

Review

The Ambiguous Aspects of Oxygen

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Abstract: For most living beings, oxygen is an essential molecule for survival, being the basis of biological oxidations, which satisfy most of the energy needs of aerobic organisms. Oxygen can also behave as a toxic agent posing a threat to the existence of living beings since it can give rise to reactive oxygen species (ROS) that can oxidise biological macromolecules, among which proteins and lipids are the preferred targets. Oxidative damage can induce cell, tissue, and organ dysfunction, which leads to severe body damage and even death. The survival of the aerobic organism depends on the development of an elaborate antioxidant defence system adapted to the normal level of atmospheric oxygen. The production of ROS in the aerobic organism can occur accidentally from exposure to pollutants or radiation, but occurs constantly during normal metabolic reactions. Cells have evolved using ROS to their advantage. Indeed, ROS are used as signalling molecules in numerous physiological processes, including muscle contraction, regulation of insulin release, and adaptation to environmental changes. Therefore, supplementation with antioxidants must be used wisely. A low level of ROS is essential for adaptation processes, so an excess of antioxidants can be harmful. Conversely, in conditions where ROS production increases, antioxidants can be useful to avoid cellular dysfunction.

Keywords: oxygen toxicity; reactive oxygen species (ROS); superoxide dismutase (SOD); pollutants; cigarette smoke; NADPH oxidase (NOX); cytochrome p450 (CYP); mitochondria; antioxidants; redox signalling; Nrf2



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1. Introduction

Aerobic organisms require the presence of oxygen to complete the processes underlying the transformations necessary to obtain the chemical energy that is useful for maintaining cellular homeostasis and supporting all activities related to life, such as growth, locomotion, and reproduction [1].

Oxygen utilisation poses challenges to living organisms, as reactive oxygen species (ROS) can form from it [1]. ROS can oxidise biological macromolecules, generating damage that can alter their functionality [2]. For this reason, the evolution of aerobiosis has evolved in concert with that of antioxidant defence systems [3]. Furthermore, in aerobic organisms, mechanisms that have enabled them to use ROS as signal molecules for cellular adaptation to environmental changes have evolved [2]. The present review briefly describes how cells use oxygen, the formation of ROS, the damage they impose on the cell, and how cells evolve using ROS to their advantage.

2. The Source of Energy

Living organisms are systems characterised by the ability to convert available external energy into work necessary to maintain their homeostatic functions [4,5]. Although, in

theory, it is possible to convert one form of energy into another, living organisms lack the conditions for such a conversion.

In essence, life energy comes from the constant stream of solar photons reaching our planet. When a photon interacts with an electron of a molecule on earth, the electron reaches a higher energy level, from which it returns to its initial state and releases energy [6]. Living organisms have developed mechanisms to capture and use the released energy to their advantage [6]. These systems consist of a gradual release of energy during the passage of electrons among the outer orbitals of atoms with different energy levels [6]. The dissipation of electronic energy is associated with the synthesis of ATP, which is a fundamental molecule for living organisms [6].

ATP consists of a base of adenine and a ribose sugar, with three phosphate groups linked by two high-energy bonds called phosphohydride bonds. The removal of phosphate groups by hydrolysis with the following conversion of ATP into ADP and AMP releases energy. The released energy can be transferred to other molecules to make unfavourable reactions in a cell favourable.

The Earth's atmosphere initially consisted of CO_2 , N_2 , and H_2O , with traces of H_2 . It is assumed that, in the prebiotic environment, oxygen did not exist in its free form. A decisive phase in the evolution of life was the appearance of photosystem II [7,8], capable of effectively capturing photons and using water to obtain the hydrogen necessary to synthesise NADPH, ATP and, therefore, for the synthesis of carbohydrates using CO_2 [9] (Figure 1). While the use of water has freed bacteria from the need to use other environmental reducing agents, such as sulphur species, it has also resulted in the release of oxygen as a side effect. The latter created the fundamental conditions for the evolution of heterotrophic organisms that depend on respiration for the synthesis of ATP. Heterotrophs are unable to capture solar photons, so they take carbohydrates and other high-energy substances by eating plants or animals and get most of the energy they need through respiration, an enormously exergonic form of metabolism that was an important driver, if not essential, to the evolution of complex life forms and eukaryotes [10,11].

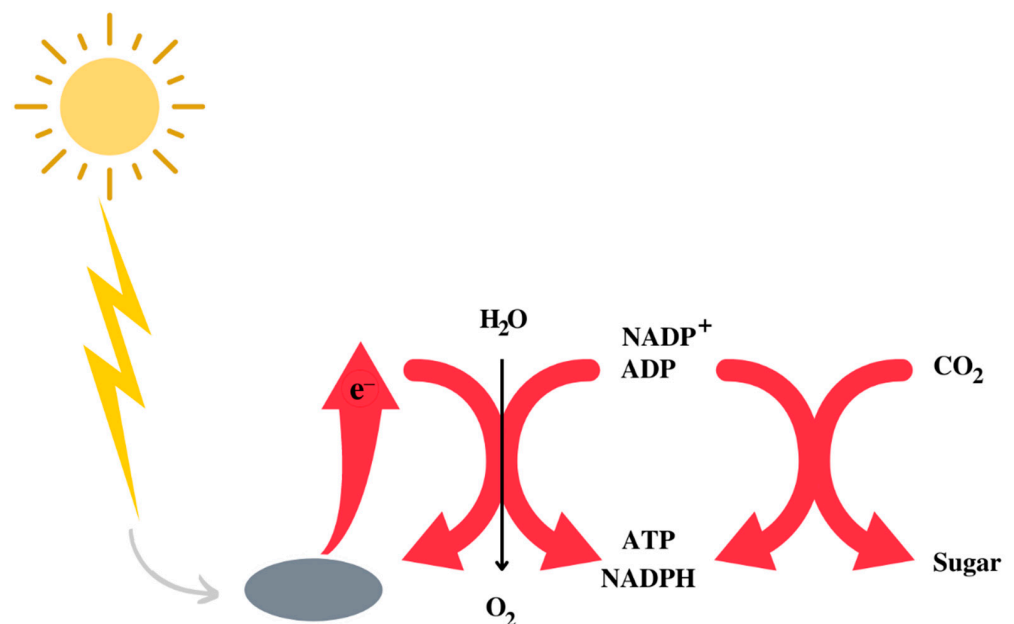


Figure 1. Schematic representation of the utilisation of the sun energy to obtain a form of metabolically utilisable energy by living beings.

Therefore, in heterotrophic aerobic organisms, most of the energy required for life processes is obtained from respiration, in which ATP is obtained by moving electrons from the high energy level of nutrient molecules, such as glucose, to a low energy level in the molecule of water through the passage among a series of electron acceptors.

In conclusion, the only energy source that heterotrophs can use is the chemical one, taken from the outside in the form of compounds with high energy content. This ability depends on the development of biochemical and physiological systems that allow for the transformation of chemical energy into other forms of energy essential for life. Ultimately, the energy supply of living systems essentially occurs in the form of an exchange of matter.

3. Oxygen Toxicity

For most living beings, oxygen is an essential molecule for survival, being the basis of biological oxidations which satisfy most of the energy needs of aerobic organisms. Although oxygen is essential for these organisms, it can also behave as a toxic agent and prove to be a threat to their existence. This inconsistent aspect of aerobic life was defined by Davies as the “paradox of aerobic life” [12]. Indeed, the organisms can survive in the presence of oxygen because they have developed an elaborate antioxidant defence system, adapting to the Earth’s oxidising atmosphere. However, this system is only adequate for the atmospheric oxygen pressure (approximately 156 mm Hg), and there is strong evidence that exposure to oxygen pressures higher than atmospheric ones causes severe damage.

The toxicity of oxygen was already known in the 1770s when Joseph Priestley, who discovered oxygen, together with but independently from Carl Wilhelm Scheele, compared its effect on the body to candle burning, observing that a candle burns out faster in oxygen than in air and wondering if “the animal powers be too soon exhausted in this pure kind of air” [13].

The deleterious consequences of breathing oxygen at elevated partial pressures were first recognised in the late 19th century. Paul Bert was the first to describe the toxic effects of hyperbaric oxygen on the central nervous system [14] and Lorrain Smith was the first to describe the effects on the lungs [15].

Another example of oxygen toxicity is the retinopathy of the prematurity (ROP). In the preterm infant, supplemental oxygen administration has been frequently used as a life-saving treatment since the 1780s [16,17] and, in the 1940s regular supplemental oxygen use was established for the preterm infant [18]. The spread of the practice of oxygen supplementation in preterm infants led to an epidemic of severe retinopathy of prematurity and blindness between the 1940s and 1950s. In 1942, the phenomenon of retrolental fibroblastic overgrowth [19] was described, and in 1950 the role of oxygen in the development of this new sudden blindness observed in preterm infants, which was called retrolental fibroplasia [20], now known as ROP, was defined. Several studies have established the role of hyperoxia in ROP [21–23].

The susceptibility of the human retina to hyperoxia is due to vasculature being not fully developed until full term of gestation, and the photoreceptors developing after the small vessels. Excess oxygen leads to the destruction and arrest of the development of the neurovascular retina, followed by abnormal neovascularisation during the vulnerable retinal development stage [24,25].

Currently, a more careful control of oxygen use (continuous monitoring, with intermittent supplementation of O₂, provided only to maintain blood O₂ levels) has significantly decreased the severity of retinopathy [26]. Administration of the antioxidant α -tocopherol to preterm infants may reduce ROP but has been shown to have harmful effects in very preterm babies [27]. Nowadays, the use of vitamin E in the prevention of the exacerbation of ROP, and of other dysfunctions related to preterm, could be reconsidered in the light of current knowledge in the field of modern neonatal medicine [28].

High partial pressures of oxygen can also be harmful to adult humans due to their effects on the central nervous system, lungs, and eyes, even if other systems can also be affected [29]. The partial pressure of oxygen in inspired air, at the sea level, is about 160 mm Hg. This value can be increased by either breathing 100% oxygen, or by increasing the pressure of the breathing mixture [30]. These conditions can occur using hyperbaric oxygen for therapeutic purposes, or during underwater diving.

The signs of oxygen toxicity are detectable in various tissues; however, the most worrying are those affecting the central nervous system and the lungs, which can be considered real target organs.

The lungs' sensitivity to the high partial pressure of O₂, or a high percentage of oxygen in the breathing air, may be due to its greater exposure to high oxygen levels compared to other tissues in the body [29].

The pulmonary oxygen toxicity onset is directly related to the partial pressure of inspired oxygen, and usually it does not occur at oxygen concentrations lower than 50% in young healthy subjects [31]. Pulmonary toxicity occurs after 4–22 h at an oxygen percentage greater than 95% at atmospheric pressure and within 3 h at 100% oxygen at 2–3 times the atmospheric pressure [32]. The first manifestation of pulmonary toxicity is tracheobronchial irritation associated with cough and progressive dyspnoea in men exposed to 98% oxygen for 30–74 h [33]. Longer exposures to oxygen may induce diffuse alveolar damage characterised by clinical symptoms and pathological signs of alveolar damage, like those of acute respiratory distress syndrome from other causes [34]. Prolonged exposure to sublethal concentrations of oxygen may result in chronic pulmonary fibrosis and emphysema with tachypnoea as well as progressive hypoxaemia that can cause, after a few days, a lung damage so severe it may, paradoxically, cause death from anoxia [35].

Central nervous system (CNS) toxicity occurs following short-term exposure to air containing highly concentrated oxygen at a pressure above atmospheric. Exposure for minutes to hours at 160 kPa of partial pressure, approximatively eight times the atmospheric concentration, is generally associated with CNS toxicity [29]. CNS toxicity does not occur during normobaric exposure to high O₂ concentrations, and conditions related to CNS toxicity can only be met in special situations such as immersion or hyperbaric oxygen treatment. The appearance time of the CNS toxicity initial symptoms (tunnel vision, tinnitus, nausea, facial twitching, dizziness, and confusion) is inversely related to the oxygen pressure and may be as short as 10 min at pressures of 4–5 atmospheres absolute [32]. Initial symptoms may be followed by tonic–clonic seizures and subsequently by unconsciousness. The onset of the initial disturbances does not follow a defined pattern before the onset of the seizures, which are the most dramatic and dangerous symptom of oxygen toxicity. However, the latter are reversible and leave no neurological damage if the partial pressure of the inspired oxygen is reduced. The onset of seizures depends on the partial pressure of oxygen and the duration of exposure [29]. Moreover, the exposure time at which symptoms begin depends on many factors and can change in the same individual day by day [30,31].

Many factors such as underwater activity, cold weather, and physical activity can reduce the time to onset of CNS symptoms [29]. Oxygen toxicity is very dangerous during diving as it may cause drowning due to an epileptic attack. Understanding that a diver exhibits symptoms of oxygen toxicity is difficult before seizure activity develops, as the first symptoms are non-specific and do not follow a typical sequence.

4. Oxygen Reactivity

The toxicity of oxygen was initially attributed to its ability to inhibit cellular enzymes [36,37]. There are several examples of oxygen-mediated enzymatic inactivation in anaerobic organisms [38]. However, most enzymes in aerobic cells are insensitive to oxygen and those that are inactivated are poorly sensitive. Furthermore, the rate at which oxygen-sensitive enzymes are inactivated is too low and does not match the rate at which the toxic effects develop [1].

Subsequently, the oxygen toxicity was attributed to the formation of oxygen radicals [39]. Despite being a free radical, O₂ does not have high reactivity. The reactions in which it is involved do not take place at ordinary temperatures or in the absence of catalysts, even if its high oxidising power makes most of the substances of biological interest thermodynamically unstable in its presence [1].

This apparent contradiction is explained by the electronic configuration of diatomic oxygen. It has two unpaired electrons, each located in a different π^* antibonding orbital [1].

The two electrons have the same quantum number of spins (they have parallel spins) in the ground state of oxygen. If oxygen attempts to oxidise another atom or molecule by accepting a pair of electrons from it, both electrons must have antiparallel spins to fit the empty space in the π^* orbitals. However, a pair of electrons in an atomic or molecular orbital does not meet this criterion since they have opposite spins according to the Pauli principle. This represents a restriction (called a spin restriction) on the transfer of electrons to oxygen, which can accept electrons only one at a time. This explains why O_2 reacts slowly with many non-radicals [39].

In aerobic organisms, the energy necessary for the vital processes derives from the oxidation reactions in which oxygen is consumed; therefore, it is evident that processes are operative in such organisms through which the spin restriction is, in some way, eliminated with a consequent increase in oxygen reactivity.

The elimination of the spin restriction could be achieved by putting enough energy in O_2 to allow the inversion of the spin of one of its electrons in the π^* orbital, thus passing O_2 from the basal to an excited state named singlet oxygen. In the singlet oxygen, the elimination of the spin restriction makes oxygen much more reactive [40]. Moreover, the two electrons may remain in different orbitals or couple in one orbital. Therefore, two species with different reactivities are generated. Of the two singlet states of O_2 , the one with unpaired electrons has higher energy, truly short life in solution and decays quickly [41]. Differently, singlet oxygen with paired electrons has a much longer lifespan, so it is believed to be the only singlet oxygen species that react in solution [41].

The energy of light in the visible spectrum would be sufficient for the formation of singlet oxygen, but, fortunately, oxygen does not absorb visible light. The formation of singlet oxygen occurs when an appropriate dye, called a photosensitiser, absorbs visible light, and then collides with molecular oxygen transferring energy to it, giving rise to singlet oxygen [41]. There are many of these dyes in nature that support singlet oxygen formation. These include photosynthetic pigments, protoporphyrin IX (a heme precursor), Bengal rose, or methylene blue [41]. Singlet oxygen participates in many biochemical processes, such as photosynthesis, cell signalling, immune responses, or the degradation of polymers. However, the transformations are generally small and, therefore, cannot explain the evidence according to which many of the major changes occurring in cells consist of the O_2 oxidation of various types of molecules.

Another way to avoid the spin restriction is to add electrons to oxygen one at a time, at a rate that allows for electronic rotational reversals between collision events [42]. The univalent oxygen reduction pathway requires the generation of oxygen reduction intermediates, which are reactive and can damage biological molecules [42].

These reactions are due to the intervention of substances that can transfer, in the right direction, one or more electrons from the molecule to be oxidised to O_2 [1]. Among such substances, there are transition metal ions of varying valence, such as iron and copper, which have unpaired electrons with parallel spins and enzymes, such as cytochrome oxidase (Cox), which have metal ions in their active sites [1].

Cox is the terminal oxidase of cell respiration, accepts electrons from the reduced form of the cytochrome c (Cyt c), promotes the four-electrons reduction in oxygen to water, completes the electron transport of the mitochondrial respiratory chain, and can protect cells from the damage due to the formation of toxic intermediate of oxygen reduction [43]. In mammalian, Cox is a bigenomic enzyme. It contains 13 subunits, 3 catalytic subunits encoded by the mitochondrial genes and the remaining 10 encoded by the nuclear genome. The latter play a role in the regulation and/or in the assembly of the enzyme. Cox contains two heme groups (heme a and a_3) and two Cu^{2+} centres (Cu^{2+} A and Cu^{2+} B) in the catalytic centre and uses more than 90% of the oxygen consumed by cells and tissues [44].

Cox transforms O_2 into water in the reduction site that contains Fea_3 and CuB , each of which accepts one electron. O_2 bounds to the reduction site when both metals are in the reduced state (Fea_3^{2+} and CuB^{1+}). The fully reduced state is named R-intermediate.

The bond of O_2 to the R intermediate forms $Fea_3^{3+}-O_2^-$ (called intermediate A). This is converted into intermediate P without electron transfer from low-potential metal sites (Fea and CuA). When the P intermediate forms, the bound O_2 accepts four electrons (two from Fea_3 , one from CuB, and the fourth from a neighbouring OH tyrosine). In the intermediate P, an oxide (O_2^-) and a hydroxide (OH^-) bond to Fea^3 and CuB, respectively. Subsequently, intermediate P receives four electrons from cytochrome c through the low potential metal sites, CuA and Fea (heme a). These equivalents are added one at a time, forming the intermediates F^- , O^- , and E, and finally reproducing the intermediate R. Each electron transfer from heme a couples with the pumping of one proton in the intermembrane space of the mitochondria, creating the driving force for ATP synthesis [43].

According to this view, the first three phases of the reaction generate, in sequence, the superoxide ion ($O_2^{\bullet-}$), peroxide ion (O_2^{2-}), which immediately protonates to hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\bullet OH$). The addition of the fourth electron leads to the hydroxyl ion, which is transformed into the water by the addition of an H^+ .

Cox, firmly bound in its active site, holds all partially reduced intermediates of O_2 until complete reduction.

These intermediates of oxygen reduction do not have the kinetic impediments of O_2 in the ground state and are much more reactive. For this reason, they are referred to as reactive oxygen species (ROS).

However, Cox is not a site of ROS release in the cells. Several experimental pieces of evidence suggest that other cellular sites are responsible for the formation of the ROS.

In the ROP, the ROS generated during the hyperoxia determine the destruction arrest of the development of the neurovascular retina as well as abnormal neovascularisation during a vulnerable developmental retinal stage [24,25].

5. Reactive Oxygen Species and Their Sources

ROS encompass a group of molecules derived from molecular oxygen, which are formed by reduction–oxidation (redox) reactions or by electronic excitation [45]. They include, other than superoxide, hydrogen peroxide, hydroxyl radical, as well as atoms such as alkoxy radicals ($RO\bullet$) and peroxy radicals ($ROO\bullet$), and radical species such as singlet oxygen and ozone (O_3) (Table 1).

Table 1. Main reactive oxygen species, their reactivity and half-life [46].

Reactive Oxygen Species		
Species	Reactivity	T 1/2
1O_2 , Singlet oxygen	+++	1–4 μs
$O_2^{\bullet-}$, Superoxide	++	1–4 μs
H_2O_2 , Hydrogen peroxide	+	1 μs
$\bullet OH$, Hydroxyl radical	++++	1 μs

The reactivity of the different ROS is very variable. Superoxide is generally the first ROS generated. It is moderately reactive and exhibits a short half-life of 2–4 μs [46]. Therefore, it is not damaging by itself, and its harmful effects depend on the products formed after dismutation. $O_2^{\bullet-}$ dismutates to H_2O_2 and O_2 spontaneously or owing to the intervention of superoxide dismutases. Therefore, it is the main source of H_2O_2 . Due to high electrostatic attraction, $O_2^{\bullet-}$ oxidises Fe–S clusters at a high rate. This oxidation leads to iron release. While $O_2^{\bullet-}$ diffuses slowly in the membrane due to its negative charge, its protonated form perhydroxyl radical (HO_2) is uncharged and may diffuse in lipids and produce a carbon-centred radical of polyunsaturated lipids. $O_2^{\bullet-}$ reacts efficiently with nitric oxide ($NO\bullet$), which leads to the formation of peroxynitrite ($ONOO^-$), a tyrosine-nitrating agent.

Notwithstanding, H_2O_2 is a strong two-electron oxidant, and the high activation energy restricts its reactivity to a few biological targets [45]. Hydrogen peroxide is relatively stable and shows a half-life of 1 ms. With glutathione, cysteine, and methionine, H_2O_2 shows a low reactivity while the reaction with cysteine in specific proteins can be very high. Reactivity with cysteine depends on the structure and environment of the protein, which could explain the selectivity and specificity of H_2O_2 in redox signalling [45].

The most reactive ROS is hydroxyl radical, which oxidises biological molecules at a diffusion-controlled rate which renders it a non-specific oxidant. Its formation is due to the reduction reaction of hydrogen peroxide in the Fenton reaction and requires the presence of a Fe or Cu metal in the reduced state [47,48]. $\bullet OH$ reacts as soon as produced with sites adjacent to the production site. For this reason, the localisation of Fe^{2+} is a determining factor for the site of toxicity. $\bullet OH$ is an initiator of lipid peroxidation.

Peroxyl radical ($ROO\cdot$) forms in the initial phases of lipid peroxidation and propagate free radical chain reactions by abstracting a proton from another polyunsaturated fatty acid, thereby creating a lipid hydroperoxide and another carbon radical. Alkoxy radical ($RO\bullet$) forms as an intermediate in lipid peroxidation during metal-catalysed decomposition of lipid hydroperoxides and can amplify lipid peroxidation chain reactions.

ROS are generated by various sources which can either be endogenous or exogenous.

6. Exogenous Sources

Exogenous or environmental sources of ROS include electromagnetic and corpuscular radiations and various types of xenobiotics.

6.1. Radiation

Various types of radiation can exert effects on living organisms, resulting in a series of biological effects that affect life processes to varying degrees. Radiations regulate several physiological processes such as photosynthesis, photoperiodism, circadian rhythms, vision, and vitamin D formation [49]. On the other hand, radiations can also be responsible for a series of pathological events in which free radicals are sometimes involved.

Ultraviolet radiation can induce biological damage involving radical intermediates. This effect is due to the capacity of some molecules to absorb quanta of energy of the ultraviolet radiation passing to an excited state, from which they can decay, dissociate, and form free radicals.

Ionising radiation can be divided into corpuscular radiation (electrons, protons, neutrons, and particles α and β) and electromagnetic radiation (X-rays and γ rays). They possess sufficiently high energy to move an electron away from a molecule, thus forming an ion that can dissociate and generate a free radical or recapture the electron passing to an excited state and then undergoing homolytic cleavage [50].

Exposure of living organisms to ionising radiation can give rise to primary radicals by transferring their energy to cellular substances, especially to the main component of cells, namely water. Following the absorption of energy, water undergoes ionisation and excitation. The electron hydrates, the radical ion decomposes, and the excited molecule splits homolytically and generates the hydrogen atom and the hydroxyl radical.

The primary radicals, which include $\bullet OH$, $H\bullet$, and e^-_{aq} , can diffuse and undergo secondary reactions with various cellular solutes, including organic macromolecules, generating new chemical species [51].

Ionising radiation, unlike ultraviolet radiation, penetrates deeply into the irradiated tissues so that damage can also occur in deeply located cells [49].

Biological systems are much more sensitive to radiation when irradiated in the presence of O_2 (oxygen effect) [52]. Radiosensitivity decreases at low levels (hypoxia) or in the absence (anoxia) of O_2 . The study of the dependence of radiosensitivity on O_2 concentration has shown a sharp decrease in radiosensitivity when the pO_2 voltage drops to values below 15 mm Hg, while for higher values, the radiosensitivity is almost constant [53]. Since in mammals the pO_2 of arterial blood is about 70 mm Hg, and that of venous blood is about

40 mm Hg, it is evident that most normal tissues show the highest degree of sensitivity towards the oxygen effect.

The oxygen effect found an initial explanation in the possibility that the primary radicals H^\bullet and e^-_{aq} react with O_2 , forming the hydroperoxyl radical and superoxide, respectively.

In this way, new free radicals are produced, amplifying the effect of the radiation. On the other hand, a free radical generated by a biological molecule, either due to the direct result of radiation or by extraction of hydrogen by the primary radical $\bullet OH$, can bind to O_2 , forming a peroxy radical which can, in turn, extract hydrogen from a biological molecule, causing a chain reaction.

6.2. Environmental Pollutants

Numerous environmental agents, such as photochemical pollutants, pesticides, solvents, anaesthetics, components of cigarette smoke and automobile exhaust gases, and aromatic hydrocarbons, can cause damage to cells.

Studies aimed at determining the mechanism underlying the deleterious effects of air pollution have shown that, although various substances are harmful to the body after their absorption, they are converted into radical species by intracellular detoxification processes, and many other environmental pollutants already exist as free radicals [54].

6.3. Nitrogen Dioxide

One of the most widespread radicals in the environment is nitrogen dioxide, a red-brown gas that irritates the mucous membranes and is responsible for specific pathologies affecting the respiratory system (irritation, bronchitis, allergies). It arises from the reaction of N_2 with O_2 during the combustion of organic material [55]. Unlike nitric oxide, dioxide is very reactive, and is emitted into the air by car and factory exhausts.

Healthy men can tolerate brief exposures at levels as low as 4 ppm without obvious lung damage. However, in asthmatics, even levels below 0.3 ppm can cause an increase in bronchial reactivity.

The dioxide is also found inside homes, where it can be produced by gas cookers, wood stoves, coal, kerosene, and by smokers, reaching levels above 1 ppm.

Nitrogen dioxide reacts with water to form nitric oxide and nitric acid. The dangerous effects of nitrogen dioxide are also related to its ability to act as a precursor to other harmful gases, such as ozone [56,57].

6.4. Ozone

Ozone forms from a photochemical reaction between O_2 and NO_2^\bullet , other than for O_2 photodissociation. Solar radiation and high temperatures activate the reaction [58]. In a simplified way, it is composed of two phases. In the first, which requires sunlight, the photochemical splitting of the dioxide into nitric oxide and atomic oxygen takes place. In the second phase, atomic oxygen reacts with molecular oxygen forming ozone.

In highly polluted urban areas during the day, there is a significant formation of ozone which is the leading cause of the so-called photochemical smog [59]. During winter, because the low temperatures inhibit the reaction, the formation of ozone is a summer-linked problem.

Parallel to the ozone synthesis reaction, there is an opposite ozone elimination reaction in which the ozone reacts with nitric oxide, reforming the starting products. This reaction is independent of light. Therefore, an almost total elimination of ozone occurs during the night. In rural areas, where the air is cleaner, nocturnal ozone depletion is much lower [59].

The gas, which in the upper layers of the atmosphere (stratosphere) is useful for protecting the Earth's surface from excessive irradiation of ultraviolet rays, in the lower layers of the atmosphere (troposphere) is instead a direct cause of damage to vegetation, buildings, and health. Ozone, being strongly oxidising, may: (i) cause the oxidation of metals and the depolymerisation and degradation of plastics and rubbers; (ii) interfere with

the function of chlorophyll and with plant growth; (iii) cause irritation and inflammation of the eyes and the upper respiratory tract.

These phenomena, dependent on short-term exposures, rapidly cease with the cessation of exposure. Conversely, prolonged exposure to high levels of ozone can cause irreversible damage to the structure of the respiratory and cardiovascular systems.

6.5. Cigarette Smoke Radicals

Tobacco smoke is a mixture of around 3000 compounds, many of which are toxic. These include nicotine, carbon monoxide, benzene, hydrogen cyanide, free radicals, cytotoxic aldehydes, and carcinogens such as nitrosamines and benzopyrene [60].

The smoke can be separated by using special filters in a gas phase and in a particulate phase; the tar residue. A smoker inhales both the gaseous phase and a large part of the particulate fraction that penetrates the body whatever filter is used. Both the gas phase and the tar are highly oxidising and are sources of free radicals. The tar, in addition to many aromatic compounds, contains about 10^{17} radicals per gram, most of which are very stable and persist for hours [61]. There are at least four distinct species of radicals, including the semiquinone, that undergo redox interconversions with quinones and hydroquinones. Aqueous tar extracts generate the superoxide radical, probably by the reaction of the semiquinones with O_2 . On the other hand, diphenol/quinone systems can function as scavengers of superoxide in dependence of the equilibrium position and pH [60].

The gaseous phase contains radicals that are both less stable and less concentrated. These include alkoxy radicals, peroxy radicals, and, to a lesser extent, carbon-centred radicals. It also contains aerosols, carbon particles containing adsorbed carcinogenic hydrocarbons, and other toxins, including polyphenols and quinones, which can generate ROS [61]. These particles are part of the so-called fine dust, also referred to as a particulate matter of the environment (PM), in which particulate matter is the set of all solid or liquid particles suspended in the air, excluding pure water, with microscopic dimensions [61].

PM10 is the atmospheric particulate with a diameter equal to or less than $10\ \mu\text{m}$, while PM2.5 is the finest fraction of PM10, made up of particles with a diameter equal to or less than $2.5\ \mu\text{m}$. The particle diameter is the most important parameter for characterising the physical behaviour of atmospheric particulate matter. Thus, PM2.5 is the most dangerous particulate for health and the environment as it can remain suspended in the atmosphere for days or weeks. The larger particles (from 2.5 to $10\ \mu\text{m}$) remain in the atmosphere for a few hours to a few days and are significantly less harmful to health and the environment. Moreover, the measurement of PM10 (expressed in $\mu\text{g}/\text{m}^3$) as a method of assessing particulate pollution provides incomplete information since it does not distinguish coarse particles from dangerous PM2.5. Paradoxically, a high value of PM10 can correspond to the presence of a few PM 2.5-type particles and many of a larger size, a more acceptable situation than a lower-value PM10 with few coarse particles and many smaller than $2.5\ \mu\text{m}$. The observations made available by the scanning electron microscope are therefore important, for they allow us to see the particles, count them, distinguish the various families, and study their chemical composition. Cigarette smoke as well as respirable fibres and dust act synergistically in increasing the production of damaging hydroxyl radicals [61].

7. Endogenous ROS Generation Sites

There is no doubt that, although the body's exposure to ROS resulting from exogenous sources can be remarkably high, exposure to endogenous sources is much more important and continuous. Free radicals are only occasionally formed by environmental factors, while they are continuously generated throughout the life span of every cell in the body, as by-products of normal cellular metabolism.

Several cellular sites of ROS generation have been identified, located on the plasma membrane, in the cytoplasm, in the peroxisomes, and on the membranes of the mitochondria and endoplasmic reticulum. However, under non-pathological conditions, most of

these species appear to arise from reactions involving components from the mitochondrial and microsomal electron transfer chains.

7.1. Mitochondria

Reactive oxygen species can be produced in the mitochondria by components of the respiratory chain located on the inner membrane, as well as by enzymes, located both on the inner membrane and on the outer membrane.

7.2. Mitochondrial Respiratory Chain

About 95% of the oxygen that animals breathe is used by the mitochondria, where the synthesis of ATP occurs. The electrons, deriving from the electron carriers, reach Cox and are used to operate a tetravalent reduction in O_2 . Cox has a very high affinity for O_2 which allows it to work even at oxygen concentrations lower than one millimetre of mercury (1 mm Hg) and when the mitochondrial energy production is at low oxygen concentrations [62]. This aspect is important because the oxygen concentration at the mitochondria level can be very low. Indeed, while in the fully oxygenated blood that leaves the lungs, the partial pressure of O_2 (pO_2) is about 100 mm Hg, pO_2 rapidly falls into the tissues when oxyhaemoglobin releases O_2 . Cells in a tissue may be in the presence of extracellular O_2 concentrations that provide a pO_2 as low as 5–15 mm Hg and a further drop in the local pO_2 around individual oxygen-consuming mitochondria verifies [62].

Cox's ability to work at very low O_2 concentrations and the fact that its activity is not associated with a ROS release may represent a means of minimising cellular O_2 damage [63].

Although Cox does not release oxygen radicals in the solution, evidence of H_2O_2 production by submitochondrial particles has been available since 1966 [64]. The observation that mitochondria contain Mn-dependent superoxide dismutase (SOD) [65] and produce $O_2^{\bullet-}$ [66] suggested that mitochondria production of H_2O_2 is due to superoxide dismutation.

Some components of the respiratory chain, although they transfer a large mass of electrons to the next component in the chain, they also transfer a small number of electrons directly to O_2 molecules, causing its reduction into superoxide (univalent reduction). Since these components are unable to retain partially reduced forms of oxygen in their sites, the superoxide radical is released and partially transformed into H_2O_2 by spontaneous or catalysed dismutation by mitochondrial SOD [67].

Theoretical and experimental reasons suggest that the production of H_2O_2 by the mitochondria is the result of the dismutation of superoxide rather than the direct transfer of two electrons to O_2 . The bivalent reduction in O_2 , although being thermodynamically favoured compared to the univalent one, is kinetically disadvantaged since it requires the inversion of the spin. $O_2^{\bullet-}$ production is detectable only using submitochondrial particles which do not contain SOD [66]. Part of the $O_2^{\bullet-}$ produced by intact mitochondria appears on the cytosolic side of the inner membrane and can be measured by following the oxidation of adrenaline or by stimulating the formation of H_2O_2 with the addition of SOD [68].

Moreover, $O_2^{\bullet-}$ can also undergo other types of reactions besides dismutation. Indeed, the reaction of $O_2^{\bullet-}$ with nitric oxide ($NO\cdot$), which leads to the formation of the highly reactive peroxynitrite, can effectively compete with SOD-induced dismutation [69], having a rate constant of one order of magnitude higher than that of the SOD. Normally, due to the low concentration of $NO\cdot$, the likelihood of this reaction occurring is low. A higher concentration of $NO\cdot$ may produce mitochondrial nitrosative damage which may be exacerbated by the MnSOD damage due to peroxynitrite [69].

The product of the superoxide dismutation, the H_2O_2 , can also be detected in intact mitochondria, and in this case the measured H_2O_2 is only a fraction of that produced and metabolised by enzymes (catalase, thioredoxin system, glutathione peroxidase) or removed by the reaction with mitochondrial components containing iron complexes which transforms it into an $\bullet OH$ radical via the Fenton reaction [67].

Thus, the measurement of H_2O_2 generation with intact mitochondria reflects the net result of the $\text{O}_2^{\bullet-}$ production, its dismutation, its reaction with NO^\bullet , and the competition between H_2O_2 destruction and diffusion. Despite these limitations, the measurements of the quantity of peroxide, which can diffuse through the mitochondrial membrane, can provide indications of the relative levels of ROS production in different metabolic conditions [70]. While Cox activity saturates at low O_2 concentration [62], the rate of electronic loss (and therefore the $\text{O}_2^{\bullet-}$ production) increases at high concentrations of O_2 [71]. In slices of rat lungs exposed to pure oxygen, about 90% of the total O_2 consumed leads to ROS formation, but when the concentration is lowered even up to 85%, ROS formation lowers to 18% of the total O_2 consumed [71].

Although the numbers of radicals produced by mitochondria *in vivo* and *in vitro* is not easily determined, it is nevertheless clear that mitochondria produce ROS at rates that depend on their metabolic state [72]. The production rate is higher during basal respiration, i.e., in the presence of respiratory substrates but in the absence of adenosine diphosphate (state 2), characterised by a low oxygen consumption, than during ADP stimulated respiration (state 3), characterised by a higher rate of oxygen consumption [72].

The rate of formation of ROS in the respiratory chain is controlled by the law of mass action and, with the same O_2 concentration, increases when the flow of electrons slows down, which increases the concentration of electron carriers in the reduced form capable of donating an electron in oxygen [72].

As electrons flow through the respiratory chain, the released energy converts into a proton gradient across the mitochondrial membrane. This gradient, in turn, is dissipated through the ATP synthetase complex that leads to the synthesis of ATP [72]. In the absence of ADP, the movement of H^+ through the ATP synthetase ceases, and the gradient of H^+ increases, causing a slowing of electron flow, an increase in the degree of reduction in the respiratory chain, and the generation of superoxide [72].

Physiological uncoupling occurs in brown adipose tissue (BAT) mitochondria, in which a mitochondrial membrane protein, named uncoupling protein 1 (UCP1), participates in the dissipation of the energy associated with the proton gradient as heat, uncoupling electron flow from ATP synthesis. This protein decreases both mitochondrial membrane potential and H_2O_2 generation [73].

At a constant pressure of O_2 , the production rate of ROS increases as a function of the degree of reduction in the auto oxidable electronic carriers.

Univalent oxygen reduction sites were initially identified in Complex I [74,75] and Complex III [76]. Subsequently, ROS production sites were identified in mitochondrial complex II [77,78] and in various mitochondrial enzymes [79,80]. Nowadays, the exact sites of mitochondrial ROS generation and their contribution *in vivo* are not a settled question.

7.3. Mitochondrial Oxidoreductases

Given the moderate redox potential of the $\text{O}_2^{\bullet-}/\text{O}_2$ pair ($E^\circ = -0.33$ V), the univalent reduction reaction of O_2 is thermodynamically favourable for numerous mitochondrial oxidoreductases. A measurable production of ROS by at least nine enzymes has been reported, the best known of which are monoamine oxidase [81,82], α -glycerophosphate dehydrogenase [83], and aconitase [84,85]. The enzymes are ubiquitously present in mammalian mitochondria, but their expression and their ability to produce ROS vary widely between tissues and animal species. Although they are sources of ROS in experiments with isolated enzymes or with mitochondria, their contribution to mitochondrial ROS production under physiological conditions is not known.

7.4. Endoplasmic Reticulum

In the endoplasmic reticulum (ER), the production of ROS is linked to the activity of two electronic transport systems with distinct functions and the folding process of proteins.

7.5. Metabolism of Xenobiotics

The first electronic transport system participates in the metabolism of xenobiotics which can be verified in two phases. The first phase (Phase I) involves a flavoprotein (NADPH-cytochrome P450 reductase), each molecule of which contains a flavin adenine mononucleotide and a flavin adenine dinucleotide, as well as cytochromes collectively known as cytochrome P450 [86]. It has been shown that subcellular fractions isolated from various tissues containing endoplasmic reticulum (microsomal fractions) rapidly produce H_2O_2 when incubated with NADPH [87,88].

Cytochrome P450 enzymes (CYPs) are a heterogeneous group of monooxygenase that play a crucial role in the oxidative metabolism of many lipophilic compounds, xenobiotics, biosynthesis of sterols, fatty acids eicosanoids vitamins, etc. [89,90], and their high concentrations are found in the endoplasmic reticulum of the liver where many chemicals are metabolised [91,92]. Significant concentrations are also found in the lungs [93], adrenal medulla [94], kidneys [95], and small intestine [96]. In any case, one oxygen atom binds to the substrate, and the other forms water, so this reaction is known as a monooxygenase reaction or mixed-function oxygenase reaction. Phase I reactions introduce a polar group into a lipophilic substrate using an electron transport system, increasing the substrate's polarity, and aiding in excretion. The process is necessary so that insecticides, hydrocarbons, and drugs are solubilised in water and then excreted in the urine or so they undergo conjugation and are excreted in the bile. During the CYP catalytic cycle, two "shunts", which can generate ROS and interrupt substrate oxidation, exist [97]. The first ROS released is O_2^- and the second is H_2O_2 [98]. Many factors can affect the coupling efficiency of a given CYP, among which the reaction with the substrate and the pH or O_2 concentration play an important role [98]. Moreover, the different CYP isoforms have different rates of reaction uncoupling, and they can also be dependent on the substrate [90].

The reactions of the second phase (Phase II) are conjugation reactions, in which an endogenous molecule is added to the product of the Phase I reaction, sometimes directly to the xenobiotic [86]. Phase II reactions, for example, are conjugations with glutathione (GSH) catalysed by glutathione-S-transferase (GST), and conjugations with glucuronic acid, catalysed by glucuronyl transferase. Other examples are sulfation (addition of a sulphate group catalysed by sulfotransferase), methylation (addition of a methyl group, usually provided by S-adenosyl-methionine), and acetylation (addition of an acetyl group catalysed by N-acetyltransferase).

Generally, the products of the reaction of the CYPs are less toxic than the substrates. However, the reaction often determines the transient formation of oxidative intermediates, such as epoxides, or the generation of highly reactive products. For example, the drug acetaminophen via CYP oxidation converts into a reactive intermediate, N-acetyl-p-benzoquinone-imine (NAPQI), that is conjugated with glutathione, by glutathione S-transferase (GST), that inactivates NAPQI [99]. Acetaminophen overdose and toxicity are caused by the generation of excessive amounts of NAPQI that are not detoxified by GST, due to glutathione exhaustion, and can be treated with the administration of N-acetylcysteine [98].

7.6. Unsaturation of Fatty Acids

The second electronic transport system, in which the flow of electrons proceeds from NADH to cytochrome b5, operates the desaturation of fatty acids, i.e., the introduction of C=C double bonds into these substances.

The system consists of three proteins: a flavoprotein (NADH-cytochrome b5 reductase), a cytochrome b5, and the desaturase enzyme [100]. The first protein, which contains FAD as a prosthetic group, catalyses the transfer of electrons from NADH to cytochrome b5 through the flavoprotein, and the reduced cytochrome b5 donates electrons to the desaturase enzyme. This enzyme, also named cyanide sensitive factor (CSF) because it is inhibited by cyanide, contains only one non-heme iron per polypeptide chain and can desaturate acyl-CoA to enoyl-CoA using one molecule of O_2 and produce two molecules of H_2O .

Both cytochrome b5 and flavoprotein can transfer electrons to O₂ to form O₂⁻, and this may be an additional source of in vivo ROS [101].

7.7. Protein Folding

The conformation of most of the proteins synthesised in the endoplasmic reticulum (ER) is stabilised by the formation of intramolecular disulphide bonds, a process that requires the oxidation of free sulfhydryl groups. The intraluminal *medium* of the ER seems to ensure the realisation of the oxidative folding of proteins maintaining a GSH/GSSG ratio lower than that of the cytosol [102,103]. The stability and functionality of the newly synthesised proteins are guaranteed by the formation, reduction, and isomerisation (i.e., the exchange of -SH groups with -S-S- groups) of disulphide bonds between pairs of cysteines. The enzyme that catalyses these reactions is the protein disulphide isomerase (PDI), a family of ER proteins characterised by the presence of thioredoxin-like domains [104].

How the ER disposes of the electrons deriving from the oxidative formation of disulphides remained unknown for a long time, until the discovery, in *S. cerevisiae*, and subsequently in mammals, of a protein that transfers disulphide bonds to PDI (Ero1p in eukaryotes and DsbB in bacteria) [105].

Ero1p functions as a PDI oxidase: oxidising equivalents flow from Ero1p to the substrate protein via PDI, through the direct dithiol–disulphide exchange between PDI and Ero1p [105]. Furthermore, Ero1p has an FAD group and is very sensitive to small changes in the physiological levels of free FAD [105]. The oxidative folding of proteins does not use oxidised glutathione (GSSG) as a source of oxidant equivalents. This role is played by molecular oxygen, which is used by Ero1p as its preferred electron terminal acceptor. Consequently, Ero1p directly couples the disulphide formation to the consumption of O₂, while its activity is modulated by the luminal levels of free FAD [105].

However, in bacteria, the oxidative folding is conveniently coupled to O₂ via the respiratory chain, while in eukaryotes, where the oxidative winding and respiration are confined to separate organelles, Ero1p uses an FAD-dependent reaction to pass electrons directly to O₂ [106]. As a result, the activity of Ero1p can generate reactive oxygen species and, thus, provide an additional source of oxidative stress, suggesting that its activity must be regulated according to the wind load [107]. Using theoretical calculations, it has been estimated that the oxidation of protein thiols via PDI and Ero1p in the endoplasmic reticulum could account for up to 25% of cellular ROS produced during protein synthesis [108].

7.8. Peroxisomes

Peroxisomes, organelles present in almost all eukaryotic cells, are other sources of ROS [109]. They can reduce oxygen to water through a two-phase mechanism that involves H₂O₂ as an intermediate [110]. In the first phase, the substrates (electron donors) include uric acid, amino acids, glycolic acid, and α-hydroxy acids according to the cell type. The electron donors in the second phase are either an H₂O₂ molecule, which is oxidised to oxygen, or a substrate such as methanol, ethanol, formaldehyde, phenol, or nitrite ions that can be oxidised by catalase [110].

Over time, more than 50 enzymes have been discovered in peroxisomes. These enzymes are involved in various metabolic pathways, including cholesterol biosynthesis, β-oxidation of fatty acids, detoxification of xenobiotics, and ROS [111]. Peroxisomes contain superoxide dismutase, which metabolises superoxide, glutathione peroxidase, which metabolises both H₂O₂ and membrane hydroperoxides.

Peroxisomes contain high concentrations of catalase, so whether a loss of H₂O₂ from these organelles contributes significantly to the intracellular concentration of the peroxide under normal conditions is not known. One of the characteristics of peroxisomes is their inducibility by xenobiotics which leads to their remarkable proliferation with a concomitant synthesis of some of their enzymes, particularly those of β-oxidation. Peroxisome proliferators include structurally diverse compounds, including hypolipidemic drugs, such as fibrates, free fatty acids, herbicides, and many environmental pollutants. The selective

transcription of peroxisomal genes by these substances is mediated by the peroxisomal proliferator-activated receptor α (PPAR- α), which belongs to the family of nuclear transcription factors [112]. While the expression of β -oxidation genes is significantly induced (10–30 times), the maximum induction of catalase does not exceed 1–2 times. Therefore, it has been suggested that the increase in H_2O_2 release linked to these disproportionate inductions is one of the causes of the increased onset of liver tumours recorded following prolonged exposure of rodents to peroxisome proliferators [113].

7.9. Cytoplasm

There is a small number of cytoplasmic oxidases capable of producing the superoxide radical. These include xanthine oxidase [114], tryptophan dioxygenase, and indolamine dioxygenase [115].

One of the most studied is xanthine oxidase, an enzyme involved in the breakdown of purines. In mammals, this enzyme is ubiquitously expressed and shows the highest specific activity in the liver and mucosa of the small intestine. The enzyme converts hypoxanthine into xanthine and xanthine into uric acid, and electrons are transferred from the substrates to the O_2 , forming $O_2^{\bullet-}$ as well as, probably, singlet oxygen [114]. In vivo, the enzyme is present as xanthine dehydrogenase, which transfers electrons from substrates to NAD^+ . Thus, the metabolism of xanthine or hypoxanthine does not produce the superoxide ion normally. However, xanthine dehydrogenase is converted to xanthine oxidase by oxidation of cysteine residues or irreversibly by limited proteolysis involving Ca^{2+} -stimulated proteases in oxidatively damaged tissues [116].

7.10. Cell Membranes

The plasma membrane is a critical site of radical reactions for several reasons. Free radicals generated outside the cells must cross the plasma membrane before reacting with other cellular components, and some of them can initiate toxic reactions at the membrane level. Hydrogen peroxide can cross membranes as easily as water. The charged superoxide molecule can cross membranes and enter cells through transmembrane anion channels. Furthermore, the superoxide can react with the H^+ ion forming the hydroxy peroxy radical HO_2^{\bullet} , which is a stronger oxidant than $O_2^{\bullet-}$ and is soluble in lipids. Thus, it can be expected to exert toxic effects on lipids and on the hydrophobic core of membrane proteins.

7.11. Lipoxygenase and Cyclooxygenase

Enzymes associated with membranes, such as lipoxygenases and cyclooxygenases, involved in the metabolism of arachidonic acid (C20:4, ω -6, AA) and of other polyunsaturated acids, can produce ROS.

The metabolism of AA leads to the formation of a large and complex family of biologically active lipids produced by almost all mammalian cells, with the exception of red blood cells. The metabolites include prostaglandins, prostacyclins, thromboxanes, and leukotrienes. These compounds are collectively known as eicosanoids, as they are all 20-carbon compounds [117]. The cytosolic phospholipase A2 mediates the release of AA from glycerolphospholipids of the nuclear envelope and plasma membrane. AA undergoes insertion of O_2 through a radical mediated mechanism by lipoxygenase (LOX, linear pathway) and cyclooxygenase (Cox, cyclic pathway) [118].

AA is metabolised by LOXs, which are non-heme iron-containing enzymes, into hydroperoxyeicosatetraenoic acids (HpETEs). HpETEs are reduced into hydroxyeicosatetraenoic acids (HETEs) [119].

The major cyclooxygenase-derived eicosanoids are prostaglandins (PGs) and thromboxane (TX). Cox enzymes catalyse the first two steps of the enzymatic reaction, including cyclooxygenase (dioxygenase) and peroxidase activity, to convert AA into PGH₂ [120]. PGH₂ is the precursor of PGs and TX, whose production depends on the differential expression of isomerases and PG synthases in different tissues [121]. Cox includes two isozymes: COX-1 and COX-2. The first is the constitutive isoform, responsible for the low PGs synthe-

sis required for cell homeostasis, and the latter is inducible by many extracellular stimuli, including cytokines and growth factors, during chronic inflammation [122,123].

The oxidation of arachidonic acid by Cox produces an oxygen (probably a haemoprotein) radical.

The metabolism of arachidonic acid by LOX also produces carbon-centred radical intermediates, which are due to the extraction of methylene hydrogen, and involved in the degradation processes of membrane lipids.

7.12. NADPH Oxidase

Currently, there is a growing body of evidence that a family of membrane-bound cellular enzymes called NADPH oxidase (NOX) is a significant source of ROS [124,125].

The primary function of NOX is to catalyse the transfer of electrons across the membrane from NADPH to molecular oxygen by generating superoxide or hydrogen peroxide.

NOX2, also known as phagocytic NADPH, is the prototype of NOX and has the role of defence against bacteria that are killed by superoxide. In addition to its presence in phagocytes, NOX2 is expressed at lower levels in many cell types, including cardiomyocytes, endothelial cells [126], neurons [127], and pancreatic β cells.

During activation, which occurs following the phosphorylation of the p47^{phox} regulatory subunit [128,129], the G protein binds GTP, migrating to the plasma membrane with the cytosolic components forming the active complex. It removes electrons from the NADPH, oxidising it to NADP⁺ inside the cell and transferring them to the FAD. Subsequently, it releases the electrons of the two heme groups, which eventually transfers them to the oxygen on the opposite side of the membrane, forming the superoxide radical.

Some isoforms of NOX, such as NOX4 which is strongly expressed in the kidney but is also present in vascular cells and neurons [130], and DUOX1 and DUOX2 which are particularly important in the thyroid [131] although they are also found in the epithelia of the digestive and respiratory tracts, mainly produce H₂O₂, while the primary product may be superoxide, which undergoes rapid dismutation to hydrogen peroxide. Furthermore, NOX4 is distinguished from the rest of the family since it appears to be constitutively active, and its activity is therefore mainly regulated by the level of expression.

8. ROS-Induced Oxidative Damage

Biological systems have an integrated antioxidant system to counteract the damaging effects of ROS (Figure 2). This system includes low molecular weight enzymes and antioxidants. The discovery of the superoxide dismutase (SOD) enzyme by McCord and Fridovich [132] allowed us to define the mechanisms underlying oxygen toxicity. SOD transforms O₂^{-•} into H₂O₂, the latter being metabolised by various enzymes such as catalase, glutathione peroxidase (GPX), and peroxiredoxin (Prx) [72].

GPX and Prx metabolise most of the H₂O₂ and their activities depend on the thiol groups of the cysteine residues of reduced glutathione (GSH) and thioredoxin (Trx), respectively, to reduce H₂O₂ [72]. The enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR) serve to maintain the sulfhydryl groups of GSH and Trx in the reduced form and require NADPH as a source of electrons for their activity [72]. Low molecular weight antioxidants include endogenous molecules and antioxidants that must be introduced with food. These molecules can be lipo- or hydro-soluble and comprise endogenously produced molecules such as GSH, or molecules introduced with food such as vitamin E and C polyphenols [72]. Vitamin E is the main liposoluble antioxidant that is localised in all cell membranes, including the mitochondrial one, protecting it from oxidative damage in several physio pathological conditions [133].

A low ROS production is important because ROS are also signalling molecules, but their high production can determine a reduction in redox signalling and/or damage to biomolecules [134]. Oxidative stress originates from the imbalance between ROS production and the ability of the antioxidant system to counteract their oxidative action. In this case,

the redox signalling is altered and oxidative damage to macromolecules increases [134]. ROS can damage lipid proteins and DNA [135].

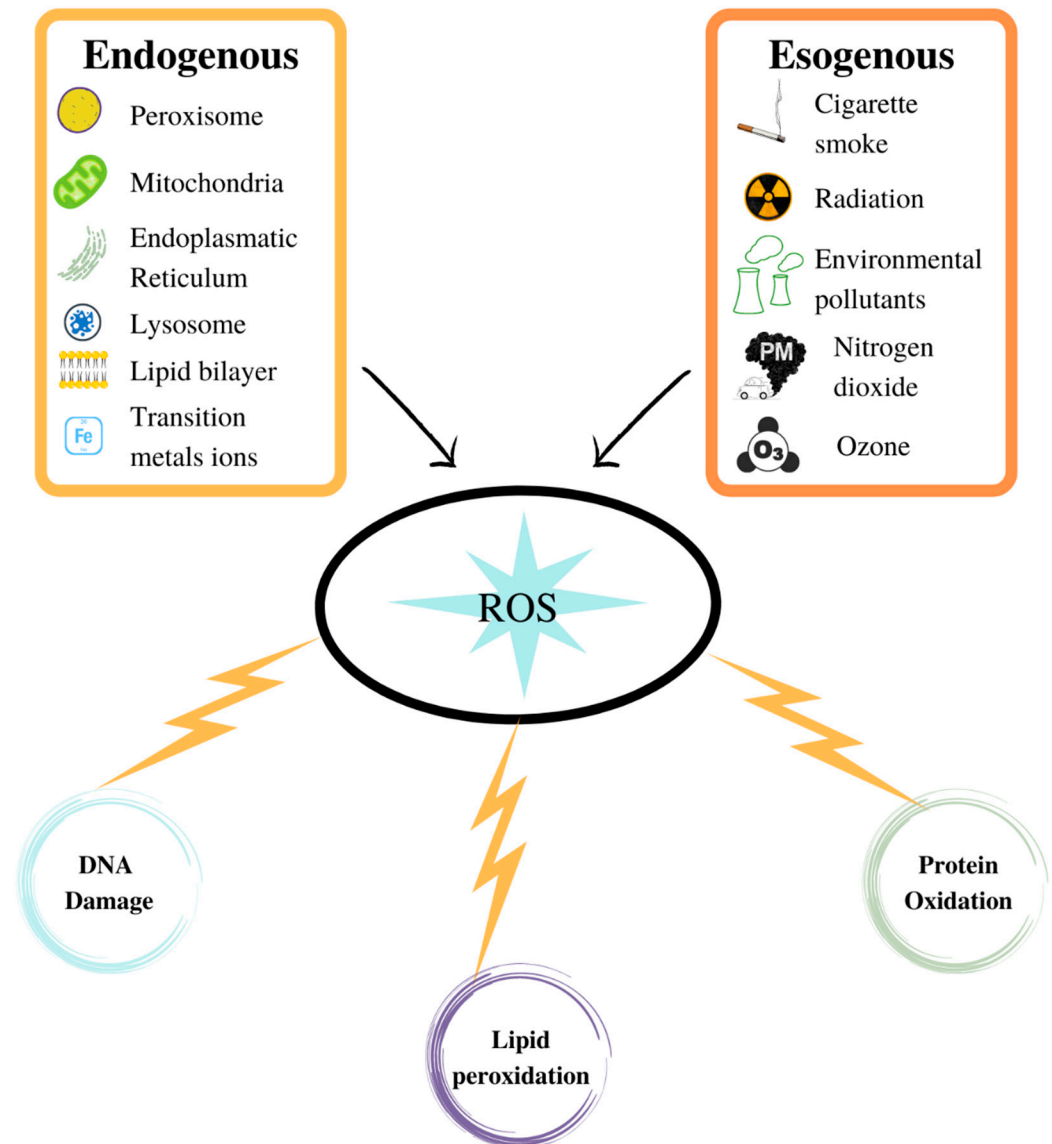


Figure 2. ROS production in aerobic organisms can be better verified via an exogenous source than through normal physiological processes. ROS can damage the biological macromolecules.

Mono- and poly-unsaturated lipids are the molecules more prone to oxidation, which results in the formation of lipid hydroperoxides (LOOHs) as primary nonradical-reaction products. LOOHs may undergo degradation into various products that have been implicated in vital biological reactions, and thus in the pathogenesis of various diseases [136]. The molecular changes caused by the reactions of products of lipid peroxidation alter the structure and function of proteins and DNA and are responsible for the cytotoxicity induced by these molecules [136].

ROS can oxidise DNA, modifying the structure of nucleotides and inducing double-stranded break formation which can favour genomic instability [137]. Moreover, ROS can directly oxidise nucleoside bases (e.g., formation of 8-oxo guanine) [138], which can determine G-T or G-A transversions if unrepaired. The base excision repair pathway recognises and repairs oxidised bases, but when oxidation occurs simultaneously on opposing strands, the base excision repair system can determine the generation of a DNA double-strand break which may be mutagenic due to chromosomal rearrangements or loss

of genetic information [139]. ROS accumulation also induces mitochondrial DNA lesions, strand breaks, and degradation of mitochondrial DNA [140].

ROS can directly or indirectly affect protein functionality. Direct action of ROS determines the nitrosylation, carbonylation, disulphide bond formation, and glutathionylation of proteins [141]. Indirectly oxidative damage to proteins consists of their conjugation with products of the breakdown of oxidised fatty acids [133]. Oxidatively damaged proteins undergo site-specific modifications of amino acids, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electric charge, and increased susceptibility to proteolysis [141].

9. ROS as Regulators of Important Physiological Processes

The discovery that a hydroxyl radical induces the activation of guanylate cyclase and increases the levels of cyclic guanosine mono phosphate [142], as well as the fact that hydrogen peroxide mimics the signalling activity of insulin [143], suggested that ROS can not only be dangerous but also involved in the regulation of important physiological processes.

More insights into this idea have been obtained thanks to the discovery in the adipose cell membrane of the enzyme NADPH oxidase, whose activity is stimulated by insulin [144] which produces the superoxide from which H_2O_2 is generated. The discovery suggested that insulin, by stimulating H_2O_2 production, induces the oxidation of membrane sulfhydryl groups that play a role in activating the insulin signalling pathway [145].

Subsequently, low levels of $O_2^{\cdot-}$ and H_2O_2 proved to activate the proliferation of both hamster [146] and human [147] fibroblasts in culture. From these initial data, several experimental pieces of evidence have suggested that ROS, other than acting as insulin-activated second messengers and stimulating cell proliferation in different cell types [148], also regulate a wide range of physiological functions. They control the activation of genes acting through the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) [149], the biosynthesis of prostaglandins [150], and embryonic development [151]. Moreover, they also function as signalling molecules inside the cell and between cells throughout life [152].

ROS are also involved in muscle contraction, as suggested by the observation that they are present at low levels in skeletal muscle under basal conditions [153] and that the antioxidant supplementation to diaphragmatic fibre bundles reducing their content determines the inhibition of muscle contraction [154].

ROS also contribute to the regulation of blood pressure [155] and are involved in the maintenance of cognitive function [156]. It has been also evidenced that, even if the increase in the rate of formation of ROS is considered an important factor involved in the determination of life span [157], some interventions able to increase longevity are linked to enhancement in the ROS production that can induce cellular pathways that, in turn, reduce cellular degeneration and promote healthy aging [158]. All these observations showed that the living being has not only adapted to the presence of free radicals but has also developed useful mechanisms to advantageously use these species in a myriad of physiological functions.

In all organisms ranging from bacteria to mammals, ROS can regulate signalling pathways initiated by factors sensitive to the cellular redox state that can induce the increased expression of antioxidant enzymes. This increase in antioxidant defences allows cells to survive to exposure to oxidants that would be lethal under normal conditions. For example, it has been found that the irradiation of cells and tissues of mice [159] and rats [160] induces the expression of antioxidant enzymes, thus activating an adaptive response that increases their resistance to radiation by increasing the antioxidant capacity.

Bacteria present a sophisticated molecular mechanism that allows them to check the cellular level of oxidant and to induce the expression of the genes coding for the antioxidant enzymes, if necessary. The regulation of genes in response to oxidative stress has been extensively studied using the bacterium *Escherichia coli* as a model. These studies have shown that the fundamental principles of the response to oxidants are conserved from

bacteria to eukaryotes, even if the molecules and mechanisms involved in ROS sensing are variable according to the organism as well as the severity and conditions of stress.

In *E. coli*, some systems stimulate an increase in the cellular content of enzymes that metabolise superoxide or peroxides, repair DNA and proteins, and reduce the deleterious effects of oxidative mutagens [161–163]. The induction of these systems is an adaptive response to oxidative stress due to exposure to oxidising agents at a non-toxic level or to ionising radiation, which makes the organism more resistant to further stress even with levels of oxidants that would be toxic under basal conditions.

In *Salmonella typhimurium*, the *oxyR* gene has been identified as a regulator of acquired resistance to oxidants. *oxyR* is involved in the expression of nine proteins, including catalase, alkyl hydroperoxide reductase, glutathione reductase, and MnSOD, in which levels increase upon exposure to hydrogen peroxide to sublethal levels [164].

In mammals, ROS can activate the transcription of genes that determine cell survival in two ways: employing transcription factors, which interact with specific DNA motifs on the promoters of target genes, or by activating cascades of mitogen-activated protein kinases, which activate transcription factors which ultimately activate the transcription of the target gene [165]. Clear evidence shows that the cellular level of ROS is correlated to the cellular antioxidant levels [166]. One of the main factors involved in this relationship is the factor 2-related erythroid nuclear factor 2 (Nrf2), which regulates the expression of genes encoding antioxidant and detoxifying enzymes [167]. Nrf2 links to a protein named small musculoaponeurotic fibrosarcoma (Maf) and acts through the binding to the antioxidant response element (ARE) of the promoter region of the target genes [168]. Its activity is linked to its subcellular localisation that, in turn, depends, at least in part, on the binding with specific residues of the cysteine of the protein Kelch ECH associating protein 1 (Keap1) [169].

Keap1 is the major negative regulator of Nrf2, regulating its ubiquitination and degradation [170,171]. The increase in ROS levels or the presence of Nrf2 activating compounds induce the oxidation of cysteine residues in Keap1, thus inducing the loss of its ubiquitin ligase activity and the release of Nrf2. Nrf2 is then phosphorylated and reaches the nucleus where it binds to the small protein of muscle aponeurotic fibrosarcoma (Maf) forming a heterodimer capable of binding to the ARE located in the promoter regions of genes regulated by Nrf2 [168,172]. Interestingly, the glycogen synthase kinase-3 (GSK-3 β), a serine/threonine protein kinase that participates in glycogen metabolism, cell proliferation, stem cell renewal, apoptosis, and development, regulates the relocation of Nrf2 to the cytosol and its degradation [173]. The activation of GSK-3 β is induced by hydrogen peroxide [174]. Thus, GSK-3 β regulation of Nrf2 activity is oxidant-sensitive as well.

ROS can activate many cellular processes, among which the proliferation and survival, interacting with signalling molecules among which mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinases (PI3K), phosphatase and tensing homolog (PTEN), and protein tyrosine phosphatases [175].

9.1. Redox Signalling

Although the role of ROS as cell-damaging and signal-transduction agents is defined, some aspects of these roles are still under study.

Variations in the concentration of ROS seem to be at the basis of their role as molecules capable of regulating cellular functions or as harmful substances. ROS act as regulators of signalling processes at low concentrations, while they act as damaging agents as they can damage and, therefore, inactivate cellular molecules at moderate or high concentrations. Shifting concentrations from positive to damaging effects is an unsolved question.

Moreover, how the specificity of ROS signalling is obtained remains unknown. In fact, ROS show indiscriminate reactivity while cell signalling verifies through the non-covalent bond between the ligand and its receptor due to the complementarity of the molecule shapes. ROS act in signalling by reactions with specific atoms of target proteins that result in the covalent modifications of the proteins. Thus, ROS recognise their targets at the atomic

and non-macromolecular level, and this extends the number of potential ROS receptors. Therefore, it is not known how the specificity of ROS signalling is achieved.

9.2. Source of ROS Acting as Signalling Agents

It is not easy to identify a single source of ROS which act as signalling agents. On the other hand, it is also not easy to identify the main cell source of ROS. Mitochondria are considered to be the most important cellular source of ROS in both physiological and pathological states [176]. They seem to play a role in various diseases and in the ageing process [177], even if the demonstration that they really are the principal source of ROS is absent [178]. On the other hand, the available data show a strong interaction between the cellular ROS sources [179–181], which makes it difficult to understand which is the main one. The control of signalling by ROS requires the colocalization of their sources and targets. This colocalization allows for the fine control of the signalling reaction kinetic by controlling the local concentrations of the reagents. For example, NOX that affects the tyrosine kinase receptor-linked signalling colocalized in the cell membrane together with its targets, phosphatases, kinases, and oxidation of other molecules, avoids, i.e., the nuclear or mitochondrial nucleotides [182,183]. The compartmentalization of ROS producers that play a role in signalling has been identified in various cell types [184]. Localization in the endoplasmic reticulum of NOX4 and of its target, the protein tyrosine phosphatase 1B (PTP1B) [185], and the generation in the phagosomes of hypochlorous acid from myeloperoxidase for defence against pathogens [186], are other examples of colocalization of ROS sources and targets.

9.3. ROS Species and Signalling

Due to their high instability and the action of the cellular antioxidant defence system, most ROS do not spread far from the production site. The hydroxyl radical, whose half-life is about 10^{-9} s for its high reactivity, has a small range of action (about 30 Å). Hydrogen peroxide has a half-life of about 1 ms and can diffuse over longer distances [187]. Thus, a hydroxyl radical generated within the mitochondrion is unlikely to exert effects on other cell sites.

Another important aspect is that while the hydroxyl radical exhibits a broad reactivity towards all biological molecules, $O_2^{\bullet-}$ and H_2O_2 appear reactive towards specific preferential biological targets. Despite having a generalized reactivity, the hydroxyl radical has been involved in the SOD-induced activation of the enzyme guanylate cyclase [188]. It has been suggested that the formation of H_2O_2 produces $\bullet OH$ due to the reaction with superoxide and that $\bullet OH$ can activate guanylate cyclase [188]. The involvement of the hydroxyl radical in the activation of guanylate cyclase by SOD has been later questioned, and it has been shown that this activation [188] depends on the elimination of superoxide. Excluding this example of $\bullet OH$ as a signalling molecule, the current idea is that this radical does not play a specific role in signalling.

9.4. H_2O_2

The major signalling molecules are $O_2^{\bullet-}$ or H_2O_2 .

Superoxide can oxidize thiols into thiyl radicals, and this can initiate a chain reaction showing a low-rate constant (approximately $10^3 M^{-1} s^{-1}$ at pH 7.4) [189], which is negligible compared to the reaction rate constant catalysed by both cytosolic and mitochondrial SOD (about $10^9 M^{-1} s^{-1}$) [190]. $O_2^{\bullet-}$ generated outside the cell from membrane NOX2, or endothelial NOX2, passes into the cell and acts as a signalling molecule [191]. Once inside the cell, superoxide is rapidly metabolized by SOD to form H_2O_2 and O_2 . No superoxide target has been identified so far and it appears to act only as a precursor of H_2O_2 . The latter can act as a second messenger due to its production and degradation, which provides specificity for the time and place of production, as well as its chemistry which provides specificity for thiol oxidation, which is finely controlled.

H₂O₂ can initiate cell signalling by modifying either molecule in target proteins or the intracellular redox state [192], although the distinction between these mechanisms is not clear.

The thiol groups of cysteine residues are the main targets of H₂O₂, and their oxidation is well described. The oxidation of sulfhydryl groups determines the genesis of some different products, including sulfenic acid derivatives (-SOH) [193], which can transform by subsequent oxidation into sulfinic (-SO₂H) and possibly sulfonic (-SO₃H) acid. Furthermore, they can determine the formation of disulphide bonds with adjacent cysteines (-S-S-) [193,194] and generate some adducts that react with NO• (S-nitrosylation) or GSH (S-glutathionylation) [195]. The modifications can be cancelled by reducing systems such as thioredoxin and peroxiredoxins, apart from sulfonic and, to a lesser extent, sulfinic acid [196].

The formation of sulfinic acid requires a further reaction with H₂O₂ that possibly occurs in oxidative stress conditions and not during physiological signalling. This reaction is also verified for sulfonic acid which does not exist under biological conditions. It can be argued that only the sulfenic acid derivative and the disulphide are relevant to signalling the oxidation state of cysteine.

The changes induced by the oxidation of the thiol groups cause structural and functional changes in the proteins and may modify the activity of an enzyme whether the critical cysteine is in the catalytic domain [197] or the affinity of a transcription factor for DNA if the critical cysteine is in the DNA binding domain [198]. Redox processes regulate many proteins, including transcription factors, molecular chaperones, and protein tyrosine phosphatases. Therefore, the redox state of the thiol groups constitutes a molecular switch that can reversibly activate or deactivate the function of a protein. This action is like the phosphorylative regulation of proteins due to the binding of a phosphate group by a protein kinase and its cleavage by a protein phosphatase [199].

Moreover, the two signalling mechanisms crosstalk each other and show a sequential partnership in some circumstances of cell regulation. The study of the system Trx/apoptosis signal-regulating kinase-1 (ASK1) provided proof of the link between the cellular redox state and phosphorylative process [200]. ASK1, a member of the family of mitogen-activated protein kinase kinase kinases (MAPKKK), is implicated in the activation of stress-activated protein kinases, among which p38 and JNK [201]. The antioxidant protein Trx forms a complex with ASK1 and inhibits its activity [200]. The increase in the levels of ROS due to tumour necrosis factor stimulation induces the dissociation of Trx from ASK1 and the increase in ASK1 activity [201].

Regarding the way changes in the cellular redox state initiate cell signalling, it should be underlined that, with respect to the extracellular environment, the cytosol is usually maintained under “reduction” conditions. Under normal conditions, the maintenance of the redox state of the cytosol is due to the ability of redox buffer systems of intracellular thiols, such as GSH and Trx, which counteract cellular oxidative stress by reducing H₂O₂.

The activities of the enzymes GSH reductase and Trx reductase serve to keep the high content of the reduced forms of GSH and Trx, respectively. GSH and Trx participate in cell signalling processes. Indeed, GSH regulates redox signalling through the modification in both total GSH levels and the ratio between its reduced and oxidised forms, while Trx regulates the activity of specific proteins by directly binding them [192].

10. Conclusions

The evolution of aerobic organisms has required the concomitant evolution of an antioxidant defence system to counteract the toxic effects of oxygen and the metabolites produced during its metabolism.

Within the organism, there are multiple sources of reactive oxygen species. Some of these are constitutively expressed, such as mitochondria and NADPH oxidase, while others are inducible. Additionally, some sources are physiological, while others, such as xanthine oxidase, are generated by pathological conditions, such as hypoxia–reperfusion.

However, it is now known that reactive oxygen species are not only harmful molecules for the organism but also necessary for the proper functionality of the biochemical machinery and communication inside and among the cells. Generally, the physiological effects of ROS depend on their low content.

There are important implications for ROS as physiological signalling molecules that impact therapeutic strategies. Antioxidants have long been considered potential therapeutics for several conditions involving oxidative stress. In general, trials using antioxidants likely failed, in part, due to the unintentional inhibition of important basal and adaptive ROS signalling pathways. It has even been argued that taking antioxidants as daily dietary supplements might also disturb these ROS pathways in ways that are not beneficial nor harmful [202].

The idea that is currently emerging is that while the administration of antioxidants in basal conditions can prevent the induction of beneficial adaptations for the organism, the administration of antioxidants under conditions of oxidative stress can prevent or mitigate the deleterious effects due to oxidative stress. For example, antioxidant supplementation to rats during a program of physical training can interfere with the training-induced metabolic adaptations in several tissues including the liver [203], muscle [204], and heart [205]. Conversely, antioxidant supplementation in a condition of oxidative stress such as those induced by thyroid hormone administration reduces the thyroid hormone-induced alteration of heart rate [206] and prevents the alteration in insulin sensitivity due to the hormones in the liver [207].

Cancer cells show an elevated level of oxidative stress, and this has been proposed to orchestrate tumorigenesis and tumour progression through direct or indirect mechanisms [208]. However, high doses of antioxidants have shown to contribute to tumour progression [209]. Cancer cells may be more sensitive to ROS than normal cells and it has been suggested that increasing oxidative stress by exogenous ROS generation therapy that selectively kills cancer cells without affecting normal cells could be a strategy against cancer progression [210]. However, the role of ROS in the cancer biology is very complex as they play both pro-tumorigenic and anti-tumorigenic roles [211].

Significant steps in the research field of antioxidant and ROS actions are being undertaken, as a lot remains to be understood. Certainly, antioxidants are not the *panacea* for all ills and their careful use is necessary to avoid incurring cell, organ, and whole organism dysfunctions.

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