatively regulate ARE-mediated gene expression, such as Mafs (MafG and MAfK), large Maf (c-Maf), c-Fos, and Fra1 [163].

Finally, in this section, we showed an overview of processes regulated by fluctuating levels of ROS and their molecular sensors. Furthermore, we showed that in response to oxidative stress and to maintain homeostasis, cells can upregulate the synthesis of antioxidant defenses (**Figure 2**).

Acknowledgements

H.U.'s research is supported by the São Paulo Research Foundation (FAPESP proj. No. 2012/50880-4).

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