Review

Free radicals in the regulation of damage and cell death – basic mechanisms and prevention

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ABSTRACT: Reactive oxygen (ROS) and nitrogen (RNS) species are known to accumulate intracellularly due to both exogenous and/or endogenous factors. In normal physiological conditions, these reactive species are maintained in an equilibrium state by the cells' antioxidant defence systems. In addition, they are recognised to play important roles in several physiological functions. However, when an imbalance in the equilibrium between oxidants and antioxidants occurs in favour of the former, we come to a situation defined as oxidative stress. ROS/RNS can cause damage to all biomolecules (namely proteins, lipids, and DNA) and ultimately participate in the regulation of mechanisms leading to cell death, being implicated in the etiology of several pathologies (like neurodegenerative and cardiovascular diseases). To cope with oxidative stress, cells possess effective enzymatic (*e.g.* superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (*e.g.* glutathione, thioredoxin, coenzyme Q) antioxidant systems. In addition, several compounds present in plants and vegetables (*e.g.* vitamins C and E, polyphenols) have been described to react with free radicals. However, some drawbacks associated to these natural compounds are in part responsible for the undergoing development of novel synthetic compounds capable of acting as antioxidants and protect cells against oxidative stress-induced cell death. Here, we review the basic mechanisms of ROS/RNS formation, as well as their interaction with biomolecules and regulation of cell death, in order to identify possible drug targets. We also report the importance of natural antioxidant systems and the ongoing research leading to the development of more powerful and effective antioxidant drugs.

Keywords: Oxidative stress, ROS/RNS, antioxidants, cell death

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1. Oxidative stress

There is a general agreement among the scientific community that significant amounts of oxygen (O_2) first appeared in the Earth's atmosphere about 2.5 billion years ago. However, the toxicity of oxygen led the existing anaerobic organisms to adapt to the new environment by developing defence mechanisms, in order to protect themselves and survive (*1*). Though some of them chose to live in an oxygen-free micro-environment, others became capable of performing both respiration and fermentation (facultative anaerobes). Only later on there was the development of new species that used respiration in an exclusive way, the aerobic organisms (*2,3*).

Initially, O_2 toxicity was thought to be due to the inactivation of enzymes, mainly the thiol group of cysteine residues. Later, toxicity was also attributed to the effects of hydrogen peroxide (H_2O_2). Ultimately, molecular biology techniques established that the toxic effects of O_2 are directly linked to its partially reduced forms, the reactive oxygen species (ROS), acting on cellular components (*4*). More than 50 years ago, Denham Harman, in his "Free Radical Theory of Aging", described these reactive species as being responsible for the most part of cellular damage and as playing a key role in the aging process [*in* (*5*)]. The discovery of the enzyme superoxide dismutase (SOD) in 1969 by McCord and Fridovich (*6*) definitively convinced the rest of the scientific community of the importance of free radicals in living systems. This led to further investigation in this field, which resulted in the finding that these free radicals also intervene in the regulation of intracellular signalling. So, as living systems evolved, they have not only adapted to a coexistence with free radicals but have developed various defence mechanisms to protect themselves against the toxicity of oxygen (*5,7*).

In normal physiological conditions, the production of free radicals and other reactive species is maintained in an equilibrium state by this antioxidant defence system (*2*). However, when an imbalance occurs between oxidants and antioxidants in favour of the oxidants, we come to a situation defined as oxidative

stress (*8*). This can result from two different factors: a decrease in antioxidants, due to mutations affecting antioxidant enzymes or depletion of antioxidants along with other essential diet constituents; or an excessive production of oxygen and nitrogen reactive species, for example, by exposure to high levels of O_2, by the presence of toxins that are metabolised to produce ROS, among others (*2*).

Oxidative stress can cause damage to all biomolecules and ultimately lead to cell death, being implicated in the etiology of several pathologies, such as atherosclerosis (*9*), neurodegenerative diseases (*10,11*), and ischemia-reperfusion injury (*12,13*).

1.1. *Formation of reactive species*

Intracellular accumulation of reactive oxygen (ROS) and nitrogen (RNS) species can be triggered by both exogenous and/or endogenous factors (*14*), as summarised in Figure 1. Mitochondria reduce to water over 95% of all the oxygen used by the cell, mostly due to the presence of an electron transport chain (ETC). However, a small percentage of the total oxygen escapes, resulting in the formation of superoxide radicals, and subsequently other reactive species, like hydrogen peroxide and hydroxyl radicals (*15,16*). In fact, these organelles are packed with several redox carriers that can potentially leak single electrons to oxygen and convert it into superoxide anion, which, by its turn, leads to the subsequential formation of other ROS. Given the moderate redox potential of the superoxide/dioxygen ($O_2^{\cdot-}/O_2$) couple ($E_{1/2} = -0.16$ V), the reaction of one-electron reduction of oxygen is thermodynamically favourable for numerous mitochondrial oxidoreductases [*in* (*17*)].

Considering that superoxide is effectively removed from the reaction by some detoxifying enzymes, like superoxide dismutase, and the possibility of highly reduced state of many redox carriers, the reaction becomes virtually irreversible. So, which of the electron carriers do become the sites of ROS production is kinetically controlled (*17,18*). Some examples of these carriers include the cytochrome *b*5 reductase and the monoamine oxidases (located in the outer mitochondrial membrane), α-glycerophosphate dehydrogenase (outer surface of the inner mitochondrial membrane), succinate dehydrogenase (complex II, situated at the inner surface of the inner membrane), aconitase (in the mitochondrial matrix), and the α-ketoglutarate dehydrogenase complex (associated with the matrix side of the inner membrane). Nevertheless, the majority of ROS are produced in complexes I and III of the mitochondrial respiratory chain (*19*). Best understood is the Complex III (ubiquinol-cytochrome *c* reductase) contribution to superoxide generation. Within this complex, the transfer of electrons from ubiquinol (UQH_2) to cytochrome *c* proceeds through a set of reactions known as the Q-cycle. However, during this process, the one-electron reduction of oxygen by the ubisemiquinone anion radical ($UQ^{\cdot-}$), an unstable intermediary of the Q-cycle, may lead to the formation of superoxide, in both the inner and outer sides of the inner mitochondrial membrane (*17,19*).

Complex I (NADH dehydrogenase, also named NADH-ubiquinone oxidoreductase), appears to be the primary source of superoxide formation in the brain,
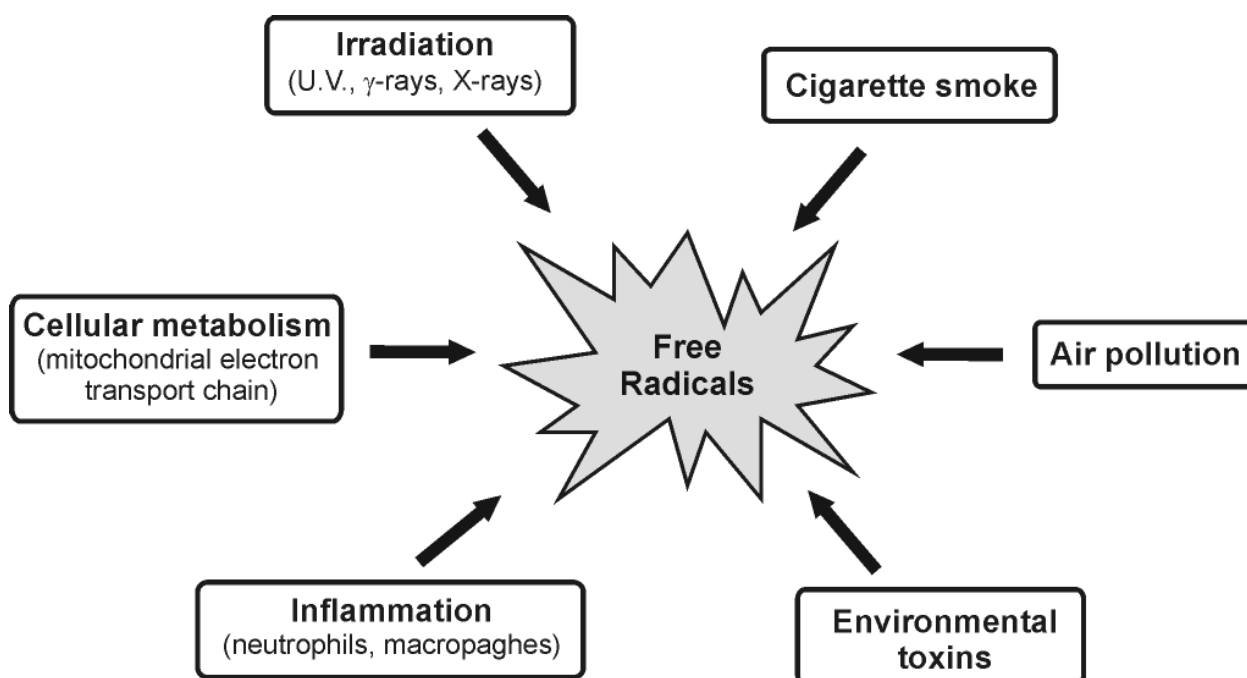

Figure 1. Summary of exogenous and endogenous sources of free radicals.

under normal conditions (*18,20*). It oxidises NADH using ubiquinone as an electron acceptor in a reversible reaction coupled with a proton pump generating transmembrane potential. To date, the increase in ROS production in this complex has been observed in three different situations: during normally functioning respiratory chain, in the presence of rotenone (an inhibitor that blocks the transfer of electrons from complex I to ubiquinone) and during reverse electron transfer (a set of reactions in the respiratory chain that allow electrons to be transferred against the gradient of redox potentials of electron carriers, from reduced ubiquinone to NAD^+, instead of oxygen).

Besides mitochondria, there are other endogenous sources of ROS. For example xanthine oxidase, a widely distributed enzyme in the tissues of mammals, during the catalisation of the reaction of hypoxanthine to xanthine and xanthine to uric acid, leads to the reduction of molecular oxygen, forming the superoxide anion in the first step and hydrogen peroxide in the second one (*21*). Additional sources of cellular ROS are inflammatory cell activation (neutrophils, eosinophils and in particular macrophages) (*22*), cytochrome P450 metabolism (namely following the breakdown or uncoupling of the P450 catalytic cycle) (*23*), and peroxisomes, which mainly lead to the production of H_2O_2 (*24*).

1.2. *Chemistry and biochemistry of ROS/RNS*

The most common intracellular forms of reactive oxygen and nitrogen species include radical ($O_2^{\bullet-}$, $^{\bullet}OH$, $^{\bullet}NO$, among others), as well as non-radical (O_2, $ONOO^-$, H_2O_2, $HOCl$, O_3) moieties, that can be deleterious to cells (*25*).

Free radicals can be defined as molecules or molecular fragments, capable of independent existence, which contain one or more unpaired electrons. The presence of these unpaired electrons confers them a considerable degree of reactivity, since they need another electron to fill the orbital and become stable (*1,26*). Free radicals are often reactive species, although the opposite is not always true. Hydrogen peroxide (H_2O_2), for example, although considered a reactive species, is not regarded as a free radical since it has no unpaired electrons in its structure (*27*). On the other hand, molecular oxygen (dioxygen, O_2) has a unique electronic configuration and is considered a reactive species.

The addition of one electron to molecular oxygen forms the superoxide anion radical ($O_2^{\bullet-}$), which, despite being a free radical, is not highly reactive, since it lacks the ability to cross lipid membranes. In this way, it stays enclosed in the compartment where it is generated (*27*). It is considered the "primary" ROS, from which "secondary" ROS can be generated (*26*), and arises mainly through metabolic processes (electron transport chain in the mitochondria, flavoenzymes like

xanthine oxidase, lipoxygenases and ciclooxygenases, and NADPH oxidase). In the presence of superoxide dismutase, two molecules of this radical can easily be transformed to hydrogen peroxide and molecular oxygen (*28*).

Singlet oxygen is also a very reactive ROS that induces various genotoxic, carcinogenic, and mutagenic effects through its action on polyunsaturated fatty acids (PUFAs) and DNA (*29*).

Hydrogen peroxide (H_2O_2) is not a free radical. Nonetheless, it is highly important, much because of its ability to penetrate biological membranes. It plays a radical forming role as an intermediate in the production of more reactive ROS molecules including HOCl (hypochlorous acid) by the action of myeloperoxidase (an enzyme present in the phagosomes of neutrophils) and, most importantly, in the formation of the hydroxyl radical *via* oxidation of transition metals (*1,27*). Once produced, H_2O_2 is removed by at least three antioxidant enzyme systems, namely catalases, glutathione peroxidases, and peroxiredoxins (*27*).

The hydroxyl radical ($^{\bullet}OH$) is highly reactive, being able to attack and damage all biomolecules: carbohydrates, lipids, proteins, and DNA (*10*). Since it has a half-life in aqueous solution of less than 1 nanosecond, when produced *in vivo*, this radical reacts close to its site of formation. Production of $^{\bullet}OH$ close to DNA could lead to this radical reacting with DNA bases or the deoxyribosyl backbone of DNA to produce damaged bases or strand breaks (*26*). It can be generated through several mechanisms, including ionising radiation and photolytic decomposition of alkylhydroperoxides, but the majority of the hydroxyl radicals produced *in vivo* comes from metal catalysed (mainly involving iron and copper) breakdown of hydrogen peroxide, according to the Fenton reaction (*21,27*). Superoxide also plays an important role in this reaction, by recycling the metal ions.

Transition metals thus play an important function in the formation of various free radicals, as is the case of hydroxyl radicals. The redox state of the cell is largely linked to an iron (and sometimes copper) redox couple and is maintained within strict physiological limits. In fact, iron, for example, is required by the human body for the synthesis of several enzymes and its adequate supply during early life is essential for normal brain development (*10,30*). However, its ability to transfer single electrons as it oscillates between the ferrous and ferric states makes it a powerful catalyst of free radical reactions (*2*). These transition metals can be released from proteins like ferritin and the [4Fe-4S] center of different enzymes of the dehydratase-lyase family, by reactions with the superoxide anion (*27,31*).

Nitric oxide ($^{\bullet}NO$) is synthesised from L-arginine by three isoforms of nitric oxide synthase (NOS): neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric

oxide synthase (iNOS) (*32*).

Like superoxide anion, $^•$NO does not readily react with most biomolecules, despite its unpaired electron. On the other hand, it easily reacts with other free radicals (*e.g.* peroxyl and alkyl radicals), generating mainly less reactive molecules (*33*), thus in fact functioning as a free radical scavenger (*27*). In addition, nitric oxide seems to be also involved in neurotransmission, regulation of vascular relaxation and in inflammatory processes (*32,34*). However, it can react with the superoxide anion, yielding peroxynitrite (ONOO$^-$). This compound has the capacity to act in a hydroxyl radical-like manner to induce lipid and protein oxidation, readily reacts with CO_2 to form nitroso peroxocarboxylate (ONOOCO$_2^-$), can become protonated as peroxonitrous acid (ONOOH) and undergo homolysis to form either $^•$OH and $^•$NO$_2$, or be rearranged to nitrate (NO$_3^-$) (*35*). In addition, peroxynitrite is known to play a key role in neuronal damage associated with excitotoxicity (*36*).

Other reactive species derived from oxygen that can be formed in living systems include peroxyl radicals (ROO$^•$). The simplest peroxyl radical is HOO$^•$, which is the protonated form of superoxide and is usually termed either hydroperoxyl radical or perhydroxyl radical. Its involvement in the initiation of lipid peroxidation has already been demonstrated (*5*).

1.3. *Physiological functions of reactive species*

Reactive species are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems (*26*). These species are maintained at low, but measurable, concentrations in the cells, through a balance between their rates of production and their rates of removal by antioxidants. Thus, each cell is characterised by a particular concentration of electrons (redox state) stored in many cellular constituents, and the redox state of a cell and its oscillation determines cellular functioning. A temporary shift of the intracellular redox state towards more oxidising conditions results in a temporary imbalance that represents the physiological basis for redox regulation (*37*).

A great number of physiological functions are controlled by redox-responsive signalling pathways (*7*). Several evidences suggest that ROS participate in the defence against intrusion of foreign bodies (*38*). Activated neutrophils and macrophages produce large quantities of ROS *via* the phagocytic isoform of NAD(P)H oxidase, in order to kill the pathogens. This massive production of ROS during an inflammatory event is called "oxidative burst" and plays an important role as the first line of defence against environmental pathogens (*5,27*). At a smaller scale, some types of non-phagocytic cells, like fibroblasts, vascular smooth muscle cells, cardiac myocytes and endothelial cells,

are also known to produce ROS by NAD(P)H oxidase to regulate intracellular signalling cascades. Thus, ROS play an important role in the regulation of cardiac and vascular cell functioning (*39*).

Nitric oxide plays several regulatory functions. In fact, its own production by iNOS, the only isoform of NOS that is not constitutively expressed, is regulated at the transcriptional and post-transcriptional levels by signalling pathways involving redox-dependent transcription factor NF-κB or mitogen-activated protein kinases (MAPKs). In addition, $^•$NO, in combination with H_2O_2, leads to the activation of the enzyme soluble guanylate cyclase (sGC), which catalyses the formation of cyclic guanosine monophosphate (cGMP) that, by its turn, is used as an intracellular amplifier and second messenger in a variety of physiological responses, such as modulation of protein kinases, ion channels, smooth muscle tone and inhibition of platelet adhesion (*7,40*).

In higher organisms, oxygen homeostasis is maintained by a tight regulation of the red blood cell mass and respiratory ventilation. It has been proposed that changes in oxygen concentration are sensed independently by several different ROS-producing proteins, including a *b*-type cytochrome. Other studies also suggested that a change in the rate of mitochondrial ROS may play a role in this oxygen sensing by the carotid bodies, which are sensory organs that detect alterations in arterial blood oxygen. Other responses to changes in oxygen pressure include the regulated production of certain hormones (*e.g.* erythropoietin) controlled by the transcription hypoxia inducible factor-1 (HIF-1) (*41,42*).

ROS also seem to be involved in cell adhesion, a mechanism that plays an important role in embryogenesis, cell growth, differentiation, wound repair, among other processes. The expression of cell adhesion molecules is stimulated by bacterial lipopolysaccharides and by various cytokines such as TNF, interleukin-1a, and interleukin-1b. The adherence of leukocytes to endothelial cells is also induced by ROS. Moreover, the oxidant-induced adherence of neutrophils is inhibited by hydroxyl radical scavengers or iron chelators, suggesting that the induction of adherence may be mediated by hydroxyl radicals generated from hydrogen peroxide within the cell (*7,43*).

Reactive oxygen and nitrogen species can directly affect the conformation and/or activities of all sulfhydryl-containing molecules, such as proteins or glutathione (GSH), by oxidation of their thiol moiety. This type of redox regulation affects many proteins important for signal transduction and carcinogenesis, such as protein kinase C, Ca^{2+}-ATPase, collagenase and tyrosine kinases, among many other enzymes and membrane receptors (*44*). In addition, ROS/RNS are known to trigger apoptotic cell death, by causing Bcl-2 (a protein located in the outer membranes of mitochondria) to activate a related protein, Bax, which

by its turn leads to the release of cytochrome *c* from mitochondria (*14,45-47*). This release then results in the activation of several other proteins, in a process that will be discussed further ahead (section 3.1.).

2. Oxidative damage to biomolecules

Due to their high reactivity, ROS are prone to cause damage and are thereby potentially toxic, mutagenic, and carcinogenic. Their targets include all biomolecules, namely proteins, lipids, and nucleic acids (*48*).

2.1. *Proteins*

Proteins can function as important targets for attack by ROS (*48*). Direct oxidation of the side chains of all amino acid residues of proteins (especially proline, arginine, lysine, and threonine) by ROS yields reactive carbonyl derivatives, particularly *via* metal-catalysed oxidation (*49,50*). Protein carbonyl derivatives can also be generated from the cleavage of peptide bonds by the α-amidation pathway or by oxidation of glutamyl residues. In addition, carbonyl groups may be introduced into proteins by secondary reaction of the primary amino group of lysine residues with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones), produced by the reaction of reducing sugars or their oxidation products with lysine residues of proteins (glycation/glycoxidation reactions), eventually leading to the formation of advanced glycation end-products (AGEs). Finally, carbonyl groups may be formed by adduction of byproducts of lipid peroxidation. These include malondialdehyde, which reacts with lysine residues and α,β-unsaturated aldehydes (4-hydroxy-2-nonenal and acrolein), which can undergo Michael-addition reactions at their C=C double bond with the sulfhydryl group of cysteine, the ε-amino group of lysine or the imidazole group of histidine residues, forming advanced lipoxidation end-products (ALEs) (*32,50*).

The introduction of carbonyl derivatives may alter the conformation, and/or even cause fragmentation, of the polypeptide chain, thus determining the partial or total inactivation of proteins. In addition, it can result in the loss of enzymatic activity, increased proteolytic degradation, altered cellular functions such as energy production, interference with the creation of membrane potentials and changes in the type and level of cellular proteins (*48,50,51*).

Protein carbonyl content is the most widely used marker for the measurement of protein oxidation. Highly sensitive assays for the detection of protein carbonyls involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which results in the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product. Stable DNP adduct can then be detected spectrophotometrically, a technique that can be coupled to protein fractionation by high-performance liquid chromatography (HPLC). Alternatively, in recent years, identification of carbonylated proteins has been performed by immunoblotting analysis, with the use of specific antibodies anti-DNP (*52*).

Protein damage is likely to be repairable and is a known non-lethal event for a cell. However, oxidation of proteins is associated with a number of age-related diseases including (but not limited to) Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, amyotrophic lateral sclerosis, and ageing (*49*). Additionally, two examples of human pathologies in which pathophysiological aspects of protein carbonylation have been extensively investigated are adult (or acute) respiratory distress syndrome (ARDS) and inflammatory bowel diseases (IBDs) (*52*).

2.2. *Lipids*

Polyunsaturated fatty acids (PUFAs), because of their multiple double bonds, are also extremely sensitive to oxidation by free radical attack. In addition, PUFAs can be formed enzymatically by the action of lipoxygenases (*53*). Arachidonic and linoleic acids are the main PUFAs in the mammalian membranes and are able to undergo both enzymatic and non-enzymatic lipid peroxidation. However, since linoleic acid is much more abundant than arachidonic acid, most of lipid peroxidation products derive from the former (*28*).

Briefly, the overall process of lipid peroxidation consists of three main stages: initiation, propagation and termination. Lipid peroxidation may be induced when a radical species (*e.g.* hydroxyl radicals generated *via* Fenton reaction) is sufficiently reactive to remove a hydrogen atom from the polyunsaturated lipid (LH), leading to the formation of a lipid radical (L·), which results in the formation of lipid hydroperoxide (LOOH) species (Figure 2). Thus, many molecules of lipids may be oxidised to lipid hydroperoxides for every initiation event. Termination reactions occur when two radical species combine to form non-radical final products or when substrate is depleted (*54,55*).

Once formed, lipid hydroperoxides can degrade rapidly into a variety of breakdown products, such as malondialdehyde (MDA), C_3-C_{10} straight chain aldehydes, and α,β-unsaturated aldehydes, including 4-hydroxy-2-nonenal (4-HNE) and acrolein (*56,57*). In addition, lipid peroxidation also leads to production of isoprostanes and neuroprostanes. Although these molecules do not show toxicity, they serve as excellent markers of arachidonic and docosahexaenoic acid peroxidation in brain and cerebrospinal fluid (*57*).

Estimates of lipid peroxidation can be obtained by measuring the formation of lipid hydroperoxides and their breakdown products, as well as by quantifying the disappearance of PUFAs. The most widely used method to assess oxidative damage to lipids is the thiobarbituric
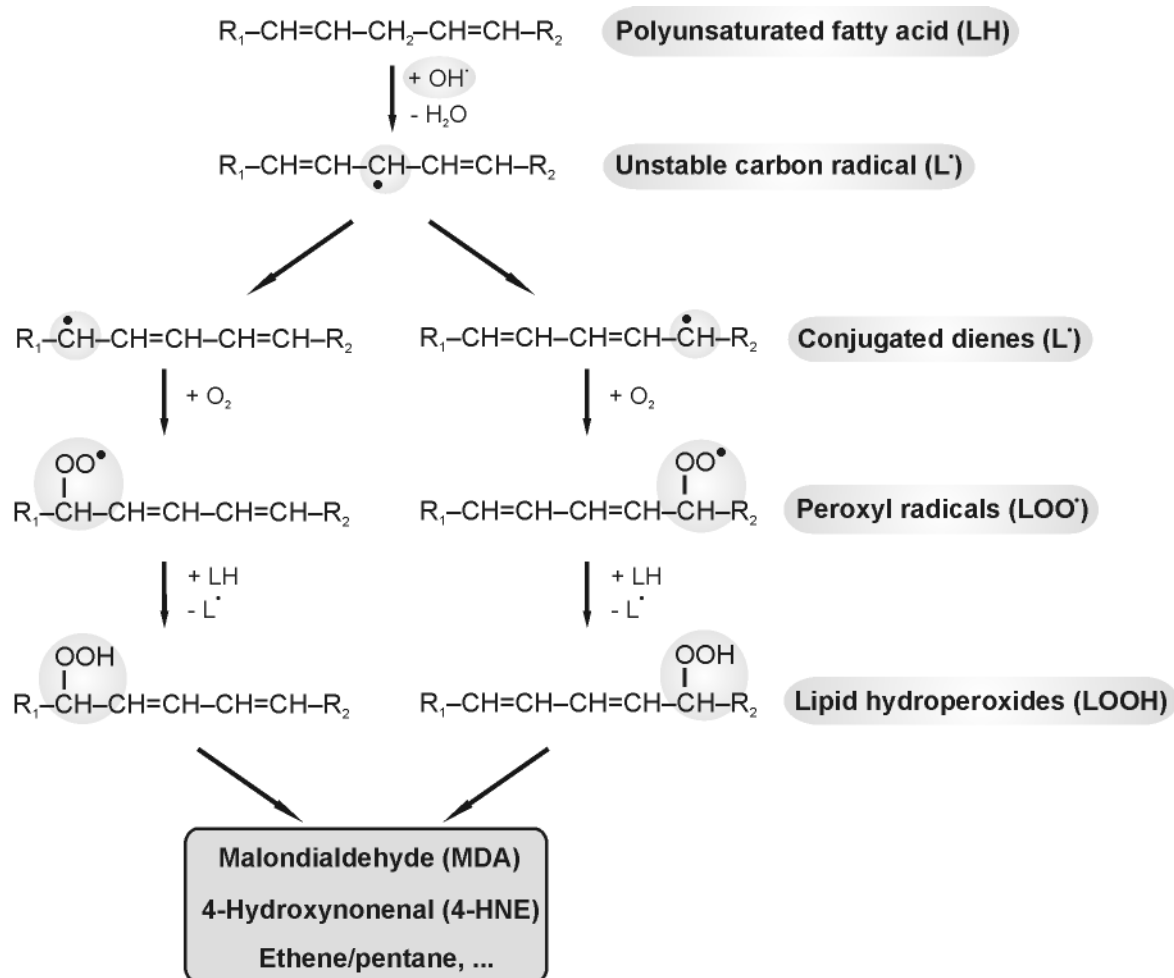
Figure 2. Basic reactions occurring during lipid peroxidation. Reactive species (*e.g.* hydroxyl radicals) abstract an hydrogen atom from a polyunsaturated fatty acid, yielding a lipid radical (L•) that may undergo some molecular rearrangements. Oxygen uptake by these radicals propagates the reaction *via* peroxyl radicals (LOO•), which leads to the formation of lipid hydroperoxides (LOOH). These can then combine and generate the final products of lipid peroxidation (*e.g.* MDA 4-HNE, acrolein, ethane/pentane, among others). Modified from Spiteller, 2001 (*28*).

acid reactive substances (TBARS) assay, which is thought to reflect the production of MDA (*56,58*).

Some byproducts of lipid hydroperoxidation have been reported to possess cytotoxic, mutagenic, and genotoxic properties (*58*). For example, MDA, 4-HNE, and acrolein can damage DNA either by reacting directly with DNA bases or by generating more reactive bifunctional intermediates, which form exocyclic DNA adducts with a five-membered ring (etheno adducts) or a six-membered ring (propane adducts) attached to DNA bases. These adducts can then induce base pair substitution mutations (*59*). Hydroxynonenal and acrolein are neurotoxic and can, among other things, inhibit enzymes critical for neuron survival (*57*). Moreover, lipid peroxidation seems to be also involved in atherosclerotic processes. The oxidation of low density lipoproteins (LDL) results in their uptake by phagocytes in the subendothelial space *via* their scavenger receptor. These phagocytic cells then accumulate in the subendothelial space, where they stimulate formation

of atherosclerotic plaques (*60*).

2.3. *DNA*

Damage to nuclear DNA has been proposed to occur through two different mechanisms: oxidative modification and DNA fragmentation mediated by endonucleases (an irreversible feature of programmed cell death). While the latter is supposed to take place during the late stage of cell death, oxidative DNA damage is believed to stand for an early event (*61,62*). Mitochondrial DNA has, by its turn, been reported to be even more susceptible to oxidation than nuclear DNA. The greater accumulation of damage may be related to: mitochondria being the main producers of ROS in the cells; repair capacity of mitochondrial DNA being limited (proteins responsible for DNA repair are expressed in lower levels in mitochondria); and mitochondrial DNA not being protected by histones (*63,64*).

The attack of ROS and RNS is responsible for

introducing several modifications to DNA, which include: oxidised bases, the most studied being 8-oxo-7,8-dihydroguanine (8-oxo-Gua), a product derived from the oxidation of a guanine by ROS; modifications to the sugar moiety of DNA, which may result in base loss – abasic (apurinic/apyrimidinic) sites – and/or strand breakage (single- and double-strand breaks); DNA-DNA intra-strand adducts and DNA-protein cross-links (*65,66*). The formation of some of these lesions is summarised in Figure 3. In addition, exposure of cells to UV radiation can result in dimerisation reactions between adjacent pyrimidine bases, yielding cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6-4)pyrimidone photoproducts (*67,68*).

DNA lesions, if left either un- or mis-repaired, may interfere with DNA-dependent processes (such as transcription, replication, recombination, and chromosome segregation), leading to mutations, chromosomal instability, and even cell death (*69*). Therefore, cells are equipped with specific and efficient repair mechanisms, which are able to repair the modifications introduced in DNA (*70*). During these repair processes, cell cycle may be arrested at any checkpoint, which allows a time window to repair damaged DNA (*69,71*). In addition, it should also be noted that oxidative stress leads to an upregulation of the expression of many repair enzymes, which may result in an enhancement of enzymatic repair mechanisms (*26,72*).

DNA double-strand breaks are repaired *via* two different mechanisms: homologous recombination (HR), in which the sister chromatid is used as a template to copy the missing information into the

broken locus (*73*), and non-homologous end joining (NHEJ), which consists in the fusion of the two broken ends with little or no regard for sequence homology, a process that may result in mutations (*74*). The mismatch repair (MMR) pathway plays an important role in the post-replicative process, by repairing replicative and recombinatorial errors that may result in mispaired bases (mismatches) (*75,76*). The nucleotide excision repair (NER) pathway recognises bulky adducts, as well as covalent linkages between adjacent pyrimidines resulting from exposure to UV radiation, and removes short DNA oligonucleotides containing a damaged base; it can be classified into global genome repair (GG-NER), which removes DNA damage from the entire genome, and transcription-coupled repair (TC-NER), which specifically removes damage from the transcribed strand of active genes, thereby releasing transcription arrest that occurs at the lesions sites (*71,77*). However, oxidatively damaged DNA is mainly removed by the base excision repair (BER) pathway, in which oxidised bases are eliminated by different DNA glycosylases, leaving abasic sites (*78,79*).

Base excision repair consists of four main steps (base removal, apurinic/apyrimidinic site incision, synthesis, and ligation), and can be divided in two sub-pathways, the short patch or single nucleotide replacing pathway and the long patch pathway (Figure 4), which involves the incorporation of up to 13 nucleotides (*64,80*).

BER follows mainly through the short patch pathway. In the first step of this pathway, a damaged base is recognised and removed from the deoxyribose phosphate moiety (*80*). Two classes of DNA glycosylases are involved in this step: Class I (bifunctional or complex), which includes, for example, 8-oxoganine DNA glycosylase 1 (OGG1), an enzyme that removes 8-oxoguanine and nicks the DNA backbone; and Class II (monofunctional or simple), including N-methylpurine DNA glycosylase (MPG) and uracil DNA glycosylase (UDG), which remove alkylated DNA bases or uracil, respectively, but without nicking the DNA backbone (*72,81*). Due to the catalytic inefficiency of OGG1's lyase activity, this enzyme remains stuck to the site of incision, limiting the overall rate of repair (*78,82*). So, following the glycosylase reaction, APE1 (apurinic/apyrimidinic endonuclease, also called APEX, HAP1 and Ref-1), easily displaces the glycosylase and cleaves the 5' terminus of the apurinic/apyrimidinic (AP) site, resulting in the loss of a base and a single-strand break with 5′-phosphate and 3′-OH termini (step 2) (*64,81*). Additionally to its role in BER, APE has also been implicated in the redox activation of transcription factors, such as p53 (*83*). In the third step of BER, the AP site that is generated by the APE1 repair activity is removed by deoxyribosephosphate hydrolase (dRPase) activity provided predominantly by DNA β-polymerase (β-pol). This is then followed by the insertion of a new base by
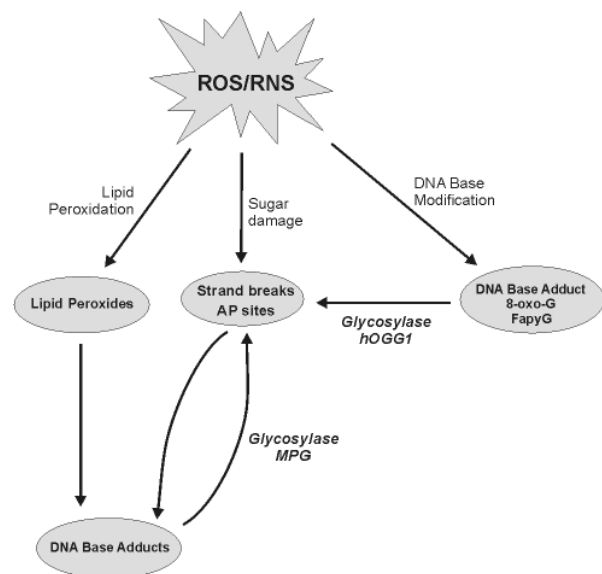
Figure 3. Types of DNA damage induced by reactive oxygen species. Abbreviations: 8-oxo-G, 8-oxo-7,8-dihydroguanine; FapyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; hOGG1, human 8-oxoguanine DNA glycosylase 1; MPG, N-methylpurine DNA glycosylase. Modified from Powell *et al.*, 2005 (*66*).
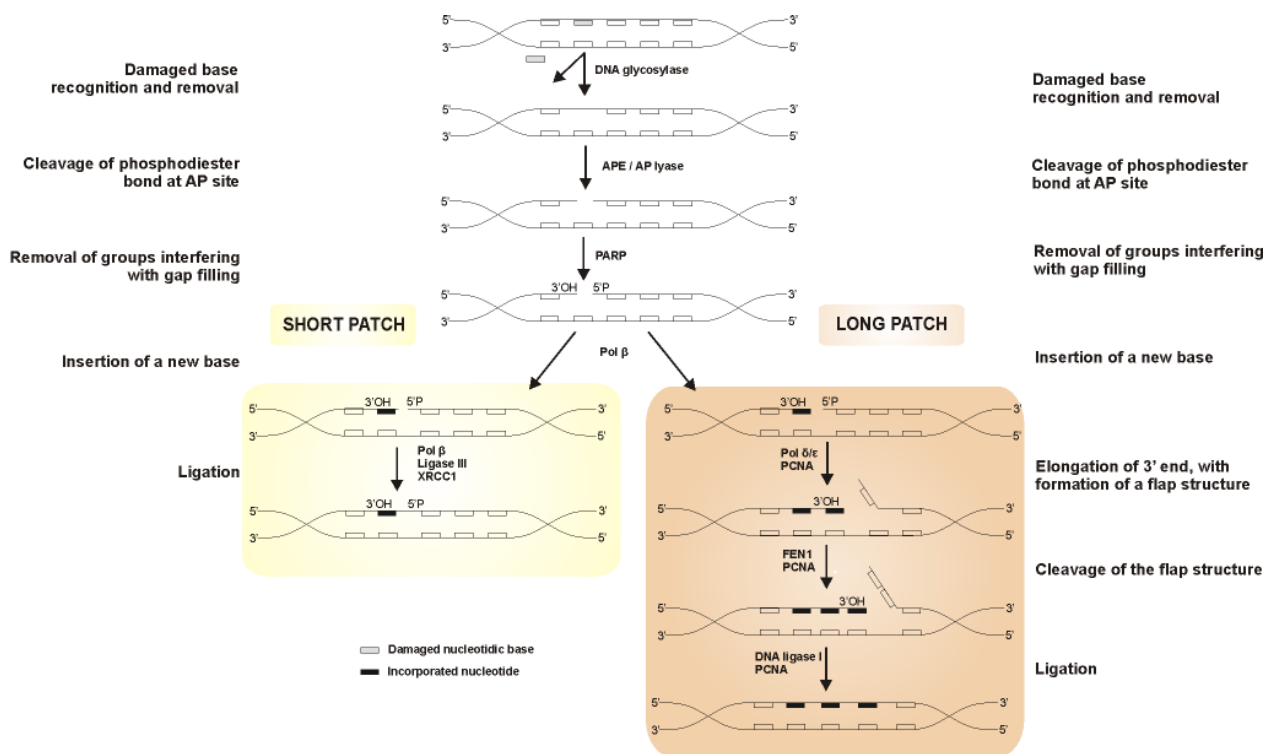
Figure 4. Base excision repair pathways. First, a damaged base is recognised and removed by DNA glycosylases, yielding an apurinic/apyrimidinic (AP) site, which is then cleaved by an endonuclease (APE), forming a strand break. Then, after some chemical groups that may interfere with gap filling and ligation are removed, different polymerases (depending whether the short patch or the long patch pathway is active) fill the gap in the DNA strand. Finally, DNA ligase inserts one (SHORT PATCH) or more (LONG PATCH) new bases. Other proteins, like poly (ADP-ribose) polymerase (PARP), XRCC1, proliferating cell nuclear antigen (PCNA), and FEN1 aid in the regulation of these pathways. The bacterial enzymes FPG and Endo III, used in the Comet assay, act on the first step of this pathways (damaged bases recognition and removal). Modified from Tudek *et al.*, 2006 (*84*).

β-pol and ligation by DNA ligase I (step 4) (*72*).

The long patch pathway is catalysed by β-pol or other polymerases like δ/ε-pol, which have a proof-reading activity associated to them. Repair follows this pathway when the terminal sugar phosphate formed after the APE1 incision (step 2) develops a complex structure that cannot be acted upon by the dRPase activity of β-pol (*e.g.* oxidised abasic site). In this case, the repair synthesis still continues, but in a strand displacement manner (*80,84*).

It should be noted that despite the presence of all these DNA repair systems, DNA damage still occurs. In fact, DNA is continuously attacked by reactive species, an event that can be attenuated by antioxidant defences. Whereas some of the damage induced by ROS attack to DNA can induce the signalling pathways leading to apoptotic cell death, the majority results in the oxidation of DNA. However, DNA repair enzymes help to maintain an equilibrium state between damage formation and repair, by repairing most of the oxidised DNA (*81*). This implies that, in normal physiological conditions, oxidative damage is maintained at a tolerable level in terms of genetic stability (*81,85*). Nevertheless, the oxidised DNA that does not get repaired can become mutated, which may then result in further damage to cells.

There are several methods that allow the

measurement of oxidative DNA damage. However, determining the background levels of the most common product of DNA damage, 8-oxo-Gua, has brought some difficulties, since damage can arise from the preparation of samples. With the purpose of normalizing the procedures to measure this kind of damage, the European Standards Committee on Oxidative DNA Damage (ESCODD) was formed (*86,87*). Although some chromatographic methods, like gas chromatography (GC) and high performance liquid chromatography (HPLC), usually coupled to mass spectrometry (MS) techniques, have been employed, they have shown inflated values, probably due to erroneous oxidation during DNA isolation. So, a set of enzymatic methods has been described as the most suitable. These methods make use of the bacterial DNA repair enzyme formamidopyrimidine DNA glycosylase (FPG) to convert 8-oxo-Gua to apurinic sites, which can then be measured by the Comet assay, alkaline unwinding or alkaline elution (*66,86*).

The Comet assay is a simple, fast, and sensitive method that detects DNA strand breaks and abasic sites, although it can detect oxidised bases *via* the use of repair enzymes, such as FPG or endonuclease III (Endo III), which nick DNA at oxidised purines and pyrimidines, respectively, at the initial step of the BER pathway (Figure 4). Moreover, this assay reduced the risk of occurring

oxidation during sample preparation (*88*).

Oxidative DNA damage can result in the arrest or induction of transcription, induction of signal transduction pathways, replication errors, and genomic instability, all of which have been implicated in cancer and neurodegenerative diseases, besides being associated to the normal process of aging (*89,90*).

3. Cell death

The exposure to oxidative stress may ultimately result in cell death, as a consequence of severe damage caused to biomolecules by reactive oxygen and nitrogen species (*27*). Cell death can occur by two main mechanisms: necrosis and programmed cell death (PCD) (*91*).

Necrosis is usually referred as an "accidental", or uncontrolled, form of cell death (*92*). During this process, there is a rapid swelling of the cell, leading to the loss of membrane integrity and consequent release of the cells' contents, which is known to evoke an inflammatory response. In this way, cell death becomes a passive consequence of irreparable damage, hence the term "accidental" (*92,93*).

Apoptosis was initially considered as the only form of programmed cell death. However, more recently, a variety of cell behaviours that may lead to active forms of cell death have been observed. These include the typical apoptotic cell death, autophagic death, mitotic catastrophe, oncosis, anoikis, excitotoxicity, Wallerian degeneration, cornification, and paraptosis. In this way, and using nuclear morphology as a distinction criterion, programmed cell death has been divided into three possible classifications: classical apoptosis, autophagic cell death, and necrosis-like PCD (*91,94,95*).

Type I PCD or classical apoptosis has been described as an active process by which dying cells are removed in a safe, non-inflammatory manner (*96*). It is a tightly regulated process, involved in many vital functions, including tissue development, carcinogenesis, immune response, and control of the balance between proliferation and differentiation (*45,97,98*). At the morphological and biochemical levels, it is characterised by shrinkage of the cell, membrane surface blebbing, oligonucleossomal DNA fragmentation, and the breakdown of the cell into various membrane-bound fragments, called apoptotic bodies. Along with these events, occurs the activation of specific cysteine proteases, named caspases, as well as the loss of membrane phospholipid asymmetry, which results in the externalisation of phosphatidylserine (*99*). Type II PCD or autophagic cell death is mainly characterised by the presence of double- or multiple-membrane vacuoles, mitochondrial dilation, as well as enlargement of the endoplasmic reticulum (ER) and Golgi apparatus (*94*). The vacuoles formed during this process, which are also called autophagosomes,

engulf portions of cytoplasm and organelles (like mitochondria and ER), then fuse with lysosomes, where the intravacuolar content is disintegrated by lysosomal enzymes (*100*). Nevertheless, it should be noted that "autophagic cell death" is a process that differs from autophagy. Indeed, autophagy is a dynamic process of protein degradation, used mainly to provide an alternative source of nutrients, which is usually associated to cell death, with either necrotic or apoptotic phenotype (*91,93*). The mammalian protein kinase TOR (which stands for "target of rapamycin") plays a major role in the regulation of the autophagic process, by inhibiting this pathway. Downstream of this protein, several other proteins encoded by *Atg* genes intervene in the execution of the autophagic process (*92,100*). The identification of these genes and the observation that their inactivation protects from this type of death, supports the evidence of this PCD as a specific type of death.

Recently, an active form of necrosis (type III PCD) was found to occur, not only under pathological conditions, but also under normal physiological conditions (*91*). It lacks caspase and lysosomal involvement and is mainly characterised by an early swelling of intracellular organelles, followed by loss of plasma membrane integrity (although nuclear disintegration is retarded). This type of PCD can also be subdivided into two sub-types: IIIA or "non-lysosomal disintegration", characterised by nuclear disintegration, and IIIB or "cytoplasmic degeneration", which displays karyolysis (complete dissolution of the chromatin of a dying cell). Moreover, programmed necrosis can occur by the induction of the tumour necrosis factor (TNF) or Fas ligand, *via* their respective death receptors, and gives an idea that necrosis is in fact a process that cells can control (*91,92,94*).

3.1. Programmed cell death pathways

Apoptosis, the most common form of programmed cell death, is a complex process involving both pro- and anti-apoptotic proteins, which can be initiated by two different signalling pathways: the death receptor (extrinsic) pathway and the stress- or mitochondria-mediated (intrinsic) pathway (*93,101*). A set of highly conserved cysteine-dependent aspartate-specific proteases, named caspases, are regarded as the central executioners of apoptosis. These caspases use a cysteine residue as the catalytic nucleophile and share specificity for cleaving their substrates after aspartic acid residues in target proteins (*102,103*).

The main intracellular signalling pathways leading to apoptotic cell death are summarised in Figure 5.

The death receptor or extrinsic pathway is activated by the binding of an extracellular ligand, such as Fas (also known as CD95 or APO-1) ligand, tumour necrosis factor (TNF) or TNF-related apoptosis induced
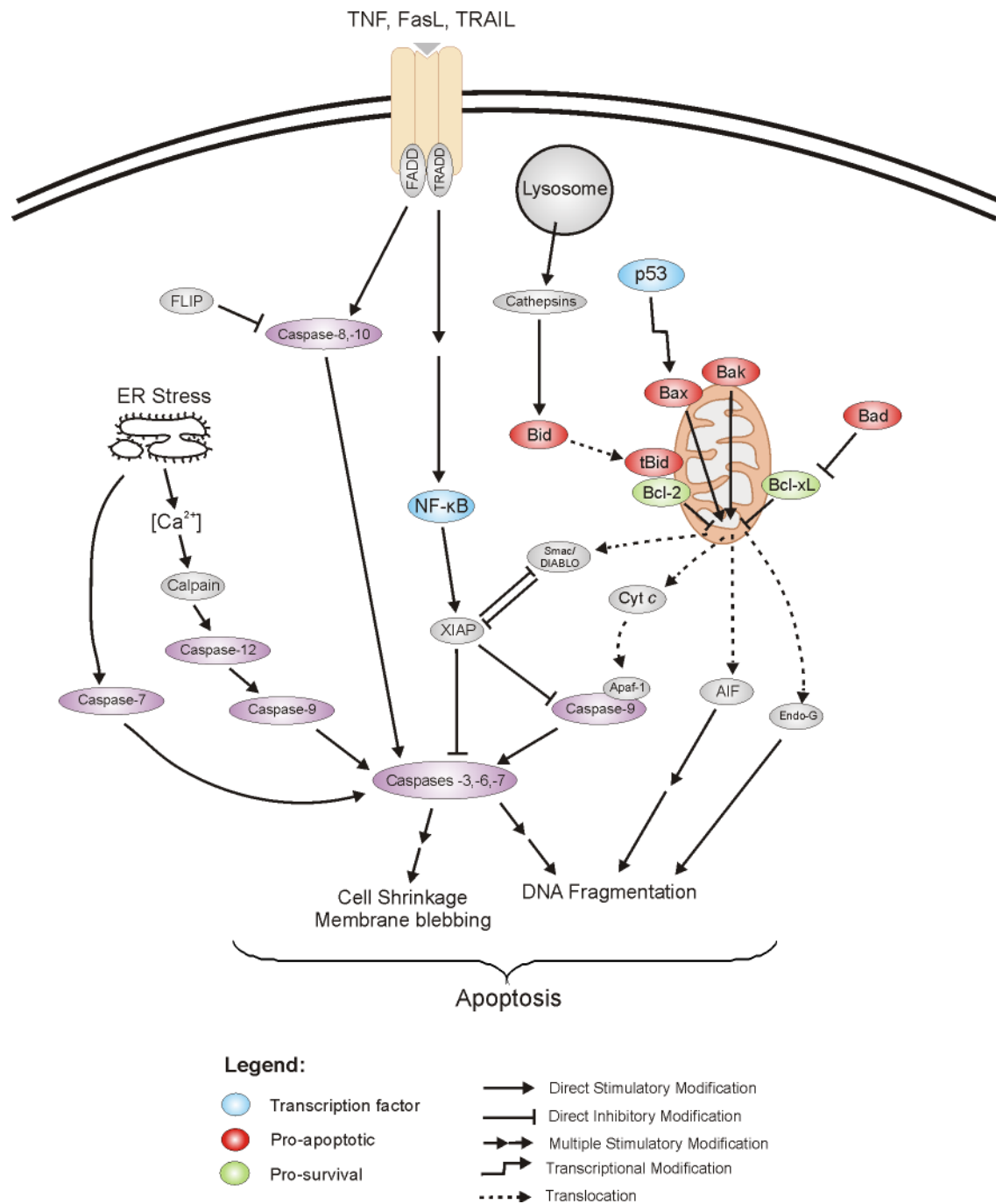
Figure 5. Main intracellular pathways leading to apoptosis and some ways by which they can be regulated. The extrinsic pathway is activated by the binding of an extracellular ligand to specific death receptors, which activate caspase-8. The mitochondrial pathway is activated by Bax or Bak, which bind the mitochondrial outer membrane, inducing the release of several pro-apoptotic factors, such as cytochrome *c*, which then forms the apoptosome upon binding to Apaf-1 and caspase-9. These two pathways can be connected by the cleavage of Bid and both of them result in the induction of effector caspases, like caspase-3, which then activate substrate responsible for the common features of apoptosis. The caspase-independent pathway, mediated by AIF, as well as the lysosomal involvement in cell death and the ER stress-mediated events are also shown in this scheme.

ligand (TRAIL), to their specific death receptors present in the cell membrane (*104,105*). This results in the recruitment of procaspase-8 and subsequent activation of caspase-8, which then leads to the activation, either directly or indirectly, of downstream caspases, like procaspase-3 (*104,106*) (Figure 5).

As shown in the same figure (Figure 5), the mitochondrial or intrinsic pathway can be initiated by the translocation of several proapoptotic proteins

from the Bcl-2 family (*e.g.* Bax, Bid or Bad) to the mitochondria (*107*). These proteins induce the permeabilisation of the mitochondrial outer membrane (MOMP), which has been considered as a point of no return in cell death (*101,108*). As a consequence of an increase in its permeability, mitochondria release several proapoptotic factors normally present in the intermembrane space, which include cytochrome *c*, the serine protease HtrA2/Omi, the Apoptosis Inducing

Factor (AIF), Smac/DIABLO (which stands for second mitochondria-derived activator of caspases/Direct IAP Binding Protein With Low pI) and endonuclease G (*98,102,107*).

The released cytochrome *c* can bind to procaspase-9 and Apaf-1 (from apoptotic protease-activating factor 1) in the presence of deoxyadenosine triphosphate (dATP)/ adenosine triphosphate (ATP). This complex, termed the apoptosome, results in the activation of caspase-9, which is mainly activated through this mitochondrial pathway (*97,109*).

The intrinsic and extrinsic pathways can be interconnected by the action of Bid, a protein from the Bcl-2 family (see Figure 5). This protein is normally found in the cytosol, where it can be cleaved by caspase-8. After cleavage, the carboxylic terminus of Bid is translocated to mitochondria, leading, directly or indirectly (by interaction with Bax) to the release of cytochrome *c* (*104,110*).

Both activated caspase-8 (as well as caspase-2 and -10, also mediators of the death-receptor pathway) and the activated caspase-9 (from the mitochondrial pathway) converge to the downstream activation of effector caspases, such as procaspase-3, -6, and -7 (*98*). These caspases activate a DNase, which is responsible for the fragmentation of oligonucleosomal DNA (*98,111*). In addition, other enzymes and/or substrates, like poly-(ADP-ribose) polymerase (PARP), fodrin, p75, actin, among others, are activated in the process, culminating in the display of several of the phenotypic characteristics of apoptotic cell death, including loss of mitochondrial membrane potential, cell blebbing, and redistribution of lipids in the outer plasma membrane (*105*).

Alternatively to the caspase-dependent pathways, the characteristic features of apoptosis can be induced by a caspase-independent way, mediated by the apoptosis inducing factor (AIF). This is a mitochondrial flavoprotein oxidoreductase that translocates first to the cytosol and then to the nucleus (*111,112*). Once in the nucleus, AIF interacts with nucleic acids, causing caspase-independent chromatin condensation and DNA fragmentation (*94*).

The endoplasmic reticulum (ER) can also act in the induction and regulation of apoptosis. ER is known to play a central role in protein biosynthesis and is the major intracellular organelle involved in calcium storage (*113*). Calcium homeostasis is maintained by some members of the Bcl-2 family, like Bax and Bak, which can also be associated to the ER membrane. Upon an apoptotic stimulus, these proteins induce calcium release through ER calcium channels, such as the inositol 1,4,5-triphosphate (IP$_3$) receptors, whose opening is mediated by the binding of IP$_3$. Calcium from the ER is then taken up by mitochondria, causing a calcium overload that subsequently results in the induction of mitochondrial membrane permeabilisation

by mitochondria-located Bax and Bak (*114,115*). Moreover, calcium release from the ER can induce the activation of calpains and caspase-12, which by their turn lead to the activation of other caspases, resulting in the propagation of the apoptotic signal (*113*). It should be noted that many factors are able to regulate these pathways, by activating or inhibiting them at specific sites (Figure 5).

Recently, a regulated lysosomal involvement in cell death has also been observed. In fact, partial lysosomal membrane permeabilisation has been described to trigger apoptosis and apoptosis-like cell death, in response to several apoptotic stimuli, such as activation of death receptors of the tumour necrosis factor (TNF) family, p53 activation, oxidative stress, among others (*116*). These stimuli can then lead to lysosomal membrane permeabilisation through either caspase-dependent or -independent mechanisms, which normally results in the release of a group of proteases named cathepsins (including cathepsins B, D, and L) from the lysosome to the cytosol (*117*). In addition, lysosomal enzymes have been found to act on mitochondria and induce the formation of mitochondrial ROS, which can then lead to further lysosomal permeabilisation (*118*). Moreover, it has been reported that mild stress triggers only a limited release of the lysosomal contents to the cytosol followed by apoptosis or apoptosis-like cell death, while elevated stress levels cause a generalised lysosomal rupture and rapid cellular necrosis (*119*). This lysosomal apoptotic signalling pathway has recently been considered as an attracting drug target, namely in cancer therapeutics (*120*).

Occasionally, deficiencies in the cell cycle checkpoints, which by its turn lead to aberrant mitosis, may result in another type of programmed cell death, termed mitotic catastrophe. This process is mainly characterised by enlarged and multinucleated cells, incomplete nuclear condensation, chromosome alignment defects, and unequal DNA separation (*93,121,122*).

3.2. *The role of ROS/RNS in the induction of cell death*

The intracellular accumulation of reactive oxygen and nitrogen species also plays an important role in the initiation of cell death processes (*123*). In fact, the amount of reactive species accumulated in the cell, and the way the cell responds to that redox imbalance, can determine that same cell's fate. For example, mild oxidative stress may activate biological responses that can either lead to survival and proliferation, or can induce apoptosis, while the accumulation of high levels of ROS may promote necrosis instead (*124,125*). Moreover, a sudden burst of ROS, resulting from the response to oxidative stress of cells already committed to apoptosis, can direct those cells towards a necrotic-like death (*14,123*).

The accumulation of reactive species has been described to precede changes in the mitochondrial membrane, nuclear condensation, and other typical apoptotic events (*14,126*). Indeed, some studies have reported that an increase in ROS induces cytochrome *c* release from mitochondria (in a voltage-dependent anion channel (VDAC)-dependent way), and caspases activation (*13,127,128*).

Many studies have shown other evidences for the induction of apoptotic pathways by reactive species. Some mediators of apoptosis (*e.g.* JNK, ERK, PTEN) have been reported to lead to increased levels of ROS (*129,130*). Similarly, it has been shown that inhibition of the mitochondrial respiratory chain at complex I (*131*), or an impairment of the electron transfer chain by mutations in mitochondrial DNA, prevent the accumulation of ROS, and consequently protect cells against apoptosis (*29,132*). In addition, lipid peroxidation has also been reported to occur following an apoptotic signal (*133,134*). Moreover, and perhaps most important, inhibition of apoptosis by the addition of antioxidants has already been described (*11,97,135,136*). For example, the antioxidant enzyme MnSOD has been reported to inhibit apoptosis during ischemia/reperfusion injury (*137*), and "classical" antioxidants such as α-tocopherol and GSH have shown to prevent apoptosis induced by ascorbate-iron (*134*). Thus, these studies point towards a wide range of actions for ROS/RNS and add further importance to the use of antioxidants to prevent apoptosis and treat several disorders.

3.3. *Intracellular regulation of cell death pathways*

The intracellular pathways leading to apoptotic cell death can be regulated at several levels, including their blockade at the death-inducing signalling complex (DISC), which is responsible for the recruitment of procaspase-8, or the inhibition of their enzymatic activity. These regulators comprise inhibitors-of-apoptosis proteins (IAPs), the FLICE-like inhibitor protein (FLIP), and calpains (*103*). IAPs are a family of cellular proteins, including eight mammalian family members with highly conserved and differential expression patterns in various tissues, which bind to the surface of caspases, blocking the catalyzing grooves of caspases. These proteins do not inhibit caspase-8, but they inhibit its substrate, procaspase-3, instead, thus arresting the death-receptor pathway. In the mitochondrial pathway, XIAP, c-IAP1, and c-IAP2 bind directly to procaspase-9, preventing its activation (*103,106*). FLIP proteins have been described mainly as inhibitors of the death receptor pathway, since they are able, when overexpressed, to inhibit the activation of procaspase-8 at the DISC (*138,139*).

Calpains represent a family of calcium-dependent, nonlysosomal, cysteine proteases that share many substrates with caspase-3, including fodrin, calcium-dependent protein kinase, and PARP. In addition, due to the presence of a calmodulin-like calcium-binding site in their structure, these proteases are involved in various calcium-regulated processes, like signal transduction, cell proliferation, platelet activation, and apoptosis (*94,103*). Moreover, calpains can also cleave Bcl-xL, an antiapoptotic protein, converting it into a pro-apoptotic molecule (*113*).

Ceramides, which represent the structural backbone of sphingolipids/sphingomyelin, are important second messengers in several cell processes, including apoptosis. Some of their targets in apoptotic signalling pathways include mitochondria, jun kinases (JNK), lysosomal cathepsin D, p38 mitogen-activated protein kinase (MAPK), Bcl-2 family members, among others (*140,141*). Although the exact mechanism is not yet fully understood, ceramides seem to induce apoptosis by inducing the release of cytochrome *c* from mitochondria to the cytoplasm (*142*), which might be related to their ability to form protein permeable channels in the mitochondrial membranes (*141,143*).

The release of cathepsins (a group of proteases that stand for "lysosomal proteolytic enzymes") from lysosomes to cytosol has also been found to be implicated in the regulation of apoptosis (*144*). This lysosomal permeabilisation seems to be an early event, occurring prior to the loss of mitochondrial transmembrane potential. Bcl-2 family members, particularly Bid, seem to be the main targets of cathepsins. Bid cleavage then leads to the activation of Bax, resulting in the consequent release of apoptogenic factors from mitochondria (*94,145*).

The activation of the tumour suppressor protein p53 can induce DNA damage. Once activated, p53 may induce the expression of genes that prevent cell division and cause apoptosis (*146*). This may lead to the activation of pro-apoptotic Bcl-2 family members, like Bax, resulting in the permeabilisation of the outer mitochondrial membrane and subsequent activation of the above mentioned mitochondrial pathway (*146-148*).

A small peptide derived from the carboxyl terminus of p21, a protein identified as a cyclin-dependent kinase inhibitor and that was known to play an important role in the regulation of cell growth and differentiation, has recently been found to also activate apoptosis by a process involving mitochondria, although the exact pathways are still unclear (*101*).

4. Oxidative stress and disease

In normal conditions, cells can deal with mild levels of oxidative stress. They do so by upregulating the expression of genes responsible for the synthesis of antioxidant defence mechanisms. However, the intracellular accumulation of high levels of ROS/RNS can result in damage to all types of biomolecules, and

ultimately lead to cell death, which has been associated with many pathological conditions (*2,5*). However, it should be noted that whereas some diseases may indeed be caused by oxidative damage to biomolecules, in others oxidative stress is a consequence (and not a cause) of the disease. However, even as a secondary event, oxidative stress is of major importance, since it can induce the aggravation of tissue damage in several disorders (*1*).

A redox imbalance in the cell can lead to oxidative DNA damage, causing mutations that may result in cancer (*149,150*). The oxidative burst induced by neutrophils and macrophages to kill pathogens may result in chronic inflammation, seen in immune disorders, such as rheumatoid arthritis and inflammatory bowel diseases (*151,152*). Glycation of proteins leading to accumulation of advanced glycation end-products (AGEs), reduced antioxidant levels, and oxidative DNA damage seem to be present in diabetic complications (*153,154*). The decline in CD4$^+$ lymphocytes (whose functioning is regulated by redox potential) counts has been described to contribute to the progress of HIV infection to AIDS (*155,156*). Moreover, oxidative stress is involved in ophthalmologic disorders, such as advanced macular degeneration (AMD) and glaucoma (*157*).

Reactive species also participates in the normal process of aging. Oxidative damage accumulates with age, which leads to an increased impairment of cellular function. This results in a markedly decreased ability for the organism to neutralise free radicals and cope with oxidative stress, as it grows older (*158*). In addition, it has been reported that the cellular response to oxidants seems to be associated with the mechanisms that regulate longevity. Three gene products, including Forkhead transcription factors, the adaptor protein p66Shc, and the hystone deacetylase Sir2, are described as being involved either in the regulation of intracellular ROS concentrations or in the increase in resistance to oxidative stress (*159*).

Oxidative stress also seems to be important in the etiology of several cardiovascular diseases, like atherosclerosis, ischemic heart disease, cardiomyopathies, and congestive heart failure, among others (*5,160*). In fact, the heart is one of the most energy demanding tissues in the body and is totally dependent upon oxidative phosphorylation to supply the large amount of ATP required for beat-by-beat contraction and relaxation, which makes it quite susceptible to oxidative stress (*161*). The enzymes xanthine oxidoreductase, NAD(P)H oxidase, and nitric oxide synthase, as well as the mitochondrial cytochromes and haemoglobin may act as the main sources of oxidative stress in these diseases (*162,163*). Furthermore, high levels of cholesterol and uptake of oxidised low-density lipoproteins (oxLDL), the main carriers of cholesterol in plasma, is a primary risk for development of atherosclerosis (*164,165*). One of the most clinically relevant cardiac problems is ischemia-reperfusion injury. This type of injury involves an impairment of the blood flow to the heart as a result of damage to the myocardium (ischemia), in which the source of oxygen is removed, causing the cessation of oxidative phosphorylation. After a short period of ischemia, blood flow restoration occurs (reperfusion), which can, paradoxically, result in the aggravation of damage occurring during the ischemic period, and can lead to both apoptotic and necrotic cell death. An intracellular calcium overload and oxidative stress are two mechanisms that have been proposed to explain the pathogenesis of ischemia/reperfusion injury. Although the origin of ROS present during these events is yet to be well understood, it has been suggested that xanthine oxidase and NADPH oxidase, as well as mitochondria (*via* the electron transport chain) are mainly responsible for the massive ROS burst occurring during ischemia/reperfusion (*161,166*). In addition, increased oxidative stress causes abnormalities in the myocyte function, including inhibition of ATPases present in the sarcolemma that, together with calcium release from the sarcoplasmic reticulum, can also contribute to the intracellular calcium overload (*5*).

To date, many studies have demonstrated the involvement of oxidative stress in neurodegenerative diseases, such as Alzheimer's and Parkinson's (*29*). For example, in the brains of patients with Alzheimer's disease, a significant amount of ROS/RNS has been detected, in association with a marked accumulation of amyloid-β peptide (the main constituent of senile plaques) and deposition of neurofibrillary tangles and neurophil threads (abnormal neurites) (*167*). Several observations support the notion that the brain is particularly susceptible to oxidative stress: 1) this organ is known to consume about a fifth of the total oxygen used by the living organism, which increases the probability of ROS formation at the mitochondrial electron transport chain level; 2) neurons are post-mitotic (non-replicating) cells and any damage to brain tissues by ROS tends to be cumulative over time; 3) the high abundance in polyunsaturated fatty acids, which are particularly vulnerable to ROS damage; 4) the presence of high levels of transition metal ions (*e.g.* iron) that catalyse ROS formation through the Fenton reaction and the reduced levels of antioxidants able to segregate these transition metal ions, like transferrin and ceruloplasmin; 5) the release of excitatory neurotransmitters, such as glutamate, that induce a sequence of events in the post-synaptic neuron, which results in the formation of ROS; 6) the release of ROS during the oxidation of dopamine by monoamine oxidase in the nerve terminals of dopaminergic neurons may produce increased oxidative stress in brain regions, such as substantia nigra, which has been suggested to be a causative role in Parkinson's disease; 7) ascorbic acid,

which is present at elevated levels in both white and grey matters, additionally to its antioxidant properties, can act as a pro-oxidant, when the free iron in brain regions increases due to intra-cerebral hemorrhage, for example; 8) in comparison with other tissues, there are low levels of antioxidant defences, such as catalase and glutathione peroxidase, in the brain (*1,29,57*).

5. Antioxidants as protectors of oxidative stress

The excess of ROS is generally inactivated by endogenous and/or exogenous antioxidant molecules (*29*).

Antioxidant is a term widely used, but difficult to define clearly. According to some authors, a generic definition should be associated with the notion of the ROS/RNS that have to be neutralised, as well as the target of damage that is measured (*2,168*). In fact, it is quite natural that an antioxidant gives protection in one system, but fails to protect, and sometimes even causes damage, in others (*169*). Taken this into account, an antioxidant could be defined as "any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate" (*2,8*). However, some authors have stated that the concept of an antioxidant *in vitro* should not be extended to cells, organs, animals or populations until the evidence has been obtained, since a molecule demonstrated to have antioxidant properties *in vitro* might have additional properties in a more complex system (*168*).

A compound might exert antioxidant actions *in vivo* by inhibiting the generation of ROS, by directly scavenging free radicals or by removing or lowering the local concentrations of metal ions, which catalyse oxidation. Additionally, it might also act by enhancing the endogenous antioxidant defences (*e.g.* by upregulating the expression of the genes encoding for the cells' natural antioxidant enzymes) (*29,169*). Some examples of antioxidants are discussed below.

5.1. *Cellular antioxidant defences*

The balance between the physiological production of ROS and their detoxification is maintained in cells by effective enzymatic and non-enzymatic antioxidant systems (*170*), as summarised in Figure 6.

As a first line of defence, preventive antioxidants act by binding to and sequestering promoters of oxidation and transition metal ions, like iron and copper, which contain unpaired electrons and strongly accelerate the formation of free radicals. Some examples of preventive antioxidants are transferrin, lactoferrin, ceruloplasmin, haptoglobins, hemopexin, and albumin (*29,171*). This class of preventive antioxidants also comprises the enzymatic antioxidant defences, like superoxide dismutase (SOD), catalase (CAT), and glutathione
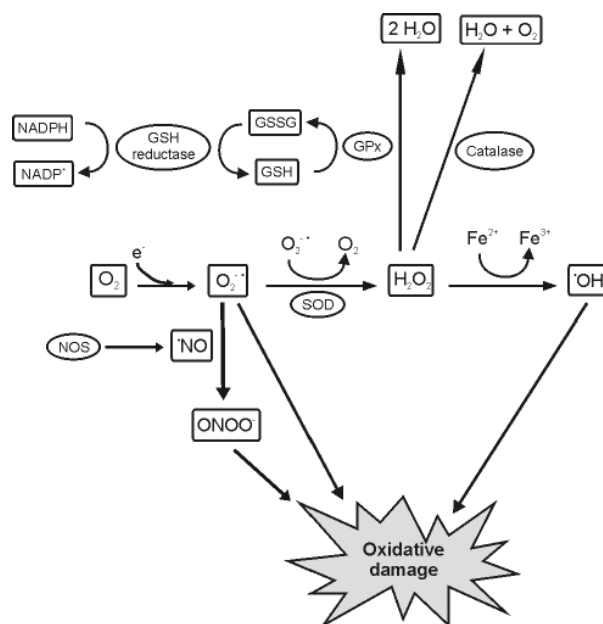
Figure 6. Formation of reactive oxygen and nitrogen species and some of the endogenous antioxidant defence mechanisms. Abbreviations: SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, oxidised glutathione; GPx, glutathione peroxidase.

peroxidase (GPx). These enzymes act on specific ROS following their formation and degrade them to less harmful products (*1,29*).

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase. This enzyme, whose activity was firstly proven in 1969 (*6*), catalyses the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. The H_2O_2 formed in this reaction is then destroyed by the action of catalases and glutathione peroxidases. In humans, there are three forms of SOD: the cytosolic Cu/Zn-SOD, which contains copper and zinc in its active centre, the mitochondrial Mn-SOD (that contains manganese in the active site), and the extracellular SOD (EC-SOD) (*172*).

Catalase is a heme-containing enzyme, mainly localised in the peroxisomes of mammalian cells, which decomposes hydrogen peroxide to water and molecular oxygen. Thus, it lowers the risk of $^{\bullet}OH$ formation from H_2O_2 *via* the Fenton reaction. In addition, catalase is involved in the detoxification of other substrates that work as H^+ donors, such as phenols and alcohols (*123*).

The selenium-containing peroxidases, of which glutathione peroxidase may be considered the most important example, catalyse the reduction of a variety of hydroperoxides (H_2O_2 or ROOH) in the presence of the reduced form of glutathione (GSH) (*123*). This enzyme (for which at least four different mammalian forms are known), oxidises two molecules of GSH to GSSG (the oxidised form of glutathione), which can subsequently be reduced to GSH again by glutathione reductase (*27*).

In addition to these enzymatic defences, cells possess low molecular mass agents that also exert an

antioxidant protection, by removing or degrading ROS to less harmful products, acting as a second line of defence. These include, among others, molecules like glutathione, thioredoxin, and coenzyme Q (*2,29*).

The tripeptide glutathione exists either in the reduced (GSH) or oxidised (GSSG) form and is ubiquitously present in all cells (distributed between the nucleus, endoplasmic reticulum, and mitochondria). Its main functions include: restoration of oxidised protein sulphydryls; detoxification of hydrogen peroxide, lipid hydroperoxides, and electrophilic compounds either directly (*via* GPx-catalysed reactions) or indirectly (through glutathione S-transferases-catalysed conjugation reactions); transport of amino acids through the plasma membrane; direct scavenging of hydroxyl radical and singlet oxygen; regeneration of some important antioxidants, like vitamins C and E, to their active forms; and protection of brain tissue from oxidative stress (*26,27,173,174*).

Along with GSH, thioredoxin, a small and multifunctional disulphide-containing polypeptide, is also important for the maintenance of cellular thiol homeostasis, contributing to the total antioxidant protection (*175,176*). It mainly functions as a general protein disulfide reductant, but is also able to provide control over several transcription factors that affect cell proliferation and death (*27,177*).

Coenzyme Q (CoQ), or ubiquinone, is a ubiquitous electron and proton carrier, playing an important role in the mitochondrial electron-transport chain. Its reduced form, ubiquinol, can function as an antioxidant, preventing lipid peroxidation by reduction of peroxyl radicals and is also able to interact with α-tocopheroxyl radical, thus regenerating endogenous vitamin E within the lipid membrane (*178*).

Other cellular low molecular mass agents, including bilirubin (*36*), lipoic acid (*179*), melatonin (*180*), and uric acid (*181*), have been reported to help cells in the removal of free radicals.

Since the processes of prevention and removal of reactive species are not completely effective, damage still accumulates in biomolecules (*8*). So, adding to the several types of antioxidants mentioned above, cells protect their critical structures by other mechanisms, such as repair and *de novo* enzymes. These act as the last line of defence, by repairing or eliminating damaged molecules, reconstituting them, and even by clearing the toxic and waste products (*29,182*). This type of antioxidants includes heat shock proteins, DNA repair enzymes, proteases, lipases, and transferases (*1*).

5.2. *Antioxidant protection by compounds derived from the diet*

Several compounds present in plants and vegetables have been suggested to have the ability of reacting with free radicals, without generating further radicals. Other compounds become oxidised after scavenging ROS, and thus need to be regenerated for further use (*174*).

Vitamins are considered to be antioxidants of major biological importance. Ascorbic acid (vitamin C) is a water-soluble antioxidant, commonly found in relatively high amounts in fruits and vegetables (*183*). It effectively scavenges several types of ROS/RNS *in vitro*, resulting in the formation of an ascorbyl radical, which can be further oxidised to dehydroascorbate. This molecule can then be regenerated, through the cellular reducing molecules of the cell, to ascorbate. In addition, ascorbic acid can regenerate other small antioxidant molecules from their respective radical species (such as α-tocopherol or GSH). Moreover, it can function as a cofactor for many enzymes, by maintaining the metal ions (such as iron and copper) present in the active centre of those enzymes, in a reduced state. However, the reduction of iron and copper by ascorbate can also result in a pro-oxidant effect, since these ions in the reduced form can be used to fuel the Fenton reaction (*178,184*).

Vitamin E is a lipophilic vitamin, synthesised only by plants, that exists in eight different forms, of which α-tocopherol is the most active. It is the major membrane-bound antioxidant employed by the cell, whose main function resides in protecting cells against lipid peroxidation (*178,185*). As mentioned above, during the antioxidant reaction, α-tocopherol is converted to an α-tocopheryl radical, which is then reduced to the original α-tocopherol form by ascorbic acid (*164*).

Vitamin A and its equivalent in animals, retinol, are also considered important antioxidants. It is present in many foods and can be formed from β-carotene transformation (*186,187*). Vitamin B comprises a family of chemically distinct water-soluble vitamins that play important roles in cell metabolism, although only some of them show antioxidant properties. Vitamin B_1 (thiamine), for example, has been shown to offer protection in different situations where high levels of oxidative stress were involved (*188*). In the same way, high levels of vitamins B_2 (riboflavin) and B_{12} (cobalamin) have also been reported to protect against oxidative injury (*189*).

Carotenoids, of which β-carotene is probably the most studied, are pigments responsible for the colour of many fruits and vegetables. These substances have been recognised as the most potent quenchers of singlet oxygen (*190*) and have been described to help in the prevention of cancer, heart disease, and stroke (*21,151,191*). Other examples of carotenoids with protective effects on oxidative conditions are lycopene (a bright red carotenoid found in red fruits) and xanthophylls (*e.g.* lutein and zeaxanthin) (*192,193*).

(Poly) phenolic compounds are secondary plant metabolites possessing one or more aromatic rings with one or more hydroxyl groups within their structure

(*194*). These comprise a wide variety of molecules, divided into several classes (like flavonoids, phenolic acids, stilbenes, and others), commonly found in fruits, vegetables, wine, tea, coffee, and some cocoa products like dark chocolate (*157,195*). Many studies have reported a wide range of biological activities for these compounds, including anti-inflammatory (*196*), anti-carcinogenic (*23*), anti-allergic (*197*), and anti-hepatotoxic (*198*), which have been described to be mostly due to their antioxidant activity and also their ability to activate endogenous defence systems (*174,199,200*).

The interaction of polyphenols with biological systems is sometimes difficult to understand. For example, the metabolisation of phenolic compounds *in vivo* may give rise to compounds that lose the original antioxidant activity (*174*). Moreover, it should be taken into account that, *in vivo*, polyphenols and their metabolites are found at lower concentrations in plasma or tissues than those of other antioxidants (ascorbic acid and α-tocopherol, for example), since polyphenols may need to undergo structure modifications (*e.g.* hydrolysis) in order to be absorbed (*195,201*).

Despite the numerous beneficial effects mentioned above for these natural compounds, it should be noted that pro-oxidant effects have been described for many of them, resulting in the induction of apoptosis, cell death, and the blocking of cell proliferation (*202,203*). For example, a high intake of vitamin C has been associated to an increased risk of disease (*204,205*). In the same way, α-tocopherol has been reported to be involved in the progression of low-density lipoprotein (LDL) oxidation and atherosclerosis, due to its pro-oxidant effects (*206*). Despite being considered an antioxidant, clinical trials regarding the use of β-carotene for cancer prevention, have demonstrated harmful effects for this compound, as reported by the Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group, in 1994 (*207*), and also by Omenn *et al.* (*208*). Some other compounds (polyphenols) have also presented these opposite effects (*202,209,210*).

5.3. *New synthetic molecules with pharmacological potential*

The above mentioned drawbacks associated to the use of some natural compounds are in part responsible for the undergoing development of novel synthetic compounds, conceived to protect cells against oxidative injury. In fact, the number of reports identifying new synthetic antioxidants has been growing rapidly (*189*).

Many studies have described the synthesis of analogues of existing natural molecules known to possess antioxidant properties, which were modified in order to improve features like their stability and cellular uptake, but maintaining the functionality of the original structure (*10*). In this way, synthetic molecules

that can mimic antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase have been reported as potential new drugs able to reduce damage caused by oxidative stress (*211*). For example, salens, which comprise a class of compounds containing manganese (III) complexes, and metalloporphyrins, a class consisting of a porphyrin combined to a metal ion (usually iron or manganese), have been indicated as mimetics of superoxide dismutase activity (*212-214*). Moreover, compounds like ebselen (a selenium-based compound), as well as diselenide and ditelluride compounds (which contain selenium, sulphur or tellurium in their structures) have been reported to possess glutathione peroxidase-like activity (*214*). In the same perspective, Ehrlich and colleagues (*215*) have recently reported the design and synthesis of a group of GSH analogues, which were named UPF peptides, that showed a higher hydroxyl radical scavenging ability and enhanced antiradical efficiency against the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical, when compared to GSH, without influencing cell viability. Structures of molecules produced by plants, with known antioxidant activity, like polyphenols (*171,216*) or chalcones (*217*) have also been modified in order to increase their biological activity and stability (*218,219*).

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butyl hydroquinone (*t*-BHQ) are synthetic antioxidants, commonly used as food additives to prevent food spoilage, which are able to block lipid peroxidation. However, probably due to their phenolic nature (presence of OH groups), these compounds have shown contradictory effects (*190,220*). In addition, other synthetic compounds, like dimethyl sulfoxide (DMSO), lazaroids (21-aminosteroids drugs structurally related to glucocorticoids), salicylates (anti-inflammatory), pyrrolidine dithiocarbamate (PDTC), and dimethylurea have been found to scavenge free radicals and have also been used in therapeutic approaches (*29,221*).

In the past few years, novel nitrogenated structures, named FMAs, have been studied in our lab (*222-224*). These compounds, which are composed of an amidine unit linked to a phenol ring and that differ mainly on the number hydroxyl groups within that ring, were synthesised based on different aspects: 1) in living organisms exist several compounds containing nitrogen atoms in their structure (*e.g.* serotonin, purinic and pyrimidinic DNA bases, catecholamines, among others), which are known to easily interact with active centres responsible for different functions in those same organisms (*225*); 2) the presence of hydroxyl groups within a phenol ring has been reported to be responsible for the antioxidant properties of a compound, since those groups are able to donate an hydrogen (*218,226,227*); and 3) it has also been acknowledged that molecules incorporating conjugated systems with

nitrogen atoms can stabilise free radicals (*228*). The combination of these factors was expected to enhance the ability of these new structures to scavenge free radicals. Experiments in cell-free systems, namely by the use of DPPH discolouration and 2-deoxy-D-ribose degradation assays, gave the first indication of the compounds' good antiradical activity. The free radical scavenging properties of the nitrogen compounds were translated into *in vitro* cell models, namely PC12 and H9c2 cells, in which they showed a strong ability to scavenge ROS/RNS and therefore show their ability to act on oxidative stress-mediated injury associated to several pathologies, including neurodegenerative and cardiovascular diseases. In fact, they were able to prevent a series of events related to oxidative stress, like intracellular ROS formation, lipid peroxidation, and oxidative DNA damage, evaluated in adequate cell models. To some extent, they also seemed to act on intracellular signaling mechanisms resulting, for example, in an increase of the capacity to repair oxidative DNA damage and in a regulation of the apoptotic cascade, specifically at the mitochondrial level. In this way, these nitrogen compounds showed superior protection profiles when compared to the traditional antioxidant trolox (a water-soluble analogue of vitamin E) (*222,223*).

Moreover, a comparative study with polyphenolic compounds, which have somehow related structures (they also possess more than one hydroxyl group), showed some advantages, of these synthetic structures, over the polyphenols, namely their lack of genotoxicity and their ability to increase the activity of DNA repair enzymes (*224*).

Additionally, novel synthetic diarylamines containing a benzo[*b*]thiophene nucleus, which are also currently being studied in our lab, have shown quite promising results, namely by protecting cells, in particular at the mitochondrial level, from oxidative-induced damage at concentrations in the nanomolar range (*229*).

6. Conclusions

Reactive oxygen (ROS) and nitrogen (RNS) species are produced as a consequence of both exogenous and/or endogenous stimuli. Depending on the amounts present inside the cells, their effects can be either harmful or beneficial. In low amounts, they act as secondary messengers and regulate intracellular signalling, being their potentially adverse effects counteracted by the cells' antioxidant defence systems. However, their prevalence in higher amounts, resulting from their overproduction, when not balanced by the antioxidant systems, leads to oxidative stress. This condition is known to cause damage to all biomolecules, including proteins, lipids, and nucleic acids. Although cells possess mechanisms to repair some of this oxidative damage (as is the case of DNA repair pathways), if not duly repaired, modifications to biomolecules can be toxic, mutagenic, and carcinogenic.

Intracellular accumulation of reactive oxygen and nitrogen species can ultimately result in the initiation of cell death processes. In this regard, it should be noted that the amount of reactive species present in the cell and the way as the cell responds to it can determine that same cell's fate: mild oxidative stress may be responsible for the activation of biological responses that can either lead to survival and proliferation, or can induce apoptosis, whereas a great accumulation of ROS/RNS can result in necrosis. Moreover, the association of oxidative stress-induced cell death with several pathological conditions (including cardiovascular and neurodegenerative diseases, among others), either as the cause or the consequence of the disease, has been widely recognized by the scientific community.

Excessive ROS/RNS can be inactivated by endogenous and/or exogenous antioxidant molecules. These may exert their antioxidant actions by inhibiting the generation of free radicals or by scavenging them, by lowering the concentrations of metal ions involved in the formation of ROS/RNS, or even by enhancing the endogenous antioxidant defences *via* upregulation of gene expression.

Usually, cells are able to maintain a balance between the intracellular formation of reactive species and their removal due to the presence of endogenous enzymatic (*e.g.* superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic (*e.g.* glutathione, thioredoxin, coenzyme Q) defences. Nevertheless, additional antioxidant protection can also be attributed to some compounds derived from the diet, which may be found in plants and vegetables. These include vitamins (namely A, C, and E), carotenoids, and polyphenolic compounds.

However, there are some drawbacks associated to the use of natural compounds, namely the pro-oxidant effects that have already been described for some of them. Moreover, metabolization *in vivo* of some of these compounds, namely polyphenols, may originate compounds that lose their initial antioxidant activity (and sometimes even yielding toxic derivatives). In addition, the structural modifications those polyphenols need to undergo to be absorbed *in vivo* may result in lower concentrations in plasma and tissues, when compared to other antioxidants (like vitamin C or E, for example).

Therefore, research on the development of new synthetic structures capable of acting beyond and/or potentiate the cells' natural defence mechanisms has dramatically increased in the last few years. These novel structures, some of which are described in this review, are often analogues of naturally occurring molecules or structurally related to them, and clearly have shown increased antioxidant activity, associated to

decreased toxicity in many cases. They look promising and so complementary research regarding their use, as active principles of pharmacological compounds, must go on.

The development of novel synthetic molecules with powerful antioxidant properties, as well as the discovery of new beneficial effects for existing ones, associated to the identification of potential new drug targets has been one of the scientists' concerns over the past years. Success in this field of research can contribute to the reduction of oxidative stress-induced damage in several pathological conditions, namely in cardiovascular and neurodegenerative diseases.

Acknowledgements

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