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REVIEW ARTICLE

Melatonin: an ancient molecule that makes oxygen metabolically tolerable

Abstract: Melatonin is remarkably functionally diverse with actions as a free radical scavenger and antioxidant, circadian rhythm regulator, anti-inflammatory and immunoregulating molecule, and as an oncostatic agent. We hypothesize that the initial and primary function of melatonin in photosynthetic cyanobacteria, which appeared on Earth 3.5–3.2 billion years ago, was as an antioxidant. The evolution of melatonin as an antioxidant by this organism was necessary as photosynthesis is associated with the generation of toxic-free radicals. The other secondary functions of melatonin came about much later in evolution. We also surmise that mitochondria and chloroplasts may be primary sites of melatonin synthesis in all eukaryotic cells that possess these organelles. This prediction is made on the basis that mitochondria and chloroplasts of eukaryotes developed from purple nonsulfur bacteria (which also produce melatonin) and cyanobacteria when they were engulfed by early eukaryotes. Thus, we speculate that the melatonin-synthesizing actions of the engulfed bacteria were retained when these organelles became mitochondria and chloroplasts, respectively. That mitochondria are likely sites of melatonin formation is supported by the observation that this organelle contains high levels of melatonin that are not impacted by blood melatonin concentrations. Melatonin has a remarkable array of means by which it thwarts oxidative damage. It, as well as its metabolites, is differentially effective in scavenging a variety of reactive oxygen and reactive nitrogen species. Moreover, melatonin and its metabolites modulate a large number of antioxidative and pro-oxidative enzymes, leading to a reduction in oxidative damage. The actions of melatonin on radical metabolizing/producing enzymes may be mediated by the Keap1-Nrf2-ARE pathway. Beyond its direct free radical scavenging and indirect antioxidant effects, melatonin has a variety of physiological and metabolic advantages that may enhance its ability to limit oxidative stress.

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Introduction

In a recent brief review, Fridovich [1] pointed out the hazards of atmospheric oxygen after it is inhaled and enters cells. The title of the report, that is, 'Oxygen: how do we stand it?' precisely reminds the reader of the high toxicity of this gas, which constitutes roughly 20% of the atmosphere on Earth. The molecular damage inflicted by oxygen is indirect and stems from the partially reduced species that are generated especially during the successive one electron reduction reactions of oxygen. These partially reduced oxygen derivatives, all of which are classified as reactive oxygen species (ROS) and some of which are free radicals (they have an unpaired electron in their valence orbital), inflict damage to critical macromolecules, that is, lipids, proteins, DNA, etc., in every cell of aerobic organisms. Thus, while oxygen is necessary for sustaining life of aerobes, such as the human, it is also consequential in contributing to diseases, aggravating degenerative processes associated with aging, and advancing disability and death.

The derivation of oxygen in the environment is a result of its production and discharge by organisms and plants that use photosynthesis as a means of generating nutrients. During this process, carbon dioxide is captured and eventually converted to molecular oxygen which is released into the environment. Some of the earliest organisms to incorporate photosynthesis into their metabolic machinery were prokaryotic cyanobacteria which evolved an estimated 3.2 billion years ago. When cyanobacteria initially discharged oxygen into the environment, it was chemically captured by dissolved iron and other organic matter in the Earth's crust, and therefore, it did not impact the oxygen concentration of the atmosphere. Once these sources became saturated, an estimated 2.5 billion years ago, atmospheric oxygen began to rise. This gradual and

eventually large increase in atmospheric oxygen is referred to as the Great Oxygen Event (GOE, Oxygen Catastrophe, etc.). It was catastrophic as free oxygen is toxic to obligate anaerobic inhabitants, the only organisms that existed at the time. Thus, the cyanobacteria may have been indirectly responsible for the early/first mass extinction on Earth. The only organisms that survived the GOE were those that had a means of protecting themselves from partially reduced oxygen derivatives, namely the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$), etc. (Fig. 1) Thus, the survivors of the GOE were those prokaryotes that had evolved means or molecules, that is free radical scavengers and antioxidants, that were capable of detoxifying the noxious oxygen by-products.

One organism that was capable of protecting itself from the toxicity of oxygen derivatives during the GOE were the cyanobacteria themselves. These photosynthetic prokaryotes have been shown to possess and likely synthesize melatonin [2, 3], an uncommonly effective molecule in safeguarding against molecular damage, so-called oxidative stress. This damage is a result of toxic oxygen-derived species, that is, ROS and reactive nitrogen species (RNS). Sometime after the GOE, aerobic prokaryotic and eukaryotic organisms evolved [4–6]. As these species consumed oxygen, an equilibrium was established in terms of oxygen use and oxygen production thereby stabilizing the concentration of free oxygen in the environment. The availability of oxygen in the atmosphere, because of its release by photosynthetic prokaryotes, was a major breakthrough for metabolic evolution since it provided an easily accessible energy supply for any organism that could use it.

The endosymbiotic legacy of melatonin transfer

When eukaryotic cells engulfed cyanobacteria, initially it was likely for the purpose of obtaining nutrients. Eventually, however, they were assimilated into the cellular machinery, that is, they established a symbiotic association with their host. Thus, the molecular biological features of the cyanobacterium were incorporated into the physiological repertoire of the eukaryote. As the engulfed cyanobacteria were already capable of melatonin synthesis, we propose the synthetic melatonin machinery which they possessed also became an integral part of the eukaryotic plant cells as well [7].

According to the endosymbiotic theory on the origin of eukaryotic organelles, engulfed nonpurple sulfur bacteria became the mitochondria while the anaerobic cyanobacteria evolved into chloroplasts. The endosymbiotic theory for the origin of these eukaryotic organelles was most ardently and successfully advanced by Margulis (nee, Sagan) [8–10] roughly 50 yr ago although it had been proposed many decades earlier [11]. Table 1 summarizes some of the common features of prokaryotes and the chloroplasts and mitochondria of eukaryotes which prompted the endosymbiotic theory.

When anaerobic photosynthetic bacteria and/or aerobic bacteria were endocytosed, some of the features of the engulfed bacteria were retained by the newly formed eukaryote (Table 1). These features were preserved because they were beneficial to and improved the function of the new eukaryote. Conversely, the engulfed bacteria which were destined to become essential organelles also received support from the eukaryotic cells; thus, the relationship became mutually beneficial (symbiotic). Collectively, the process of being engulfed and assimilated into the new organism is referred to as endosymbiosis (Fig. 2) [8–10].

As the engulfed bacteria already possessed melatonin and the ability to produce it, we surmised that this function was likewise retained by eukaryotic cells [7]. Likewise, the organelles that developed from the engulfed bacteria never relinquished their capacity to produce melatonin

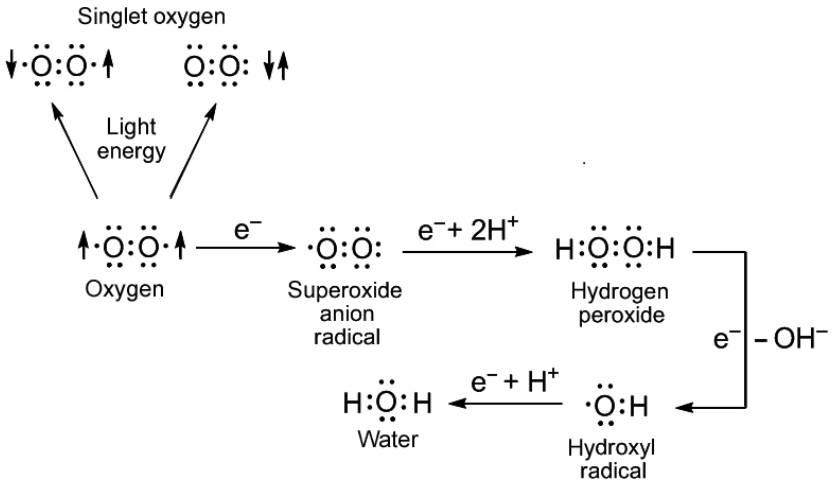

Fig. 1. Oxygen in its ground state has two unpaired electrons, which is unconventional. Based on the Pauli exclusion principle, when two electrons are in the same orbital, they must have an opposite spin (spin state) thereby canceling their respective magnetic fields. In the case of oxygen, the unpaired electrons occupy different orbits, and moreover, they have the same spin direction. These features not only contribute to oxygen's paramagnetism but also contribute to its ability to form a free radical, with the addition of a single unpaired electron, that is, the superoxide anion radical ($O_2^{\cdot-}$). This figure illustrates the generation of toxic oxygen derivatives, that is, $^1O_2$, $O_2^{\cdot-}$, H_2O_2, and the $^{\cdot}OH$, and its four electron reduction to water.

Table 1. This table summarizes the features of prokaryotes and eukaryotes which suggest they have a common origin. The eukaryotes include plants and animals including man

	Ancestral eukaryote (prokaryotes)	Eukaryotes	Mitochondria of eukaryotes	Chloroplasts of photosynthetic eukaryotes
Genetic material	Single circular chromosome	Multiple linear chromosomes in nucleus	Single circular chromosome	Single circular chromosome
Replication	Binary fission	Mitosis	Binary fission	Binary fission
Ribosomes	'705'	'805'	'705'	'705'
Location of electron transport chain	Plasma membrane around cell	Cellular mitochondria and chloroplasts	Inner mitochondrial membrane[a]	Inner chloroplast membrane[a]
Size	~1–10 μm	~50–500 μm	~1–10 μm	~1–10 μm
Appearance on Earth	Anaerobic bacteria – 3.8 billion years ago Photosynthetic bacteria – 3.2 billion years ago Aerobic bacteria – 2.5 billion years ago	1.5 billion years ago	1.5 billion years ago	1.5 billion years ago

[a]The inner mitochondrial and inner chloroplast membranes were originally the plasma membranes of the bacteria that were engulfed.

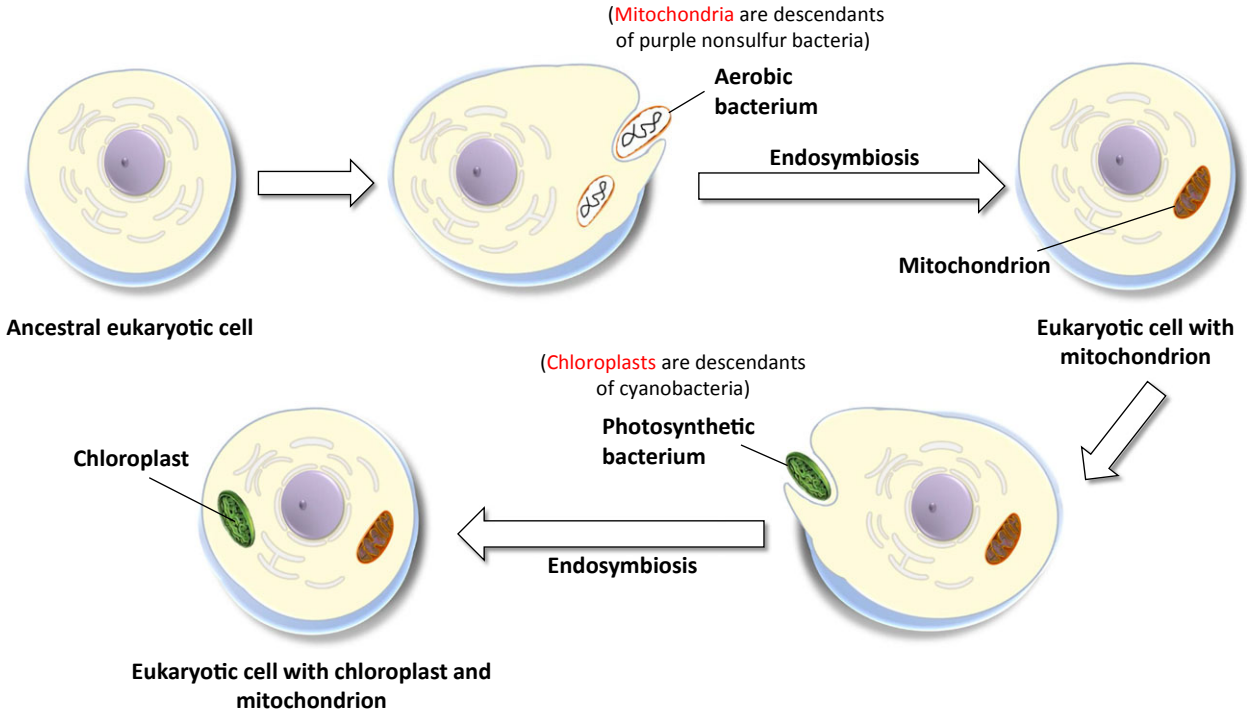

Fig. 2. The endosymbiotic theory of the origin of mitochondria and chloroplasts, as most ardently espoused by Margulis [8–10], poses that these organelles derived from bacteria that were initially engulfed and digested for their nutrient value but eventually evolved into the mitochondria and chloroplasts. As a result, all existing eukaryotes have organelles that were originally derived from bacteria. As the engulfed bacteria already had the ability to synthesize melatonin, we surmise that this function was retained such that the mitochondria and chloroplasts of existing eukaryotes may have the machinery to produce melatonin.

throughout evolution such that all mitochondria-bearing cells of present-day unicellular and multicellular organisms presumably still possess the capacity to generate melatonin [12]. The preserved association of melatonin with mitochondria and chloroplasts is particularly critical as highly toxic-free radicals are profusely formed at these sites. Thus, the presence of melatonin in these organelles allows this highly effective free radical scavenger to quickly and efficiently detoxify these partially reduced brigands before they structurally and functionally mutilate neighboring molecules, that is, lipids, proteins, DNA, etc. If melatonin was not situated in these organelles, the damaged molecular fallout would be greatly magnified and the functional deterioration of these organelles would be accelerated. Slowed or failed mitochondrial function, which occurs when critical molecules in this organelle are malfunctional, is widely believed to contribute to debilitation, that is, aging [13, 14].

Due to the selective pressure resulting from the increasing oxygen in the atmosphere, the evolution of organisms that used oxygen as a basis of their metabolism was enhanced. Their evolution provided these organisms with a tremendous metabolic advantage as 36 molecules of ATP are produced as a result of the combustion of each molecule of glucose. Virtually all oxygen utilized by aerobic organisms is reduced to water by the addition of four electrons via cytochrome oxidase in mitochondria. The use of oxygen to produce ATP, however, comes with a rather high metabolic toll because the successive univalent reduction of oxygen produces free radicals (molecules or portions of molecules that have an unpaired electron in their valence orbital), which readily inflict damage on other adjacent molecules [15].

While melatonin predictably evolved in primitive bacteria, during evolution, it persisted in all organisms including multicellular species where it was repurposed for a number of other critical functions (Fig. 3). Nevertheless, its original action that allows it to directly detoxify ROS/RNS was retained; this function persists in present-day mammals including man.

Melatonin: benefits for plants

Like animals, plants also assimilated melatonin into their metabolic framework and took advantage of its free radical-neutralizing actions. In animal tissues, melatonin reduces the toxicity of a large variety of free radical-generating toxins and/or processes, for example, bipyridyl herbicides, heavy metals, and hypoxia–reoxygenation [16–20]. While the latter process is not an issue in plants, paraquat and diquat are in widespread use in certain parts of the world [21, 22]. Although their ability to generate ROS varies among plant species, there is certainly extensive evidence that the toxicity of these herbicides is a consequence of damage inflicted by these oxidizing agents. Inasmuch as these herbicides are purposefully used to kill unwanted plants, there is no reason to test the ability of melatonin to prevent their toxicity. On the other hand, the varying sensitivity of plants to these herbicides may relate to their different levels of the antioxidant melatonin that they contain [23].

In a single published report, however, melatonin was shown to resist the growth-stunting action of copper in the pea plant (*Pisum sativum*) [24]. Because of observations such as this and others, we suggested that since melatonin increases the resistance of some plants to soil toxins, melatonin may find utility in phytoremediation. As envisioned, plants treated with melatonin could be grown in highly contaminated soils and, after taking up the toxins, the plants could be removed resulting in the remediation of the soil [24].

Environmental pollutants are frequently strong oxidizing agents; examples include ozone (O_3) and nitrogen dioxide ($NO_2^{\cdot}$). The former, in particular, is believed to account for more plant damage than any other airborne pollutant [25]. When melatonin was initially discovered in land plants in 1995 [26, 27], we speculated that the differential sensitivity of tobacco plants (*Nicotiana tabcum*) to O_3 may relate to the specific concentrations of melatonin they contain [26], an observation supported by recent findings that melatonin does reduce ozone toxicity in rats. Whether melatonin changes the sensitivity of plant tissues to $NO_2^{\cdot}$ has yet to be tested.

Since plants are sessile, they are exposed to more environmental stresses than are mobile species, that is, animals. As in mitochondria, the electron transport chain of chloroplasts also 'leaks' electrons onto oxygen with the formation of free radicals [28]. Chloroplasts are surrounded by a bilayered envelope that encloses the stroma. The stroma is the location at which CO_2 is converted into carbohydrates via the Calvin cycle, an ATP- and NADPH-requiring process [29]. The internal membrane structure of the chloroplast is complex and consists of thylakoids (closely stacked membranes) and an interconnecting series of membranes referred to as stromal thylakoids. These membranes are the location of the green photosynthetic pigments (chlorophylls a and b), which are sensitive to blue and red wavelengths of the electromagnetic spectrum, and yellow carotenes and xanthophylls which also absorb blue light. The thylakoids generate both the ATP and NADPH required to drive CO_2 fixation via the Calvin cycle [30].

Chloroplasts sustain molecular damage due to several means. The absorbance of light energy by chlorophylls

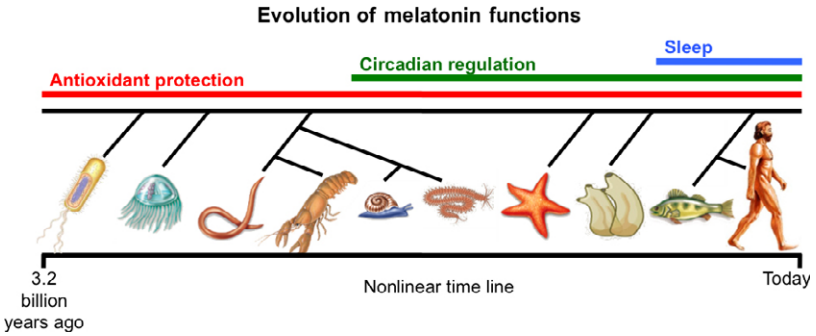

Fig. 3. The original function of melatonin is proposed to have been as an antioxidant [7]. Throughout subsequent evolution, melatonin and its rhythm in the blood were repurposed for other functions, for example, as a circadian rhythm regulator and sleep promotion. Its functions, however, extend beyond those listed in this figure. Melatonin was retained with its original molecular structure (the melatonin in cyanobacteria is identical to that in current-day humans) during evolution. All animals and plants that have been studied seem to contain melatonin. Modified from Schippers and Nichols [228].

promotes the formation of singlet oxygen ($^1O_2$) (Fig. 1); this excited state of oxygen is especially damaging to polyunsaturated fatty acids (PUFA) which are an essential component of the thylakoid membranes, as well as to proteins [31]. The leakage of elections onto oxygen also causes its reduction to $O_2^{\cdot-}$. $O_2^{\cdot-}$ produced in chloroplasts is dismutated, as in animal cells, by superoxide dismutases (SODs) resulting in the formation of H_2O_2 (Fig. 1). Plant SODs are either bound to thylakoids or free in the chloroplast stroma. H_2O_2 has a major negative impact as it inactivates the chloroplast enzymes, and also, it suppresses the Calvin cycle by interfering with its enzymes [31]. The chloroplast, however, has no means to enzymatically remove H_2O_2 as the chloroplasts of most plants are devoid of catalase and selenoprotein glutathione peroxidase. Also, although glutathione transferases are present in a variety of plants and are normally used to reduce organic hydroperoxides, their significance for this process in plants is uncertain [32]. Melatonin likely plays a critical role against the toxicity of H_2O_2 as it reportedly detoxifies this reactive species [33] as well as its major toxic derivative, the $^{\cdot}OH$ [34–37]. This function persists in present-day plants and animals, and moreover, both categories of organisms have evolved melatonin-influenced enzymes which detoxify harmful oxidizing species [38–42]. This combination of actions of melatonin, along with others outlined below, accounts for melatonin's high efficiency in reducing the molecular carnage that results from ROS/RNS [18–20, 43–48].

Melatonin in mitochondria: in the right place at the right time

If melatonin is proven to be synthesized in mitochondria and chloroplasts as we proposed [12], it would certainly be an advantage given that these organelles are major sites of free radical generation [49, 50] and melatonin has a potent capacity to limit the damage these toxic oxygen derivatives leave in their wake [51, 52]. While originally thought to be produced exclusively in and secreted from the vertebrate pineal gland [53], it is now known that the indole is present in many, perhaps all, vertebrate organs [54] and in organs of all plants that have been investigated [48, 55, 56]. That melatonin is not relegated solely to the pineal gland is also emphasized by the reports that it is present in invertebrates [57–59], which lack a pineal gland and some of which consist of only a single cell.

If melatonin is synthesized in both mitochondria [12, 60] and chloroplasts [61, 62], it could explain the generally much higher concentrations of this indole in plants than in animals, as the former have both organelles while the latter only possess mitochondria, except for some single-celled 'animals'.

There are very few studies in which mammalian mitochondrial melatonin levels have actually been measured. One recent investigation did assay melatonin in fractionated rodent cerebrocortical cells [60] and noted that the concentration varied among organelles in the following order: mitochondria >membranes >nuclei >cytosol (Fig. 4). The levels of melatonin, determined by HPLC, in these organelles fluctuated over a 24-hr period, but the variations were not correlated with the light: dark cycle. The concentrations of melatonin in the mitochondria and membranes were much higher than in the blood and, moreover, these remarkably high levels did not diminish after the animals had been pinealectomized, a procedure that eliminated the nighttime increase in plasma melatonin concentrations. These findings strongly suggest that the subcellular melatonin was not of pineal origin and was probably locally produced. In addition, particularly the

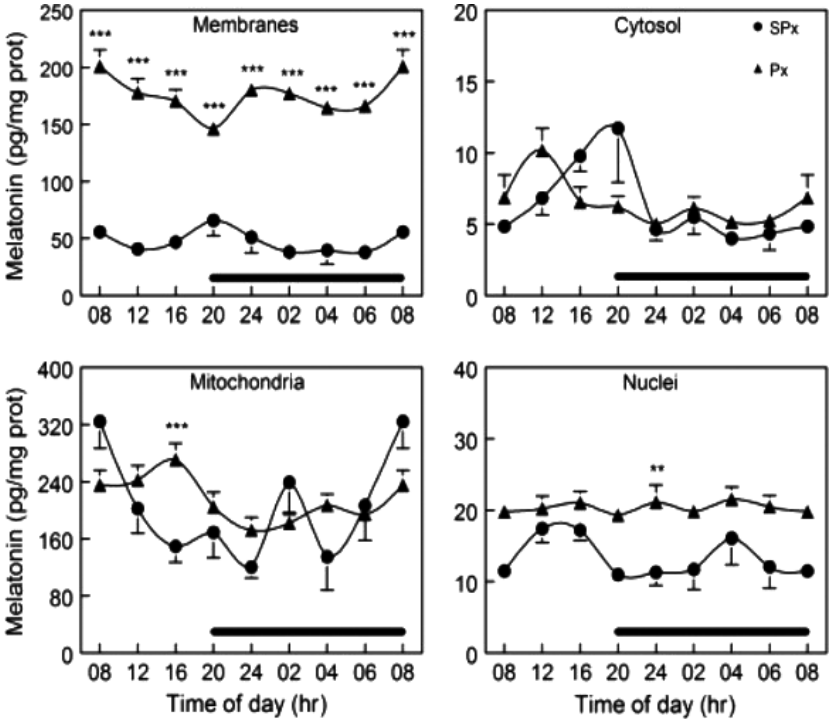

Fig. 4. Levels and changes in melatonin over a 24-hr period in organelles of cerebrocortical cells isolated from intact (SPx) and pinealectomized (Px) male rats. Pinealectomy causes circulating levels to plummet to almost undetectable levels even at night. This, however, did not cause a drop in intracellular melatonin concentrations. Each point represents the mean value for the animals killed at that time point. The black bar signifies the period of darkness. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus SPx values. From Venegas et al. [60].

mitochondria and membranes were capable of maintaining a high level of melatonin against a concentration gradient (relative to levels in the blood). The time-related variations in the levels of melatonin in these organelles may be linked to its utilization, for example, as a radical scavenger, rather than to differential changes in its synthesis.

These findings are of particular interest for several reasons. First, the subcellular organelles obtained from cerebrocortical cells had higher melatonin levels than the same organelles from hepatocytes [60]. This is noteworthy as the brain has a higher requirement for oxygen than any other organ [63, 64]. Hence, it would be expected that its cells would generate exceptionally high levels of free radicals, and therefore, they would require higher concentrations of radical scavengers such as melatonin to fend off the impending damage. This may be even more critical given that the brain has lower antioxidant enzyme capability than some other organs [65, 66].

The high concentrations of melatonin in the mitochondria retrieved from the brain are also consistent with the much greater levels of melatonin in the cerebrospinal fluid (CSF) than in the peripheral circulation [67, 68]. It has recently been postulated that these values are especially elevated because the primary route of secretion of melatonin from the pineal gland is directly into the CSF [69, 70]. This allows melatonin to readily penetrate the surrounding neural tissue to afford the brain the extra protection it needs to ward off free radical mutilation; however, the results of the study of Venegas et al. [60] summarized above proved that intracellular melatonin levels are not reliant solely on that delivered to the cells via the circulation.

It is essential that the mitochondria avoid as much oxidative damage as possible as failed mitochondria compromise ATP production [71, 72] resulting in massive cellular loss due to apoptosis [73]. Indeed, a large number of diseases associated with aging are the basis for what are known as 'mitochondrial diseases' [74, 75] and the 'mitochondrial theory of aging' [76, 77]. Thus, it seems the special association that melatonin seems to have with mitochondria is very fortuitous [78].

Melatonin levels in brain cells are clearly higher in the mitochondria than in the cytosol or nucleus [60] suggesting that the latter areas are less aggressively defended by melatonin against free radical attack. This, however, may not be the case as melatonin is also a prodrug for some other molecules that are also highly effective radical scavengers [79]. Some of these derivatives may be more polar than melatonin, and thus, they would more readily permeate the aqueous environments of the cells to repel free radicals [80].

One example of the highly protective actions of melatonin relative to even synthetically produced, mitochondrial-targeted antioxidants comes from the work of Lowes et al. [81]. Using a model of septic shock in which rats were injected with two highly toxic agents, lipopolysaccharide (LPS) and peptidoglycan G (PepG), the ability of MitoE and MitoG was compared with melatonin relative to their abilities to arrest the inflammatory response, to curtail oxidative stress, and to maintain mitochondrial respiration. MitoE and MitoQ are commercially produced antioxidants that are linked to a highly lipophilic triphenylphosphonium cation which allows them to concentrate in mitochondria in very high concentrations (several hundred-fold more than normal); they allow the delivery of high concentrations of tocopherol and ubiquinol, respectively, to the sites of maximal free radical generation.

Based on oxidative damage to hepatic proteins (measured as liver protein carbonyls) and plasma levels of peroxidized lipids (measured as lipid hydroperoxides), melatonin performed better than either MitoE or MitoQ in resisting oxidative damage (Fig. 5). Likewise, a comparison of plasma alanine transaminase and aspartate transaminase also revealed that melatonin was equal to or better than the synthetic molecules in restricting oxidative damage [81]. The relative abilities of the three antioxidants to safeguard against toxic levels of a pro-inflammatory cytokine (interleukin-6) and to restrict the depression of mitochondrial respiration were broadly similar.

The results reported by Lowes et al. [81] are quite remarkable considering that using the specialized delivery system allowed for very high concentrations of tocopherol and ubiquinol to enter the mitochondria [82, 83] where they could function as antioxidants. Despite the several hundred-fold higher levels of these antioxidants in the mitochondria, their performance still did not exceed that

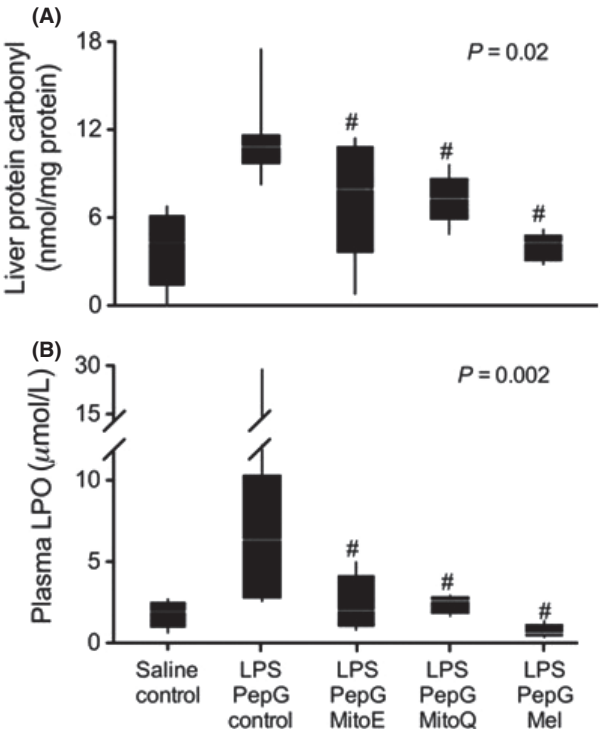

Fig. 5. Hepatic free radical-damaged proteins (protein carbonyl, A) and oxidized lipid in the plasma (LPO, lipid peroxidation, B) after treatment of rats with two highly toxic agents [lipopolysaccharide (LPS) and peptidoglycan (PepG)]. Also shown are the relative efficacies of two synthetic antioxidants (MitoE and MitoQ) and a naturally occurring antioxidant (melatonin, mel) in reducing oxidative mutilation of proteins and lipids. On the basis of these measurements, the authors concluded melatonin is superior to MitoE and MitoQ in arresting oxidative stress. From Lowes et al. [81].

of melatonin, and, in fact, relative to the degree of induced oxidative damage, they were less protective than melatonin.

In view of melatonin's high efficacy in the LPS/PepG treatment, a reasonable conclusion is that melatonin itself is a mitochondria-targeted antioxidant. There also is another consideration. In plants, external stresses are a potent inducement for melatonin synthesis [48, 56, 84, 85]. This may also apply to animals where there is evidence that stress promotes elevated endogenous melatonin production [86, 87]. Thus, the LPS/PepG treatment used by Lowes et al. [81] may have upregulated melatonin production (in mitochondria?) thereby providing improved protection against free radical damage. In any case, the statement at the head of this section that melatonin, as an antioxidant, is 'in the right place at the right time' seems valid.

Melatonin reduces oxidative stress: the machinery and the mechanisms

In aerobes, oxidative stress is inevitable and, perhaps fortunately, cannot be completely prevented. Some free radicals escape detoxification and have essential functions in organisms, for example, the killing of invading bacteria by leukocytes and macrophages [88, 89]. Clearly, quenching all radicals with direct scavengers or enzymatic antioxidants would be detrimental. The ability of any antioxidant to detoxify free radicals must be context specific.

Context specificity is a functional feature of melatonin as well. Thus, while melatonin is commonly classified as an antioxidant, under appropriate circumstances, it also functions as a beneficial pro-oxidant, for example, in cancer cells [37, 90]. This is also apparent when the actions of melatonin are evaluated in reference to its ability to modulate apoptosis. In normal cells, melatonin has anti-apoptotic actions allowing these cells to survive, while in cancer cells, melatonin is often pro-apoptotic, that is, it rids tissues of cancer cells [91–93]. Although diametrically opposite, both these functions are essential actions of melatonin.

In this section, we review the multitude of processes that contribute to the ability of melatonin and its metabolic kin to function in the reduction of oxidative stress. This protection applies to DNA, proteins, and lipids [45, 94–96]. There is certainly no shortage of studies that have unequivocally documented the ability of this molecule to attenuate the accumulation of free radical-damaged molecules, for example, in ischemia–reperfusion injury [20, 97–100], toxin exposure [17–19, 101], inflammation [102–105], and many other clinically relevant situations [106–108]. These benefits are apparent in both plants and animals [46, 48, 109, 110].

The antioxidant machinery

The fact that not only melatonin but many of its metabolic kin likely work in sequence or in concert to neutralize ROS/RNS makes this cache of molecules highly efficient in reducing oxidative stress (Fig. 6) [87, 111–113]. While it is obviously easy to measure the marked reduction in redox-damaged molecules under both in vitro and in vivo conditions when melatonin is used as a treatment, it is unlikely that all of the protection afforded is exclusively a result of melatonin per se. This becomes readily apparent when the scavenging activity of melatonin on the $ABTS^{\cdot+}$ is compared with that of other radical scavengers, that is, vitamin C, trolox, glutathione, NADH, and NADPH [114]. When incubated with the $ABTS^{\cdot+}$, each of these molecules rapidly scavenged the radical (within <1 min); then, the curves plateaued and no additional scavenging occurred over the next 11 min. By comparison, melatonin also rapidly reduced the $ABTS^{\cdot+}$ but continued to scavenge this radical for up to 12 min (Fig. 7). This prolonged period of scavenging was judged to be a consequence of the sequential generation of melatonin metabolites that are also capable of neutralizing the $ABTS^{\cdot+}$. Beyond these derivatives, some other related compounds including 6-hydroxymelatonin [115], 5-hydroxytryptophan, and 5-methoxytryptamine [116] are also reported to be radical scavengers.

Assuming that melatonin, the derivatives shown in Fig. 5 and other structurally similar indoles all efficiently neutralize free radicals in vivo, they could be involved at any level of oxygen reduction, that is, $O_2^{\cdot-}$, H_2O_2, etc. Table 2 lists the radicals and oxidizing agents that have

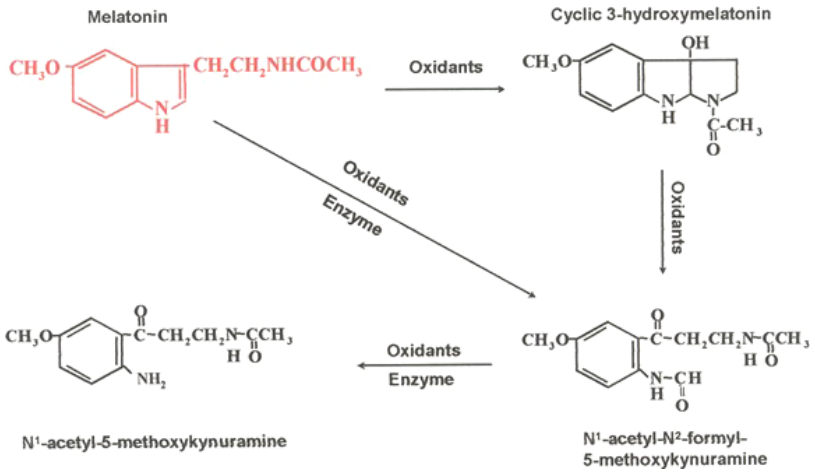

Fig. 6. This diagram outlines what has come to be known as the antioxidant cascade of melatonin. In this scheme, the primary (melatonin), secondary (cyclic 3-hydroxymelatonin), tertiary (*N*1-acetyl-*N*2-formyl-5-methoxykynuramine), and the quaternary (*N*1-acetyl-5-methoxykynuramine) molecules all function as free radical scavengers. Thus, a single melatonin molecule along with its metabolites can scavenge numerous reactive oxygen species/reactive nitrogen species. This scheme is based on observations reported by Tan et al. [87].

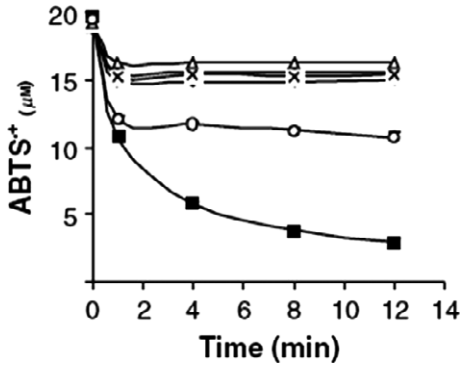

Fig. 7. This figure illustrates the scavenging of the $ABTS^{\cdot+}$ by several antioxidants (NADH, NADPH, vitamin C, trolox, glutathione and melatonin). In this system, 20 μM $ABTS^{\cdot+}$ was incubated with 10 μM concentration of radical scavengers. Due to the antioxidant cascade, melatonin and its metabolites continued to neutralize the cation radical well after the other antioxidants had completed scavenging and were depleted.△ = NADH; X = NADPH, vitamin C and trolox (water soluble vitamin E); O = glutathione;■ = melatonin. From Tan et al. [114].

Table 2. Some of the publications related to the ability of melatonin to scavenge reactive oxygen species and reactive nitrogen species. These studies were performed in vitro, some in purely chemical systems and some were computational. As with any radical scavenger, proof that these reactions occur in vivo is difficult to determine. Thus, while melatonin is a potent inhibiter of oxidative stress, how this relates to the direct scavenging actions of this molecule remains unknown. There is some evidence showing that melatonin is pro-oxidant, particularly in cancer cells

Oxidizing agent	References
Superoxide anion radical	[117–119]
Singlet oxygen	[117, 120, 121]
Hydrogen peroxide	[33, 117]
Hydroxyl radical	[34, 35, 122–130]
Nitric oxide	[126, 131]
Peroxynitrite anion	[132–134]
Peroxyl radical	[135–139]
Alkoxyl radical	[136, 140]
Hypochlorous acid	[141, 142]

been reported to be somehow detoxified by melatonin. In reality, these damaging agents could have been scavenged by melatonin itself or by any of its metabolic by-products; this could not be determined by the authors of the associate publications. As previously mentioned, in some cases, perhaps melatonin is primarily a prodrug which gives rise to more efficient scavenger molecules [79].

Using cyclic 3-hydroxymelatonin (c3OHM) synthesized by Siwicka et al. [143], Tan et al. [144] found it to be at least as effective as melatonin itself in scavenging the $^{\cdot}OH$. It also reduced oxidative degradation of cytochrome C when it was exposed to H_2O_2. As c3OHM proved so effective in combating oxidative damage, the authors surmised that at least some of melatonin's ability to reduce molecular destruction by free radicals stems from the action of its metabolite, c3OHM. In a follow-up report [145], the same group used density functional theory to examine the reactions of c3OHM with the $^{\cdot}OH$ and the $ROO^{\cdot}$ (hydroperoxyl radical). Again, they report that c3OHM reacts with the $^{\cdot}OH$ at a diffusion-limited rate and was better in doing so than melatonin itself of other metabolites in the antioxidant cascade, AFMK and *N*1-acetyl-5-methoxykynuramine (AMK). When interacting with the $ROO^{\cdot}$, c3OHM was several orders of magnitude faster than melatonin; AFMK or AMK in an aqueous solution was almost 100 times faster than trolox (water soluble vitamin E). The results obtained by both Tan et al. [144] and Galano et al. [145] point to the validity of melatonin's antioxidant cascade and identify the important role of c3OHM in this process.

Other metabolites in the cascade, that is, AFMK and AMK, have also been tested in terms of their ability to function as scavengers and to reduce oxidative stress. The findings, published by several laboratories, confirm that kynuramines are capable of providing antioxidant protection. Thus, AFMK clearly functioned in the detoxification of ROS [146–149] and RNS [150–152] and reduced oxidation of biological molecules [150, 153–156]. Moreover, some additional products were identified that may also participate in the antioxidant cascade (Fig. 8) [151, 157, 158].

Besides directly neutralizing free radicals, melatonin enzymatically removes them from the cellular environment either by metabolizing them to inactive species or inhibiting enzymes that result in their formation. Some of these processes are summarized in Table 3. AMK has some similar actions as melatonin by inhibiting nitric oxide synthase [178, 179]. The mechanisms by which oxidative stress stimulates the activities of ROS detoxifying enzymes may involve the Keap/Nrf2/ARE pathway [180, 181]. Thus, oxidative stress enhances the release of Nrf2 (nuclear factor erythroid 2-related factor 2) from Keap (Kelch-like ECH-associated protein) allowing the former to translocate into the nucleus where it binds to the ARE (antioxidant response element) on the promoter region of the detoxifying enzymes, for example, superoxide dismutase and glutathione peroxidase; this leads to the increased mRNA expression and activities of these enzymes [182, 183] (Fig. 9). Besides removing toxic agents from the intracellular environment, high Nrf2 levels are associated with reduced cancer growth and increased life span [184–186], both of which are documented or proposed actions of melatonin [187–192]. We suggest that the role of melatonin in regulating the Keap/Nrf2/ARE sequence is the ability of melatonin to suppress the ubiquitin/proteasome pathway [193, 194]. This action helps to preserve high Nrf2 levels which aids in stimulating antioxidant enzyme activities and other processes mediated by Nrf2 binding to the ARE. Finally, Nrf2 is also activated by the extracellular signal-regulated kinase (ERK) pathway, which is likewise regulated by melatonin [181].

Interestingly, Nrf2 is normally upregulated in calorie-restricted rats [185]; this procedure reproducibly prolongs the life span of rodents [195–197]. Similarly, Nrf2 is highly constitutively expressed in the long-lived naked mole rat [196, 197]. As melatonin upregulates Nrf2, the collective findings prompt the suggestion that the anti-aging actions of melatonin may also involve the Keap/Nrf2/ARE pathway. Normally, aging leads to a marked reduction in

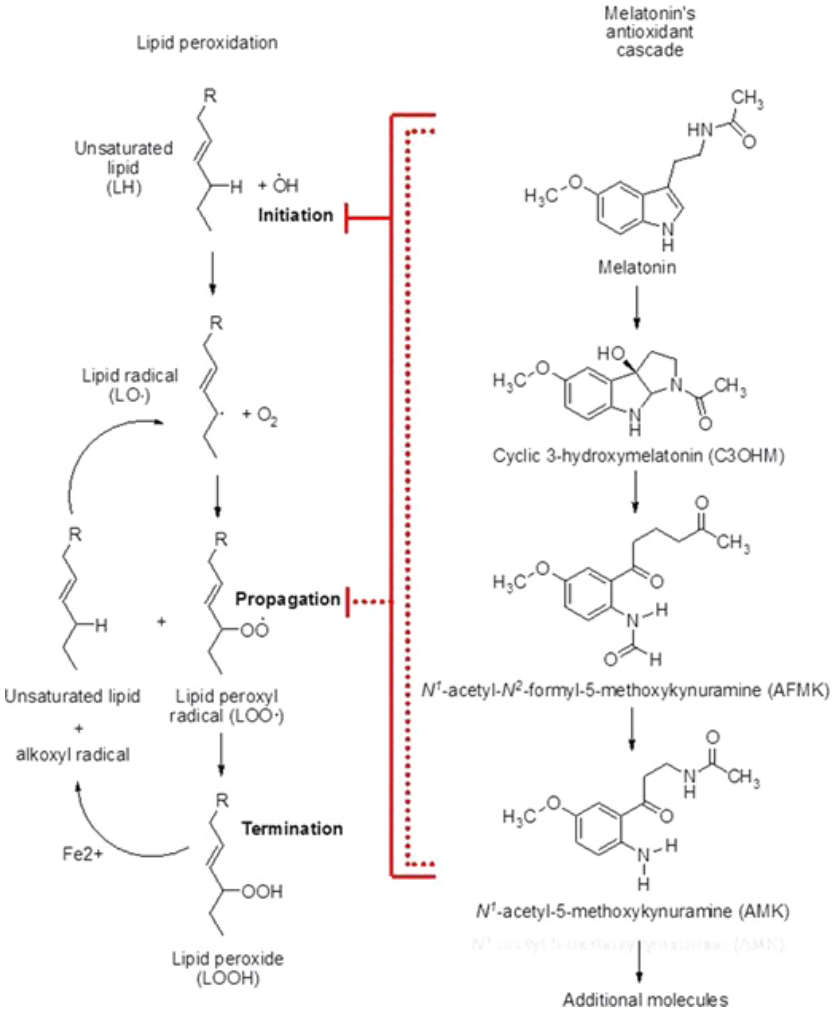

Fig. 8. This figure illustrates the self-propagating process of lipid peroxidation (left) which, once the initial unsaturated lipid is oxidized (in this case by the ˙OH) (Initiation), the resulting lipid peroxyl radical (LOO˙) continues the chain reaction by oxidizing an adjacent unsaturated lipid molecule (Propagation). On the right are melatonin and its metabolites which function in an antioxidant cascade. Melatonin and its metabolites scavenge radicals (in this illustration the ˙OH and LOO˙) with different efficiencies at both the Initiation and Propagation phases. From Reiter et al. [156].

Table 3. Some of the enzymes that are modulated by melatonin which alter the concentration of free radicals within cells are enumerated in the table. Changes in the activities of these enzymes either metabolically destroy reactive oxygen species or they reduce their generation

Enzyme	References
Superoxide dismutases	[159–161]
Glutathione peroxidase	[38–40, 160, 161]
Glutathione reductase	[40, 162]
Catalase	[160, 161]
Glutathione S-transferase	[161]
Glucose-6-phosphate dehydrogenase	[163]
Glutamyl cysteine ligase	[164, 165]
Cyclo-oxygenase	[166, 167]
Heme-oxygenase	[161]
Nitric oxide synthase	[166, 168, 169]
Myeloperoxidase	[170, 171]
Lipoxygenase	[172, 173]
Paraoxonase	[174, 175]
Quinone reductase	[176, 177]

melatonin levels [198–200] which are preserved in calorie-restricted rats [201]. So the highly preserved Nrf2 concentrations and the prolonged life span may, in part, be related to the preserved melatonin values in the diet-restricted animals.

Other factors that may contribute to melatonin's ability to limit oxidative stress

In addition to the reported direct scavenging actions, most frequently via electron donation, and the indirect antioxidative effects due to modulation of enzyme activities, melatonin has other means to reduce oxidative stress. Two reports have confirmed that melatonin binds transition metals that participate in the Haber Weiss/Fenton reactions which generate the ˙OH [202, 203]. Accordingly, Galano et al. [203] modeled two processes, that is, the direct chelation mechanism and the coupled-deprotonation-chelation mechanism (CDCM), to explain how melatonin renders transition metals inactive. They determined that under physiological conditions the CDCM pathway was the most likely means by which melatonin binds Cu^{2+}. The chelating action of melatonin was also shared by melatonin's metabolites, c3OHM and AFMK with c3OHM being the most effective. Thus, besides the antioxidant cascade [87], there seems also to be chelating cascade as well. Obviously, reducing ˙OH formation via the

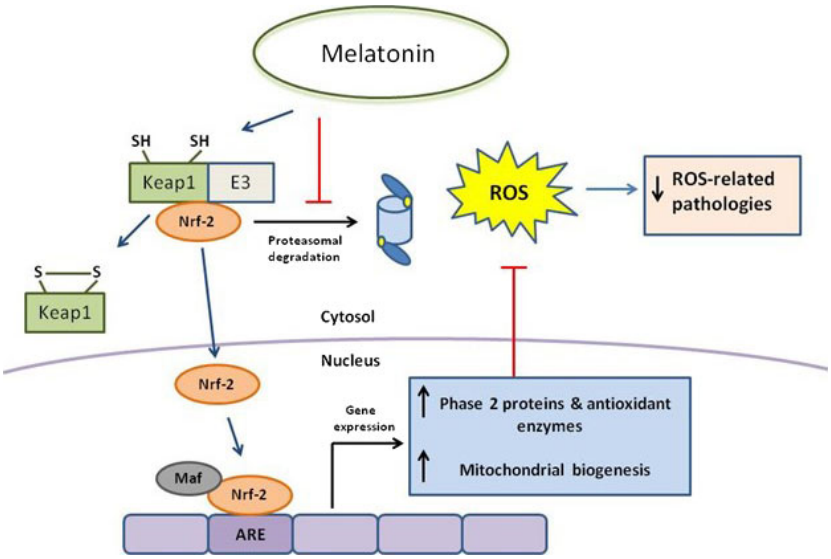

Fig. 9. The proposed means by which melatonin stimulates antioxidant enzymes, etc. Oxidative stress releases Nrf2 from Keap1 leading to its phosphorylation and translocation into the nucleus. At the same time, melatonin may inhibit the ubiquitination of Nrf2, thus reducing its degradation by the proteasome. In the nucleus, Nrf2 couples with Maf allowing it to bind the antioxidant response element (ARE). This causes the transcription of a number of enzymes which convert reactive oxygen species/reactive nitrogen species to unreactive products thereby controlling oxidative stress.

chelating actions of melatonin and its metabolic kin would limit the molecular havoc that leads to accumulated oxidative damage and physiological inefficiency.

There are a wide variety of other features that may contribute to melatonin's high efficacy in abating oxidative stress. Melatonin seems to be in higher concentration in the mitochondria than in the blood [60]; this would be particularly fortuitous as these organelles are a site of high free radical generation. Besides directly scavenging ROS in these organelles, melatonin may also reduce electron leakage from the respiratory chain complexes [204] which results in higher ATP production [205–207], both of which benefit the cell. Reducing electron leakage also limits formation of free radicals, a process referred to as radical avoidance [202].

Besides mitochondria, some fluids have very high melatonin concentrations relative to those in the general circulation. One example is the CSF [67–69]; this is considered important as in this fluid melatonin would be in the position, after it is absorbed, to protect the brain from the high free radical damage it normally sustains because of its high utilization of oxygen [70]. In the bile as well, melatonin levels are likewise highly elevated [208]. Perhaps this protects the biliary epithelium from the toxic actions of bile [209]. The fact that melatonin probably is present in every cell [210] and in every species, both plant [211] and animal [54], also emphasizes its importance as a ubiquitously acting antioxidant. Finally, melatonin strengthens circadian rhythms which, when disrupted, aggravate free radical production [212, 213].

Epilogue

Dealing with the toxicity of oxygen is a full-time job for all organisms as its derivatives, that is ROS and RNS, are the scourge of healthy macromolecules. On the other hand, oxygen is a molecule that aerobes cannot live without yet living with it is likewise a major burden. Because of the high toxicity of oxygen, both plants and animals have had to evolve an efficient and versatile defensive system to shield themselves from oxidative/nitrosative stress. Despite having a variety of antioxidants to fight against the damage caused by oxygen derivatives, the system is by no means perfect and some mutilation always occurs which compromises physiology and metabolism.

Melatonin is an antioxidative agent that predictably evolved very early in the most phylogenetically distant organism, the cyanobacterium. In the three billion years since its evolution, melatonin has had ample time and opportunities to hone its ability to combat oxidative damage; the experimental data that have accumulated indicate it has done an excellent job in developing a variety of defensive strategies. In spite of this, however, and even with the aid of many other antioxidants, melatonin cannot prevent all molecular damage as some ROS/RNS always go unintercepted and succeed in causing molecular carnage. This damage, which accumulates over time, is widely accepted as contributing to many diseases and to aging.

Among the vast number of antioxidants that have been identified, melatonin seems to have some unique features. One noteworthy feature is what has been described as the antioxidant cascade wherein melatonin and several of its metabolites sequentially function in the detoxification of radicals, possibly with different efficiencies and specificities. Also, melatonin has molecular actions that lead to the modulation of both antioxidative and pro-oxidative enzymes, the results of which is a diminished free radical output in normal cells. In contrast, in cancer cells, melatonin enhances free radical generation, that is, it becomes pro-oxidative. Thus, the actions of melatonin are context specific allowing it to preserve the life of normal cells while killing cancer cells [91].

For humans, at least, antioxidants are either consumed in the diet, for example, vitamins C and E, or produced in vivo, for example, glutathione. Melatonin is available from both sources. As melatonin has been identified in plants, in some cases, in very high concentrations, when plants containing melatonin are ingested, it changes blood melatonin levels [214–216] and its levels in the circulation correlate with the total antioxidant capacity of the blood

[217]. As some plants have been genetically engineered to produce enhanced amounts of melatonin [218, 219], it is likely these and similarly engineered products will eventually become available to the public as a dietary source of melatonin.

Melatonin has proven itself as a high potency antioxidant in numerous experimental paradigms. Research on its potential in staving off human [220–224] and plant [225] diseases is still in the early phases, but these data suggest melatonin will be a highly practical and safe molecule for use over a wide range of doses. Certainly, compared with the early assumptions that melatonin's primary usefulness would be as a treatment for circadian and sleep disorders [226, 227], its benefits clearly exceed these expectations [78].

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