Melatonin: Helping cells cope with oxidative disaster

Sergio D. Paredes[1] and Russel J. Reiter[2]

[1] Department of Physiology (Neuroimmunophysiology and Chrononutrition Research Group), Faculty of Science, University of Extremadura, Badajoz, Spain

[2] Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas, USA

[1]**Corresponding Address**
Dr. Sergio Damián Paredes Royano,
Department of Physiology,
Faculty of Science, University of Extremadura,
Avda. de Elvas, s/n, 06006, Badajoz, Spain.
Phone : +0034 924 289 388.
Fax : +0034 924 289 388.
E-mail : sparedes@unex.es

Abstract

Melatonin possesses the capability of donating electrons, consequently reducing the reactivity of molecules with an unpaired electron in their valence orbital, i.e. free radicals. A plethora of studies support the role of this indoleamine in diminishing molecular damage associated with massive free radical generation both *in vitro* and *in vivo.* Melatonin protects against neurodegenerative disorders, ischemia/reperfusion injury, acts as a radio protector, and counteracts herbicide and metal toxicity due to its essential role in antioxidant protection. At the intracellular level, it reduces electron leakage from the mitochondrial respiratory chain complexes as well as scavenging radicals generated in the cytosol (exclusive of its actions in mitochondria) and nucleus. Comparative studies with other well-known naturally occurring antioxidants show that melatonin's efficacy is equal to or better in neutralizing highly toxic oxygen and nitrogen-reactants. However, not only melatonin but a series of its metabolites are also capable of detoxifying free radicals and related species in what is referred to as the antioxidative cascade. Thus, melatonin may be actually seen as a prodrug for a family of other molecules that also have the capability of reducing oxidative/nitrosative stress. Taken together, the results reviewed here indicate that melatonin is a key element in antioxidative medicine in the context of the antioxidative defense system.

Keywords

Antioxidant, free radical, lipid peroxidation, melatonin, oxidative stress.

Received 06.12.2010
Accepted 09.12.2010

Introduction

Melatonin is a tryptophan derivative found throughout the animal kingdom from unicellular organisms to humans (Reiter, 1991a, Hardeland and Poeggeler, 2003). It is also present in plants (Dubbels et al., 1995, Hattori et al., 1995, Kolar and Machackova, 2005, Reiter et al., 2007, Paredes et al., 2009). The nocturnal secretion of melatonin by the pineal gland, a pine cone-shaped structure attached to the posterior wall of the third ventricle in the vertebrate brain, serves as the chemical expression of darkness (Reiter 1991b). Using the fluctuating endogenous melatonin signals, vertebrates synchronize both their circadian rhythms and their circannual reproductive activities (Reiter et al., 2009a).

Free radicals are molecules with an unpaired electron in their valence orbital. These damaging agents include both oxygen-based (reactive oxygen species or ROS) and nitrogen-based reactants (reactive nitrogen species or RNS). The resulting tissue destruction is referred to as oxidative or nitrosative stress, respectively (Reiter et al., 2008a). The most damaging ROS is the hydroxyl radical (•OH) while the most destructive RNS is the peroxynitrite anion ($ONOO^-$) (Stankovska et al., 2006). These molecules together with their related oxygen and nitrogen-based oxidizing agents persistently pulverize and damage molecules in the vicinity of where they are formed. Apart from its daily and seasonal signaling role, melatonin is also a potent free radical scavenger and broad-spectrum antioxidant (Tan et al., 1993a, Tan et al., 2002). This highly novel concept was suggested for the first time in the early 1990s when two reports yielded data that suggested that melatonin may scavenge free radicals (Chen et al., 1993, Tan et al., 1993a). Tan et al., (1993b) tested this then new attribution to the indoleamine; the data, in fact, revealed that melatonin is a highly efficient scavenger of the destructive •OH. This seminal observation has been repeatedly confirmed in pure chemical systems in both *in vitro* and *in vivo* studies (Matuszak et al., 1997, Bromme et al., 2000, Li et al., 2002, Sofic et al., 2005, Valko et al., 2005, Fukutomi et al., 2006). Subsequent investigations have documented that this indoleamine also neutralizes other reactive oxygen and nitrogen-based reactants (Gilad et al., 1997, Zhang et al., 1998, Ceraulo et al., 1999, Noda et al., 1999, Blanchard et al., 2000, Tan et al., 2000, Reiter et al., 2001, Turjanski et al., 2001, Tan et al., 2002, Reiter et al., 2003, Rosen et al., 2006, Reiter et al., 2008a).

Melatonin also has receptor-mediated actions which contribute to the capability of this molecule in eradicating radicals and reducing oxidative stress (Reiter et al., 2000, Rodriguez et al., 2004, Tomas-Zapico and Coto-Montes, 2005). Thus, melatonin stimulates a number of antioxidative enzymes which metabolize reactive products to innocuous agents. The enzymes whose activities have been shown to be upregulated by melatonin include both Cu/Zn and Mn superoxide dismutases (SOD), glutathione peroxidase (GPx) and glutathione reductase (GRd) (Barlow-Walden et al., 1995, Pablos et al., 1995, Rodriguez et al., 2004). Signaling effects of melatonin also include the down-regulation of prooxidant enzymes, including nitric oxide synthase (NOS) and lipoxygenase (Hardeland, 2005).

Here, we discuss several important issues related to the antioxidant actions of melatonin. A concise summary of the investigations that have been performed in relation to the beneficial effects of the indoleamine in disease states which reportedly involve free radicals and oxidative stress are presented. Hopefully, this review will contribute to the consideration of this molecule as an antioxidant protective drug for mainstream antioxidative medicine.

Melatonin as a promising key element in antioxidative medicine

Due to its low toxicity and high efficacy, melatonin has repeatedly been used in clinical trials to treat conditions that are associated with elevated free radical damage, including septic shock (Gitto et al., 2001), certain neurodegenerative conditions (Pappolla et al., 2003, Ishido, 2007), ischemia/reperfusion injury (Reiter et al., 2005), ionizing radiation (Vijayalaxmi et al., 2004, Manda et al., 2007), and toxin (Matsura et al., 2006, Xu et al., 2007) and heavy metal (Qi et al., 2000, El-Sokkary et al., 2003) exposure, among others. In the following sections, some of the abundant literature on the protective actions of melatonin against various disease states and disorders related to the exacerbated generation of ROS and RNS are described.

Melatonin and Alzheimer's disease

Alzheimer's disease is the largest unmet medical need in neurology. Current drugs improve symptoms, but do not have profound disease-modifying effects. However, in recent years, several approaches aimed at inhibiting disease progression have advanced to clinical trials. Among these, strategies targeting the production and clearance of the amyloid-β peptide - a cardinal feature of Alzheimer's disease that is thought to be important in disease pathogenesis - are the most advanced. Approaches aimed at modulating the abnormal

aggregation of tau filaments (another key feature of the disease), and those targeting metabolic dysfunction, are also being evaluated in the clinic (Citron, 2010).

The development of β-amyloid-based senile plaques in the vicinity of neurons and the generation of intracellular neurofibrillary tangles are malformations related to free radicals. β-amyloid deposits generate ROS that lead to the oxidation of essential macromolecules in neurons eventually causing them to undergo apoptosis. Neurofibrillary tangles are formed when the cytoskeletal protein, tau, is phosphorylated. The tangles also induce free radical generation that compromises the function of neurons (Reiter et al., 2008a).

The toxicity of amyloid-β has been repeatedly shown to be reduced by melatonin (Pappolla et al., 2000). Similarly, the hyperphosphorylation of tau is ameliorated when melatonin is present (Yin et al., 2006); melatonin arrests tau metabolism by inhibiting one of the enzymes that causes its phosphorylation, i.e., glycogen synthase kinase-3 (Deng et al., 2005). Additionally, in mice transfected with the human amyloid precursor protein gene, melatonin limits the accumulation of amyloid-β in the brain and forestalls the death of the animals (Matsubara et al., 2003). In humans, melatonin has been shown to reduce mild cognitive impairment in elderly subjects suffering with Alzheimer's disease (Furio et al., 2007).

Melatonin and Parkinson's disease

Parkinson's disease is among the most common neurodegenerative disorders. It is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the midbrain and a subsequent loss of dopamine. It is clinically manifested by defective motor function, a decline in cognitive function, and depression. Histologically, its features include the presence of Lewy bodies and cytoplasmic inclusions that are composed predominantly of fibrillar-synuclein (Spillantini et al., 1998). In addition to a few specific mutations, oxidative stress and generation of free radicals from both mitochondrial impairment and dopamine metabolism are considered to play critical roles in Parkinson's disease etiology. Thus, most biochemical studies suggest that, directly or indirectly, ROS and RNS are important mediators in the pathogenesis of Parkinson's disease. In this regard and taking into account the powerful antioxidant properties of melatonin, the treatment with the indoleamine has proven successful in both *in vivo* and *in vitro* models of the disease (Mayo et al., 2005a). Of particular importance are the actions of melatonin against toxins proven useful in inducing Parkinson-like neural damage in experimental models including those caused by rotenone (Coulom and Birman, 2004, Saravanan et al., 2005, Saravanan et al., 2007), 6-hydroxydopamine (Mayo et al., 1998), glutamate excitotoxicity (Herrera et al., 2001, Cheng et al., 2008), and especially 1-phenyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Reiter et al., 2008b). In each of these studies, it was presumed that the antioxidative actions prevented or limited the severity of experimental Parkinsonism.

MPTP has been widely used to induce experimental Parkinson's disease. This toxin readily crosses the blood-brain barrier, is taken up by astrocytes where it is metabolized to the 1-methyl-4-phenyl pyridinium ion (MPP^+) by monoamine oxidase B, after which it is released and taken into dopaminergic cells via the dopamine transporter. Inside the dopaminergic neurons it concentrates in the mitochondria and poisons complex I leading to excessive free radical production and apoptosis of the cells. After loss of the dopaminergic neurons, which are concentrated in the substantia nigra, the signs of Parkinson's disease appear. The treatment with melatonin has been reportedly shown to counteract MPTP toxicity. In an attempt to suppress the neural toxicity of the toxin, MPTP-injected mice were sacrificed four hours after the challenge and their brains collected; the striatum and the hippocampus showed increased levels of lipid peroxidation (Acuna-Castroviejo et al., 1997). These increments were not observed in the mice treated with melatonin. More importantly, immunocytochemically-detected tyrosine hydroxylase activity in the dopaminergic nerve terminals in the striatum was lost as a result of the injection of MPTP; again, this was prevented by melatonin. Although this study did not observe changes in the substantia nigra (likely due to the brief treatment period), the findings are consistent with a protective action of melatonin at the level of nigral dopaminergic neurons.

To promote a gradual reduction in the loss of dopamine-containing neurons in the substantia nigra, Antolin et al., (2002) treated mice with a low daily dose of MPTP for 35 days; in half of the animals each injection of the complex I inhibitor was preceded by co-administration of melatonin. MPTP by itself caused a dramatic reduction in the number of Nissl-stained neurons and immunoreactive tyrosine hydroxylase-positive cells in the substantia nigra. In mice given melatonin in conjunction with MPTP, the measured parameters were essentially indistinguishable from those of the control mice. These findings, along with others published soon thereafter (Chen et al., 2002, Khaldy et al., 2003) strongly support the idea that melatonin protects

against mitochondrial complex I dysregulation induced by MPTP.

That melatonin does not modify the conversion of MPTP to MPP^+ was shown by the observation that the indoleamine did not alter the level of monoamine oxidase B (Thomas and Mohanakumar, 2004); this enzyme is responsible for the metabolism of MPTP to MPP^+. The most direct evidence that melatonin prevents the toxicity of MPTP/MPP^+ at the level of the mitochondria was provided by Chen et al., (2005). This group measured mitochondrial DNA (mtDNA) damage as a result of MPTP administration to mice. They observed a rapid increase in immunoreactive 8-hydroxy-2-deoxyguanosine, a reliable marker of DNA damage, in mtDNA of the substantia nigra. Melatonin pre-injection, in a dose-response manner, reduced the damage. Likewise, using cultured SH-SY5Y cells Chen et al., (2005) also found melatonin was protective of mtDNA against MPP^+ toxicity and, moreover, they observed that MPP^+ time-dependently elevated mitochondrial free radical generation and a reduction in the mitochondrial membrane potential over a 24 hour period. Furthermore, 72 hours after MPP+ exposure, 49% of the cells had undergone apoptosis. When cells were co-incubated with a combination of MPP^+ and melatonin, however, mitochondrial free radical generation was reduced, mitochondrial membrane potential did not collapse and cellular apoptosis was averted.

Protection of melatonin against ischemia/reperfusion injury

Anoxia occurs during ischemia; during reperfusion oxygenated blood flows into tissue when the previously obstructed vessel is reopened. These two events result in large-scale free radical-mediated damage and leaves enormous amounts of molecular debris in its wake. A major portion of the mutilation is a result of free radicals generated during these processes. Organs in which this damage is of greatest concern include the myocardium (as in a heart attack) and in the brain (as a stroke, or brain attack) because of the high morbidity and mortality associated with interruption of the blood supply to these organs.

Experimentally, the administration of melatonin has been shown to highly significantly reduce tissue damage and abnormal physiology resulting from ischemia/reperfusion both in the heart (Tengattini et al., 2008) and in the brain (Reiter et al., 2005), including the spinal cord (Nesic et al., 2008, Samantaray et al., 2008). In relation to the heart, melatonin has been widely tested for its ability to abrogate cardiomyocyte malfunction and tissue loss during an episode of ischemia/reperfusion (Reiter et al., 2010). Altogether, the studies related to the ability of melatonin to limit cardiac damage and restore function of the ischemic/reperfused heart support the notion that melatonin has significant cardioprotective actions. Melatonin treatment has been shown to greatly improve the hemodynamic parameters (coronary, aortic, and cardiac output and heart rate) and reduced post-ischemic arrhythmias during the reperfusion period in rat hearts rendered globally ischemic for 30 minutes (Dobsak et al., 2003). In ischemic hearts not treated with melatonin, all developed fatal ventricular fibrillation. Moreover, when heart tissues were compared for the incidence of apoptotic cells (TUNEL assay), melatonin had markedly reduced cardiomyocyte death. Melatonin in a dose-dependent manner also protected the heart against peroxyl radical damage and reduced lipid peroxidation. The beneficial effects of melatonin in this ischemia/reperfusion model were primarily attributed to its potent anti-oxidant activities. Oxidative stress is considered a major damaging agent in cardiac diseases (Lefer and Granger, 2000).

The ability of melatonin to modulate mitochondrial function during anoxia and reperfusion has been thoroughly examined (Petrosillo et al., 2006, 2009, Paradies et al., 2010) due to the fact that free radicals are operative in ischemia/reperfusion injury to the heart and mitochondria are a major source of reactive oxygen species. During reperfusion especially bio-energetic parameters in heart mitochondria including oxygen consumption, activities of complexes I and III, H_2O_2 production, as well as the degree of lipid peroxidation, cardiolipin content, and cardiolipin oxidation were negatively influenced (Jou et al., 2010, Paradies et al., 2010). Each of these alterations was significantly rectified by the infusion of melatonin. The protective actions of melatonin at the mitochondrial level improved the post-ischemic hemodynamic function of the heart as well. Melatonin diffuses into cells easily and is known to scavenge radicals at the mitochondrial level (Jou et al., 2007, Reiter et al., 2008b). In relation to the ability of melatonin to ameliorate mitochondrial malfunction in the ischemic/reperfused isolated rat heart, the action of the indole in preventing mitochondrial permeability transition pore opening has been tested. Opening of the mitochondrial permeability transition pore is considered a major event in the sequence of changes leading to cell death of cardiomyocytes. Melatonin has been found to significantly improve functional recovery and, in the most recent paper, to limit infarct volume and reduce

necrotic cell death as evidenced by lower levels of lactate dehydrogenase in the perfusate. In addition, melatonin is also able to limit NAD release, cytochrome c escape from mitochondria, and oxidation of cardiolipin, all of which are normally associated with ischemia/reperfusion (Petrosillo et al., 2009, Paradies et al., 2010). These studies suggest that the use of melatonin may be a beneficial strategy for the treatment of cardiac reperfusion injury as well as other cardiovascular disorders in which free radicals are involved.

Protection of melatonin against ionizing radiation

Clinical, experimental or accidental exposures to ionizing radiation are classic means that result in the generation of free radicals within cells and tissues. Because of this, radiation is used to kill cancer cells; however, at the same time, normal cells in the path of the radiation are also damaged. Additionally, there are some situations where individuals may be accidentally exposed to toxic or even lethal levels of ionizing radiation. Due to its very low toxicity over a wide range of doses, melatonin may be a highly effective protector against molecular damage due to ionizing radiation exposure. Many studies point to this. In one of them, human lymphocytes were exposed for 20 minutes to 150 cGy gamma radiation. The treatment with melatonin limited the number of abnormal cells expressing genetic damage, i.e. exchange type of aberrations, eccentric fragments and the formation of micronuclei which are usual consequences of high energy radiation exposure (Vijayalaxmi et al., 1995a, 1995b). In general, the abnormal changes were reduced by an estimated 60–65% when melatonin was used as a radioprotective agent. In another *in vivo/in vitro* study, half of a group of adult humans was given melatonin orally, after which a blood sample was collected; lymphocytes were harvested and then exposed to 150 cGy gamma radiation (Vijayalaxmi et al., 1995b). Moreover, serum concentrations of melatonin were measured in both groups of subjects. Those who had received melatonin had much higher circulating levels of the indoleamine; this correlated with reduced levels of lymphocytic DNA damage as estimated by the lower numbers of chromosomal aberrations and micronuclei.

It has been repeatedly observed that cells lining the intestinal cysts are readily destroyed by ionizing radiation. This leads to sloughing of the gastroendothelial lining cells of the gut which causes severe diarrhea and possibly mortality. Monobe et al., (2005) showed that orally administered melatonin protected against intestinal damage following the exposure of male mice to doses of radiation (CS^{137} gamma-rays; 0.98 Gy min^{-1}) ranging from 7 to 21 Gy. The doses of melatonin used ranged from 1 to 20 mg kg^{-1} with the degree of protection of the epithelial lining cells positively correlating with the dose.

Melatonin also protects DNA from ionizing radiation damage. Pre-treating rats intraperitoneally with the indoleamine (50 mg kg^{-1}) counteracted the elevation of hepatic 8-hydroxy-2-deoxyguanosine levels observed after whole body radiation (800 cGy). Likewise, lipid peroxidation products were also depressed in the liver due to melatonin administration and the fluidity of microsomal membranes was preserved; membranes become rigid when their intrinsic polyunsaturated fatty acids are oxidized (Karbownik et al., 2000). Importantly, melatonin also preserves hippocampal neurogenesis and cognitive functions in rats exposed to ionizing radiation (Manda et al., 2007, Manda and Reiter. 2010).

Protection of melatonin against herbicide and metal toxicity

The bipyridyl herbicides, paraquat and diaquat, are notorious for their toxicity to plants where they are involved in redox cycling (Reiter et al., 2008c). Paraquat is especially toxic to the lungs since several respiratory cells take up this agent against a concentration gradient. Also, the lung has a high concentration of oxygen lending itself to elevated oxidative stress. For diquat, lung is not a major target but rather the intestine and liver suffer the greatest damage.

Melatonin is able to suppress the oxidative damage provoked by both herbicides. Particularly, melatonin co-administered with a high dose of paraquat (5 mg/kg and 50 mg/kg, respectively) abolished the elevation in the levels of lipid peroxidation products (malondialdehyde and 4-hydroxyalkenals) in the serum and lungs of rats observed when paraquat was administered alone (Melchiorri et al., 1995, 1996). Similarly, melatonin reversed loss of reduced glutathione concentrations. Finally, melatonin co-treatment with paraquat raised the LD50 of the rats from 75 mg/kg to 251 mg/kg paraquat.

Similar observations were made when diquat was used. Xu et al., (2007) reported that 50 mg/kg diquat severely damaged the liver and kidneys of mice as evidenced by the rise in hepatic and renal F2-isoprostance levels and serum concentrations of alanine aminotransferase, an enzyme which escapes from damaged hepatic cells into the serum. Melatonin (20 mg/kg), given 30 min in advance of diquat treatment, counteracted each of the changes measured after diquat-only treatment. Moreover, melatonin reduced the acute 24 hour death rate in diquat-treated mice from 91% to 57%.

The pathologies associated with iron [e.g., hemachromatosis or copper (e.g., Wilson's disease] overload are well known. As a result of unusually high levels of these metals, free radicals are generated in excess and individuals with these conditions exhibit elevated lipid peroxidation endproducts and protein carbonyls while conventional antioxidants, e.g., vitamins C and E, are typically depressed in these subjects. Thus, oxidative/nitrosative stress is considered a contributory factor to these diseases.

Melatonin has been found to be protective in terms of reducing oxidized products generated by aluminum, iron, titanium, arsenic, lead, vanadium, cadmium, mercury, molybdenum, chromium, uranium, nickel, copper, and cobalt (Reiter et al., 2008c). In most cases the number of publications on each of the metals in relation to melatonin is small and the endpoints few (Qi et al., 2000, El-Sokkary et al., 2003, Alonso-Gonzalez et al., 2007, Belles et al., 2007, Flora et al., 2007, Lin et al., 2007). Uniformily, however, the findings are consistent with a reduction of metal-promoted free radical damage when melatonin is present. Melatonin also reportedly binds several of the afore-mentioned metals which would reduce their ability to participate in reactions that generate free radicals (Limson et al., 1998).

Comparative efficacy of melatonin vs. vitamin E and other antioxidants

Melatonin has been most frequently compared with vitamin E in terms of its relative efficacy in protecting against free radicals and the accompanying molecular damage (Reiter et al., 2009b). Both melatonin and vitamin E are highly lipid-soluble molecules and, as a result, they would be expected to be especially effective antioxidants in the lipid-rich portions of cells. In fact, in most of the comparative studies performed, melatonin was equal to or better than vitamin E in curtailing the breakdown of lipids. Some examples of these comparisons were in relation to the cardiotoxicity of doxorubicin (Wahab et al., 2000), cholestasis induced by extra hepatic duct ligation (Montilla et al., 2001), erythrocyte toxicity mediated by chlorpyrifos-ethyl (Gultekin et al., 2001), hepatic damage resulting from ethanol administration (Mansouri et al., 2001), tissue damage due to phenylketonuria (Martinez-

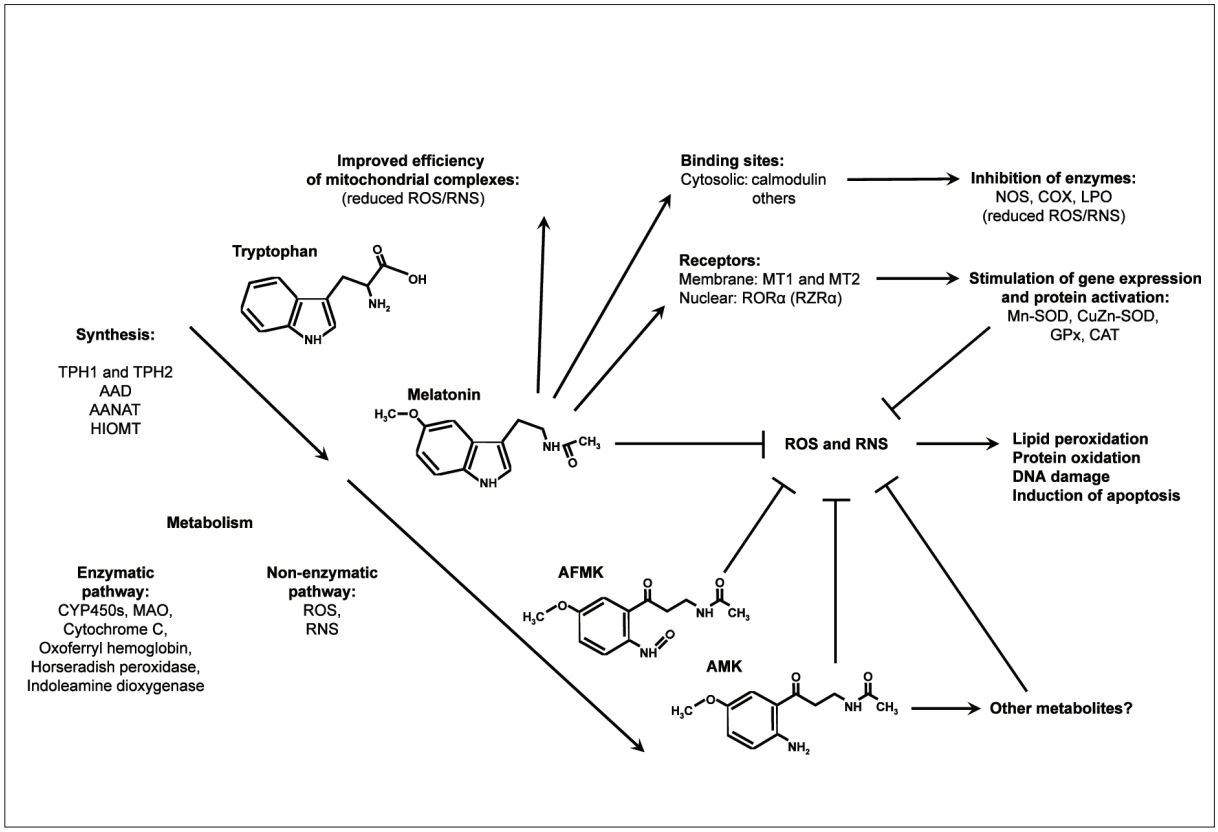

Figure 1. Melatonin and its metabolites detoxify free radicals. The amino acid tryptophan is the precursor of melatonin. Melatonin scavenges the •OH to generate cyclic 3-hydroxymelatonin; this latter molecule also functions as a scavenger and produces N^1-acetyl-N^2-formyl-5-methyoxykynuramine (AFMK). AFMK scavenges radicals to generate N1-acetyl-5- methoxykynuramine (AMF). Melatonin can also be converted to AMFK directly either enzymatically or via radical scavenging. This sequence of reactions is referred to as melatonin's antioxidative cascade (Tan et al., 2002).

Cruz et al., 2002), streptozotocin-induced diabetes (Baydas et al., 2002a), basal levels of lipid peroxidation (Baydas et al., 2002b), retinal ischemia-reperfusion injury (Yilmaz et al., 2002), brain lipoperoxides induced by amyloid-β peptide (Rosales-Corral et al., 2003), neural and hepatic lipid peroxidation and changes in GSH levels mediated by thioacetamide (Tunez et al., 2007), caerulein-induced pancreatic and hepatic oxidative damage (Esrefoglu et al., 2006), malondialdehyde levels and alterations in antioxidative enzymes resulting from exposure to 720 cGy ionizing radiation (Yilmaz and Yilmaz 2006), mitochondrial damage induced by fetal hyperphenylalaninemia (Martinez-Cruz et al., 2006), etc.

Melatonin has been also compared with a number of other molecules with antioxidant properties, including vitamin C, mannitol, glutathione, N-acetylcysteine, 2-lipoic acid, xanthurinic acid, reseveratrol, NADH, NADHP, etc. (Reiter et al., 2009b). For example, Cadenas and Barja (1999) attempted to reduce the destruction of DNA caused by potassium bromate ($KBrO_3$), an oxidizing agent commonly used as a food additive, by giving a variety of antioxidants (melatonin, resveratrol, vitamin E, butylated hydroxytolvene (BHT) and 2-mercaptoethylamine) or a spin trapping agent, α-phenyl-N-tert-butyl nitrone (PBN). When $KBrO_3$ is given orally or when it is injected intraperitoneally into rats, elevated levels of the DNA damaged product 8-hydroxydeoxyguanosine (8-OHdG) as well as byproducts of lipid peroxidation are found in the kidney (Kurokawa et al., 1983, Sai et al., 1991). It is thought that the metabolism of this additive results in the generation of free radicals, causing the observed renal damage. With the exception of 2-mercaptoethylamine, and considering only the mean renal 8-OHdG, all agents roughly equally reduced damage to kidney DNA induced by $KBrO_3$, although statistical analysis suggested resveratrol provided the best protection and it was somewhat better than that provided by melatonin. In this case, however, resveratrol had been given at a dose of 16 mg kg^{-1} BW for 7 days (112 mg kg^{-1} total) while the total melatonin dose was 48 mg kg^{-1} (four doses of 12 mg kg^{-1} each over a 24 hour period); thus, the claim that resveratrol was the most potent antioxidant may be premature. Melatonin was also compared to glutathione, ascorbic acid and vitamin E (trolox, water soluble vitamin E) in cell-free *in vitro* experiments, resulting in the most potent •OH scavenger of the four (Sofic et al., 2005).

It has been known for almost two decades that oxidative stress is a major contributor to the development of the systemic complications of malaria (Clark et al., 1989, Siddiqi and Pandey, 1999). In fact, in the liver of *Plasmodium*-infected mice, elevated levels of the activity of xanthine oxidase, a free-radical generating enzyme, and lipid peroxides are known to occur (Guha et al., 2006, Dey et al., 2009). Based on these findings, it was surmised that melatonin may be useful to reduce the obvious oxidative damage that occurs during malarial infections (Guha et al., 2007). Once parasitemia was established, infected mice were treated with either melatonin, vitamin E or vitamin C. Melatonin, in a dose-response manner scavenged •OH generated in the liver and markedly reduced lipid hydroperoxides and protein carbonyl that were a consequence of the malarial infection while elevating hepatic glutathione concentrations. The effective dose of melatonin required to achieve these beneficial changes was roughly 20-fold lower than those of either vitamin C or E. Moreover, melatonin provided hepatoprotection by almost completely suppressing the mitochondrial apoptosis pathway including restoration of the mitochondrial membrane potential, preventing caspase 3 activation, limiting the over-expression of Bax, preventing the down regulation of Bcl-2, and reducing DNA fragmentation and apoptosis (evaluated using the TUNEL assay). Considering the marked protective effects of melatonin on all aspects of liver function during malarial infection, Guha et al., (2007) suggest that melatonin, in preference to either vitamin C or vitamin E, may well be the most effective agent to combat free radical-mediated molecular mutilation resulting from a malarial infection.

The free radical scavenging cascade

The rapid drop in circulating melatonin under the conditions of excessive stress previously described in this review may be considered a protective mechanism for organisms against highly damaging oxidants. Thus, the reduction in melatonin occurs presumably because it is being used to scavenge free radicals. In this sense, melatonin can be categorized as a first line of defensive molecule. However, melatonin's interaction with ROS/RNS is a prolonged process that also involves many of its derivatives. The process by which melatonin and its metabolites successively scavenge ROS/RNS is referred as the free radical scavenging cascade (Figure 1) (Tan et al., 2002). This cascade reaction is a novel property of melatonin and explains how it differs from other conventional antioxidants. This cascade reaction makes melatonin highly effective, even at low concentrations, in protecting organisms from oxidative stress (Tan et al., 2007).

Participating kynuric metabolites of melatonin in the free radical scavenging cascade include the molecules N^1-

acetyl-N^2-formyl-5-methoxykynuramine and N^1-acetyl-5-methoxykynuramine; these are currently also identified by the acronyms AFMK and AMK. The perception of these kynuramines to be potentially important molecules started when they were discovered to represent major brain metabolites (Hirata et al., 1974). Subsequently, AFMK and AMK were also found to be metabolites of melatonin (Tan et al., 2001, Hardeland et al., 2009). Prior to that discovery, melatonin was believed to be almost exclusively metabolized to 6-hydroxymelatonin and its excretion product, 6-sulfatoxymelatonin. After the discovery of Hirata et al., it required more than three additional decades before the relative importance of AFMK and AMK was revealed. Thus, it was not until the wide distribution of tissue melatonin was uncovered and the potent radical scavenging capacity of this 5-methoxylated indolamine was discovered (Reiter et al., 1993, Tan et al., 1993a, Poeggeler et al., 1996) that interest in these compounds was augmented considerably. Presently, along with the increasing awareness of the importance of melatonin, AFMK and AMK significance has come into focus due to their melatonin-like protective actions, i.e., they reduce the damage provoked by oxidative/nitrosative stress as well as upregulating antioxidant enzymes and downregulating pro-oxidative and proinflammatory enzymes (Leon et al., 2006, Hardeland et al., 2009).

AFMK protects DNA (Burkhardt et al., 2001, Tan et al., 2001) and lipids (Tan et al., 2001) from attack by the •OH. This is not surprising considering the high reactivity of this oxygen derivative. In the case of DNA protection, the efficacy of AFMK is roughly one-fifth that of melatonin (Tan et al., 2001). However, AFMK turned out to be much more resistant to other oxidants, such as carbonate radicals (Hardeland et al., 2004, Hardeland, 2005), protoporphyrin IX cation radicals (Hardeland et al., 2004), quinoxaline-2-oxyl radicals (Behrends et al., 2007), and also singlet oxygen (Schaefer and Hardeland, 2009), whereas all these agents efficiently destroyed AMK. The lower reactivity of AFMK towards free radicals is associated with the preference of this molecule for two-electron transfer reactions (Hardeland, 2005), as demonstrated by cyclic voltammetry (Tan et al., 2001). As radical reactions represent single electron transfer reactions, potent radical scavengers exhibit a preference for one-electron exchange (Hardeland, 2005), as shown for melatonin by cyclic voltammetry (Tan et al., 2002); this is evident for AMK based on its efficient interaction with the ABTS cation radical (Ressmeyer et al., 2003), a radical species of comparably low reactivity.

In terms of scavenging ROS and preventing protein oxidation, AMK has greater efficiency than does its precursor, AFMK (Ressmeyer et al., 2003). In addition to its direct antioxidant capacities, AMK effectively inhibits neuronal nitric oxide synthase activity and reduces intracellular NO levels (Entrena et al., 2005, Leon et al., 2006). Its ability to reduce NO formation has been referred to as free radical avoidance activity (Hardeland, 2005). AMK, like its precursor melatonin, promotes mitochondrial complex I activity to elevate ATP production by lowering electron leakage and inhibiting opening of the mitochondrial permeability transition pore (Acuna-Castroviejo et al., 2003, Hardeland, 2005).

The anti-inflammatory and immunoregulatory activities of AFMK and AMK have also attracted significant attention. AMK was observed to inhibit the biosynthesis of prostaglandins (Kelly et al., 1984). AFMK (0.001^{-1} mM) inhibits tumor necrosis factor-alpha and interleukin-8 formation caused by lipopolysaccharide in neutrophils and peripheral blood mononuclear cells. The formation of AFMK during melatonin oxidation was speculated to be an important event in the cross-talk between neutrophils and monocytes (Silva et al., 2005). A mechanistic study has revealed that AFMK and AMK selectively inhibit gene expression of a proinflammatory enzyme, cyclo-oxygenase 2 (COX-2) (Mayo et al., 2005b). AFMK also regulates the cell cycle of malaria parasites as does its precursor melatonin (Budu et al., 2007).

The antioxidative actions of AFMK and AMK summarized here indicate that some functions of melatonin may be mediated or amplified by its metabolites. A major gap in the understanding of the functions of AFMK and AMK, however, is the lack of information on the molecular mechanisms of action, as far as they exceed the purely chemical reactions with free radicals and other aromates. Investigators should not only study the interference of kynuramines with receptors for other ligands, but also identify binding sites of possible physiological relevance. In particular, a search for AFMK and AMK receptors might be worth the effort (Hardeland et al., 2009).

Concluding remarks

Since melatonin was discovered to be an indirect antioxidant and direct efficient free radical scavenger, its ability to reduce oxidative stress has been repeatedly documented. It has also been shown that part of melatonin's ability to quell the oxidation of key molecules stems from its conversion to metabolites, i.e., AFMK and AMK, when it incapacitates free radicals and their related products. This review has considered briefly a few of the

many reports related to the role of melatonin in protecting against free radical-generating molecules. The literature in this area is massive despite the fact that melatonin has only been known to be an antioxidant for roughly 17 years.

In general, melatonin functions in all parts of all cells and improves physiological infrastructure. As a result, it enhances cell function and optimizes the ability of cells to survive in hostile environments as well as help them cope with the oxidative disaster that characterizes many disorders and diseases. Considering its broad spectrum of antioxidative actions and its remarkable lack of side effects, it is an ideal candidate to be considered in mainstream antioxidative medicine.

Acknowledgements

This work was supported by a research grant from Consejería de Economía, Comercio e Innovación (Junta de Extremadura, PRI07A024). S.D. Paredes was beneficiary of a grant by Junta de Extremadura-Fondo Social Europeo (REI09009).

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