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Review

Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans

Russel J. Reiter[1✉], Dun-xian Tan[1], Juan C. Mayo[1], Rosa M. Sainz, Josefa Leon[1] and Zbigniew Czarnocki[2]

[1]*Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas, U.S.A.;* [2]*Department of Chemistry, Warsaw University, Warszawa, Poland*

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This brief resume enumerates the multiple actions of melatonin as an antioxidant. This indoleamine is produced in the vertebrate pineal gland, the retina and possibly some other organs. Additionally, however, it is found in invertebrates, bacteria, unicellular organisms as well as in plants, all of which do not have a pineal gland. Melatonin's functions as an antioxidant include: a), direct free radical scavenging, b), stimulation of antioxidative enzymes, c), increasing the efficiency of mitochondrial oxidative phosphorylation and reducing electron leakage (thereby lowering free radical generation), and 3), augmenting the efficiency of other antioxidants. There may be other functions of melatonin, yet undiscovered, which enhance its ability to protect against molecular damage by oxygen and nitrogen-based toxic reactants. Numerous *in vitro* and *in vivo* studies have documented the ability of both physiological and pharmacological concentrations to melatonin to protect against free radical destruction. Furthermore, clinical tests utilizing melatonin have proven highly successful; because of the positive outcomes of these studies, melatonin's use in disease states and processes where free radical damage is involved should be increased.

✉Corresponding author: Russel J. Reiter, Ph.D., Department of Cellular and Structural Biology, Mail Code 7762, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900 U.S.A.; phone: 210/567-3859; fax: 210/567-6948; e-mail: Reiter@UTHSCSA.EDU

Abbreviations: AD, Alzheimer's disease; AFMK, N^1-acetyl-N^2-formyl-5-methoxykynuramine; CAT, catalase; DMPO, 5,5-dimethylpyrolidine oxide; ETC, electron transport chain; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GRd, glutathione reductase; LOO, peroxyl radical; MPTP, 1-methyl-4-phenylpyridinium; RDS, respiratory distress syndrome; RNS, reactive nitrogen species; ROS, recative oxygen species; SOD, superoxide dismutase; t-BHP, *t*-butyl-hydroperoxide.

N-Acetyl-5-methoxytryptamine, commonly known as melatonin (Fig. 1), is a synthetic product of the vertebrate pineal gland as well as of other select organs. The biochemical pathway concerned with the synthesis of melatonin has been well described as have been the neural mechanisms governing pineal melatonin production (Reiter, 1991). The indoleamine was initially found to function as a mediator of circannual reproductive rhythms (Reiter, 1980) as well as of circadian cycles (Kennaway & Wright, 2002). Subsequently, however, melatonin was shown to have significantly broader actions including oncostatic effects (Blask *et al.,* 2002), immune system stimulation (Guerrero & Reiter, 2002) and anti-inflammatory functions (Cuzzocrea & Reiter, 2002).

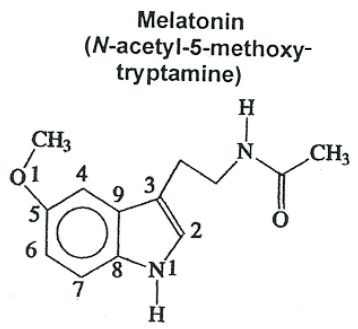

Figure 1. Molecular structure of the antioxidant melatonin.

This molecule was discovered to be a direct free radical scavenger roughly a decade ago and subsequently there has been a vast amount of research documenting its potent and diverse antioxidant capabilities.

Even more recently, and somewhat unexpectedly, melatonin was identified as a powerful direct free radical scavenger (Tan *et al.*, 2002) and indirect antioxidant (Reiter *et al.*, 2000c; Rodriquez *et al.*, 2004). What seems particularly unusual is the high efficacy of melatonin as a protector against reactive oxygen (ROS) and reactive nitrogen species (RNS). This field of research has witnessed an explosive expansion in the last decade and whereas all of the mechanisms of melatonin's effects as an executioner of free radicals and related products have not yet been identified, there is no doubt concerning its ability to restrain the molecular damage resulting from toxic oxygen and nitrogen-based reactants (Acuña-Castroviejo *et al.*, 2002; Reiter *et al.*, 2002b). This review summarizes some of the mechanisms of melatonin's protective actions as well as documents that it significantly reduces oxidative stress at many levels. It is noted, however, that this brief resume cannot do justice to the massive number of reports that have been published on these subjects and the reader is urged to consult other reviews for additional details and information (Hardeland *et al.*, 1995; Reiter *et al.*, 2000b, 2001; Tan *et al.*, 2002; 2003b).

DIRECT ANTIOXIDANT ACTIONS OF MELATONIN

Melatonin seems to function *via* a number of means to reduce oxidative stress. Thus, the experimental evidence supports its actions as a direct free radical scavenger (Hardeland *et al.*, 1993; 1995; Allegra *et al.*, 2003), as an indirect antioxidant when stimulating antioxidant enzymes (Reiter *et al.*, 2000c; Rodriquez *et al.*, 2004), its stimulation of the synthesis of glutathione (an essential intracellular antioxidant) (Urata *et al.*, 1999), its ability to augment the activities of other antioxidants (or *vice versa*) (Gitto *et al.*, 2001a), its protection of antioxidative enzymes from oxidative damage (Mayo *et al.*, 2002; 2003), and its ability to increase the efficiency of mitochondrial electron transport chain (ETC) thereby lowering electron leakage and reducing free radical generation (Acuña-Castroviejo *et al.*, 2002; Okatani *et al.*, 2003a). While melatonin has proven highly effective in lowering molecular damage under conditions of elevated oxidative stress (Reiter, 1998; Reiter & Tan, 2003), the contribution of each of the above-mentioned processes to the ability of this indole to restrain the resulting molecular mutilation that accompanies exag-

gerated free radical generation remains unknown.

Melatonin as a direct scavenger of oxygen-based free radicals and related species

There is now a vast literature documenting melatonin's interaction with both ROS and RNS (Reiter *et al.*, 2001; Poeggeler *et al.*, 1997; Bandyopadhyay *et al.*, 2002; Brömme *et al.*, 2002; Li *et al.*, 2002) and, furthermore, a potential product of that interaction has been identified to be cyclic 3-hydroxymelatonin (Tan *et al.*, 1998). In the proposed scheme, each molecule of melatonin scavenges two ·OH; this study also showed that cyclic 3-hydroxymelatonin is excreted in the urine (human and rat) and the quantity of this by-

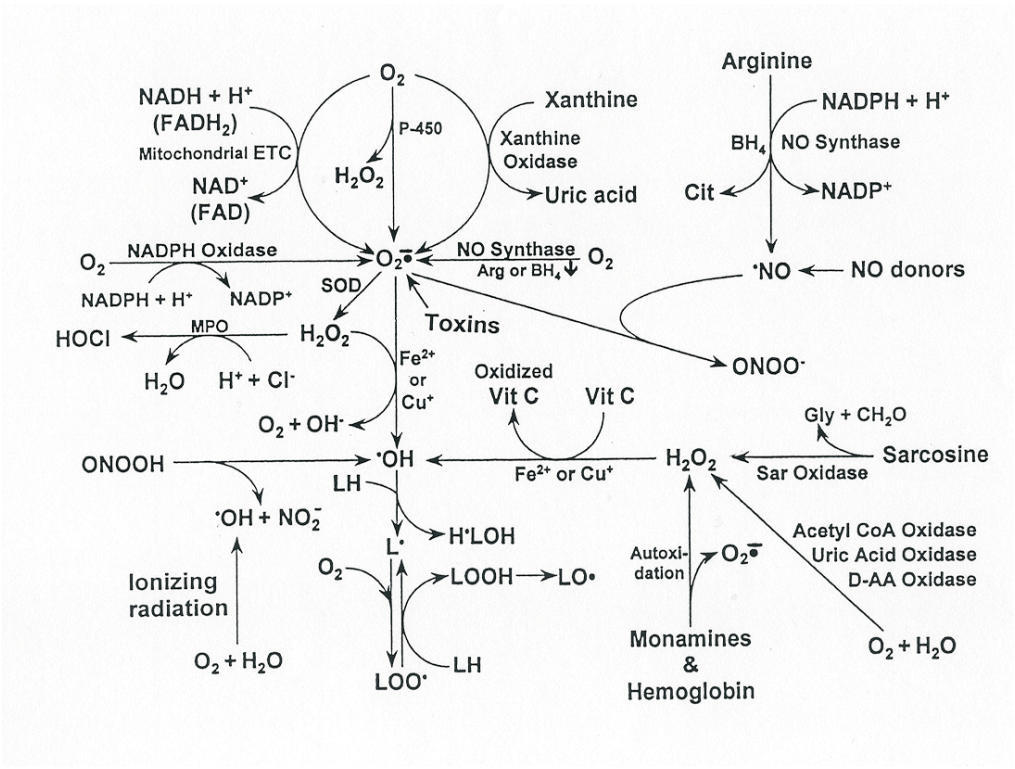

Figure 2. Oxygen and nitrogen-based free radicals and associated reactants that are generated in cells by various processes.

Free radicals are defined as molecules that have an unpaired electron in their valence orbital. Free radicals and the related reactants are not equally toxic. It is generally conceded that the most reactive, and therefore damaging, products are the oxygen-based hydroxyl radical (·OH) and the nitrogen-based peroxynitrite anion ($ONOO^-$). Arg, L-arginine; BH_4, 5,6,7,8-tetrahydro-L-biopterin; Cit, L-citruline; ETC, electron transport chain; FAD, flavin adenine dinucleotide (oxidized); $FADH_2$, flavin adenine dinucleotide (reduced); Gly, glycine; MOP, myloperoxidase; NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); P-450, cytochrome P-450.

2002; Tan *et al.*, 2002; Allegra *et al.*, 2003). The initial evidence illustrating melatonin's ability to neutralize the highly toxic hydroxyl radical (·OH) (Fig. 2) appeared roughly 10 years ago (Tan *et al.*, 1993). Since then, numerous reports have appeared which confirm this action of melatonin (Poeggeler *et al.*, 1994; Hardeland *et al.*, 1995; Matuszak *et al.*, product is proportional to the amount of melatonin administered to an animal and to the degree of oxidative stress the animal has experienced. The findings also indicate that cyclic 3-hydroxymelatonin is a footprint molecule that appears in the urine and that it is an index of *in vivo* ·OH scavenging by melatonin. Finally, cyclic 3-hydroxymelatonin it-

self has free radical scavenging activity (Tan *et al.*, 2003b; Lopez-Burillo *et al.*, 2003).

Stasica and co-workers (2000), using a computational approach, determined the most likely probable site on the indole ring of melatonin that may bind a ·OH; the C2 carbon was proposed as the likely site of attack. Additional details concerning the structural properties of melatonin that make it an efficient scavenger of the ·OH as well as the potential reactions of the indole as a radical scavenger are reviewed by Tan and colleagues (2002).

Hydrogen peroxide (H_2O_2), a non-radical ROS, is generated *in vivo* by several enzyme systems and, additionally, it is produced intracellularly by the dismutation of the superoxide anion radical ($O_2^{\cdot -}$) (Fig. 2). *In vivo*, H_2O_2 is a weak oxidizing and reducing agent. Also, no electric charge allows H_2O_2 to traverse cell membranes and is therefore accessible to sites significantly removed from its point of generation. Although H_2O_2 is weakly reactive, its major toxicity derives from its conversion to the highly toxic ·OH *via* the Fenton or Haber-Weiss reactions.

Melatonin as a scavenger of H_2O_2 in a pure chemical system was initially documented by Tan *et al.* (2000). A mechanism of the oxidation of melatonin by H_2O_2 was suggested on the basis of the major resulting metabolite, i.e., N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK). AFMK was confirmed using electron ionization mass spectrometry and proton and carbon nuclear magnetic resonance. Whether intracellular melatonin neutralizes H_2O_2 in a manner as described by Tan *et al.* (2000) is unknown. If it does, it would act like the H_2O_2-metabolizing enzymes, i.e., glutathione peroxidase (GSH-Px) and catalase (CAT), in the removal of this oxidizing agent and, importantly, reduce the generation of the ·OH. That AFMK is a byproduct of the interaction of melatonin with H_2O_2 has been reported by others as well including under *in vivo* conditions (Burkhardt *et al.*, 2001; Carampin *et al.*, 2003; Rozov *et al.*, 2003). One recent report questioned whether melatonin interacts directly with H_2O_2 (Fowler *et al.*, 2003). Why this group failed to document what other reports found is not apparent but could be related to the fact they tested a single dose of melatonin.

Recently, significant attention has been focused on AFMK as a scavenger of oxygen-based reactants as well. Cyclic voltametry has shown that AFMK is capable of donating two electrons; furthermore, the kynuramine reduces damage to DNA and lipids in a high free radical environment and lowers neuronal death when these cells are exposed to either H_2O_2, glutamate or amyloid β_{25-35} (each of these is known to generate free radicals) (Tan *et al.*, 2001). This indicates that not only the parent molecule, i.e., melatonin, but also the resulting products, i.e., cyclic 3-hydroxymelatonin and AFMK, may also function as scavengers of toxic reactants. This cascade of scavenging actions may be one reason accounting for the unexpectedly high efficacy of melatonin in reducing free radical damage *in vivo*. Finally, another product, *N*-acetyl-5-methoxykynuramine (AMK), is likewise capable of neutralizing some oxygen-based reactants (Tan *et al.*, 2002; Ressmeyer *et al.*, 2003) as is the chief hepatic enzymatic metabolite of melatonin, 6-hydroxymelatonin (Qi *et al.*, 2000; Hara *et al.*, 2001).

$O_2^{\cdot -}$ (Fig. 2) is generated during respiration in mitochondria when electrons leak from the ETC and during the respiratory burst of phagocytic cells. Relative to the ·OH, $O_2^{\cdot -}$ has low toxicity but it rapidly couples with nitric oxide (NO·) to produce a non-radical nitrogen-based reactant, the peroxynitrite anion ($ONOO^-$) (Fig. 2); this product is considered to be almost as damaging as is the ·OH. In addition to its inherent toxicity, $ONOO^-$ *via* peroxynitrous acid (ONOOH) may be metabolized to the ·OH *in vivo*.

The efficacy of melatonin in neutralizing the $O_2^{\cdot -}$ is only poorly defined. Melatonin reportedly scavenges this reactant in a pure chemical system where a hypoxanthine/ xan-

thine system was used to generate the $O_2^{\cdot -}$ (Marshall *et al.*, 1996); the ability of melatonin to quench the $O_2^{\cdot -}$ is also supported by evidence that melatonin modestly diminished the electron spin resonance (ESR) signal produced by the adduct, 5,5-dimethylpyrroline oxide (DMPO)-$O_2^{\cdot -}$ (Zang *et al.*, 1998). However, the role, if any, of melatonin in neutralizing the $O_2^{\cdot -}$ is unclear, particularly *in vivo*.

Macromolecular damage *in vivo* is also a consequence of singlet oxygen ($^1O_2$), an energy-rich form of oxygen; $^1O_2$ is usually produced in photosensitizing reactions of a variety of substrates including dyes and biological pigments. Poeggeler and co-workers (1996) were the first to show that melatonin neutralized $^1O_2$, during which AFMK was generated. This quenching ability of melatonin was confirmed by Zang *et al.* (1998) and by Roberts and colleagues (2000). That AFMK is the product formed when melatonin is oxidized by $^1O_2$ has been confirmed (De Almeida *et al.*, 2003). In light of these findings, it appears that AFMK is a product common to several interactions of melatonin with oxygen-based reactants.

The evidence that melatonin functions as a chain breaking antioxidant by scavenging the peroxyl radical (LOO·) remains problematic. The first reports on this subject placed melatonin among the very best scavengers in terms of its ability to neutralize the LOO· (Fig. 2); thus, the claim was made that melatonin is twice as effective as vitamin E, the primier chain breaking antioxidant, in interfering with the propagation of lipid peroxidation (Pieri *et al.*, 1994; 1995). These reports, however, have not been universally confirmed. Using a markedly different system to perform their tests, Livrea and co-workers (1997) also analyzed the ability of melatonin in terms of its chain breaking activity. Using either non-peroxidable unilamellar dimirystoyl phosphatidycholine liposomes or peroxidable soybean phosphatidylcholine liposomes, this group reported that melatonin was not a particularly effective chain breaking antioxidant. The data accumulated by Livrea *et al.* (1997) are consistent with those of Antunes and colleagues (1999) who thoroughly evaluated the lipoperoxyl-trapping efficiency of melatonin and concluded that it had limited ability to neutralize directly the LOO·. Given that melatonin is a heterocyclic aromatic amine, this group noted that molecules containing an NH group in a 5-membered pyrrole and carbazole ring do not typically function as highly effective chain breaking antioxidants.

Despite the controversy regarding the ability of melatonin to interact with the LOO·, *in vivo* melatonin has consistently been found to be highly efficient in limiting the peroxidation of lipids (Reiter *et al.*, 1998). Curtailing the progress of self-propagating lipid breakdown by melatonin may be a result of its ability to scavenge the initiating agents, e.g., ·OH, $ONOO^-$, etc., rather than being due to its apparent limited capability as a direct LOO· scavenger, i.e., to function as a chain breaking antioxidant.

Due to the electron-deficient nature of halide ions, haloperoxyl radicals are significantly more reactive than the alkylperoxyl radical; accordingly, the trichloromethylperoxyl radical ($CCl_3OO\cdot$) was found to be potently trapped by melatonin (Marshall *et al.*, 1996). In a pulse radiolysis study, this finding was also reported by Mahal and co-workers (1999). This latter group also showed, like Scaiano (1995), that melatonin traps the *tert*-butoxyl radical.

Hypochlorous acid (HOCl) formation is catalyzed by myloperoxidase (MPO) in activated neutrophils (Fig. 2). HOCl is a powerful oxidizing agent and it damages a wide variety of biomolecules. Presumably by electron donation, Dellegar *et al.* (1999) reported that melatonin detoxifies HOCl. In the process, melatonin is theoretically converted to the melatoninyl cation radical which could scavenge another radical. This melatonin radical intermediate has also been proposed in other

scavenging actions of the indoleamine (Tan *et al.*, 1993; 1998).

Melatonin as a direct scavenger of nitrogen-based free radicals and related species

NO· (nitrogen monoxide), a molecule widely produced in mammals where it has a variety of beneficial functions is a rather weak free radical. NO· is involved in a number of inflammatory processes that can lead to extensive tissue injury. Additionally, much of the toxicity of NO· may be a consequence of its coupling with $O_2^{\cdot -}$ which results in the formation of the highly reactive $ONOO^-$ (Fig. 2).

That melatonin detoxifies NO· has been reported by several groups (Mahal *et al.*, 1999; Noda *et al.*, 1999; Blanchard *et al.*, 2000). The latter group, however, showed that melatonin interacts with NO· only in the presence of molecular oxygen, a finding suggesting that melatonin may in fact react with a molecule derived from nitric oxide, possibly $ONOO^-$. The requirement for O_2 in the reaction of melatonin with NO· was confirmed by Turjanski *et al.* (2000a; 2000b). The chief product of the melatonin/NO· reaction is reportedly *N*-nitrosomelatonin. Semiempirical AM1 computations are consistent with the nitrosation of melatonin by NO·/O_2 (Turjanski *et al.*, 2000c).

The coupling of two relatively unreactive species, i.e., NO· and $O_2^{\cdot -}$, generates the potently oxidizing $ONOO^-$ (Fig. 2); this combination reaction has a very high rate constant (5.0×10^9 $M^{-1}s^{-1}$). The toxicity of peroxynitrite could relate to any of three reactive species, i.e., ground state peroxynitrous acid (ONOOH), the activated form of the acid (ONOOH*) as well as to its conjugated base ($ONOO^-$). Zhang and colleagues (1998; 1999) documented that melatonin is a substrate for peroxynitrite. Melatonin was shown to react with $ONOO^-$ with first-order kinetics; however, the rate constant for the reaction of melatonin with ONOOH was considerably higher (a reaction unimportant at physiological pH). Blanchard *et al.* (2000) also found that melatonin interacted with $ONOO^-$ but with the formation of different products than those reported by Zhang *et al.* (1998; 1999). These differences cannot currently be explained.

INDIRECT ANTIOXIDANT ACTIONS OF MELATONIN

Besides its ability to directly scavenge oxygen and nitrogen-based reactants, melatonin has a number of indirect means by which it may reduce oxidative stress. The relative importance of the direct and indirect antioxidative processes of melatonin *in vivo* remain unknown.

Melatonin stimulation of antioxidative enzymes

Antioxidative enzymes provide a major defense mechanism against free radical damage either by metabolizing them to less reactive species or to non-toxic byproducts (Fig. 3). The important antioxidative enzymes that have been investigated relative to melatonin are the superoxide dismutases (SOD), both MnSOD and CuZnSOD, catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRd) and glucose-6-phosphate dehydrogenase (G6PD) (Reiter *et al.*, 2000c; Mayo *et al.*, 2002; Rodriquez *et al.*, 2004).

The initial reports documenting melatonin's stimulatory effect on GPx appeared almost 10 years ago when it was shown that pharmacological doses of melatonin given to rats (Barlow-Walden *et al.*, 1995) and chicks (Pablos *et al.*, 1995) *in vivo* resulted in a marked augmentation in the activity of this enzyme. GPx reduces free radical damage because it metabolizes H_2O_2 (and other peroxides) to water; in the process, however, glutathione (GSH) is oxidized to its disulfide, GSSG (Fig. 3). GSSG is then quickly reduced back to GSH by GRd, an enzyme which has

also been shown to be stimulated by melatonin (Pablos *et al.*, 1998). The recycling of GSH may well be a major action of melatonin in curtailing oxidative stress. The ability of melatonin to regulate the GSH/GSSG balance by modulating enzyme activities appears to involve an action of melatonin at a nuclear binding site (Pablos *et al.*, 1997). The other GSH metabolizing enzyme, i.e., CAT, also increases its activity in response to melatonin (Naidu *et al.*, 2003).

Subsequent studies showed that the stimulation of the activities of GPx and GRd is achieved with physiological levels of melatonin. Thus, Pablos *et al.* (1998) observed that the normal nighttime rise in the activities of these enzymes in a number of tissues is extinguished if chicks are kept under constant light; light exposure at night, of course, prevents the nocturnal rise in physiological melatonin concentrations which, because of the loss of this stimulatory signal, prevents the activities of GPx and GRd from rising.

GRd requires the co-factor NADPH which is generated by the antioxidative enzyme G6PD (Fig. 3). Although the amount of data is limited, there is one report claiming that melatonin also stimulates G6PD (Pierrefiche & Laborit, 1995). This would be important in GSH recycling since NADPH is a necessary cofactor for G6PD.

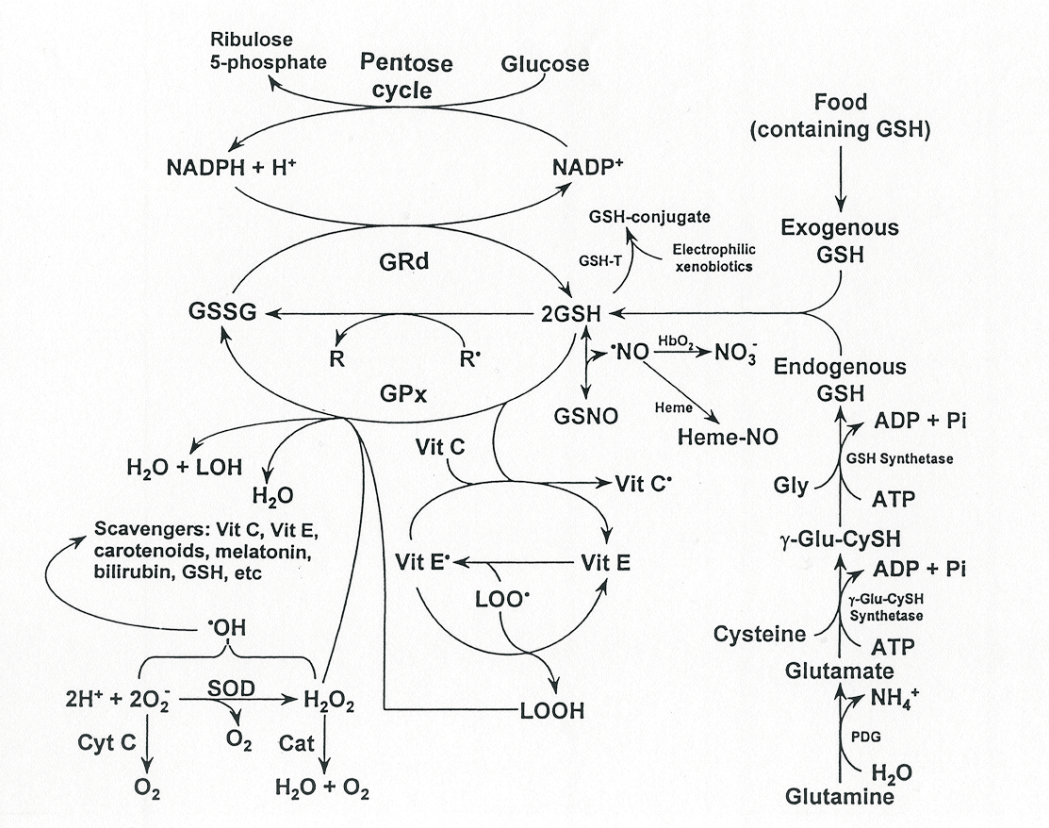

Figure 3. Free radicals and other reactants are enzymatically removed from cells by a series of antioxidative enzymes.

Melatonin stimulates the activity of several of these as summarized in the text. Melatonin also promotes the production of glutathione, an important intracellular antioxidant. Arg, L-arginine; GH_4, 5,6,7,8-tetrahydro-biopterin; Cat, catalase; Cyt c, cytochrome *c*; GSSG, glutathione (oxidized; glutathione disulfide); GSH, glutathione (reduced); GPx, glutathione peroxidase; GRd, glutathione reductase; GSH-T, glutathione *S*-transferase; GSNO, nitrosylated glutathione; HbO_2, oxyhemoglobin; PDG, phosphate-dependent glutaminase; SOD, superoxide dismutase.

Subsequent to this early series of studies, numerous reports have confirmed and extended the evidence concerning the promotional effects of melatonin on the antioxidative enzymes (Rodriquez *et al.*, 2004) including, not only GPx and GRd but SOD as well. Furthermore, due to the ease with which it crosses the placenta, melatonin, when administered to pregnant rats, results in a rise in GPx and SOD activities in the brain of the fetuses (Okatani *et al.*, 2000). Also, in the human chorion melatonin has been found to increase GPx activity (Okatani *et al.*, 2001).

In addition to estimating enzyme activities, gene expression for antioxidative enzymes have been studied following melatonin administration. For example, Mayo *et al.* (2002) found that the depressions in gene expression for neural GPx, CuZnSOD and MnSOD that occurred after treatment of rats with the neurotoxin 6-hydroxydopamine were prevented by melatonin. Similarly, others (Kotler *et al.*, 1998; Antolin *et al.*, 2002) have also observed that melatonin enhances gene expression for antioxidative enzymes either under basal conditions or after their inhibition by neurotoxic agents. While the direct free radical scavenging properties of melatonin are independent of any receptor for the indole, its ability to alter the activity of antioxidative enzymes likely requires an interaction of melatonin with either membrane or nuclear receptors.

While melatonin clearly functions as a direct free radical scavenger and indirectly reduces oxidative stress *via* the stimulation of antioxidative enzymes, the relative importance of each of these processes in reducing tissue damage due to free radicals in an unresolved issue. The high efficacy of melatonin in preventing oxidative mutilation of essential biomolecules suggests that both mechanisms must be important and, in fact, other processes, as summarized below, may likewise contribute to melatonin's unexpectedly strong antioxidative capabilities.

Melatonin stimulation of glutathione synthesis

GSH is very abundant intracellular free radical scavenger and antioxidant (Fig. 3). A single report has shown that melatonin stimulates its rate limiting enzyme, γ-glutamylcysteine synthase, thereby increasing intracellular GSH concentrations (Urata *et al.*, 1999). This action of melatonin, unlike the direct free radical scavenging function of the indoleamine, is likely mediated by specific receptors. The stimulation of GSH synthesis by melatonin could be a major antioxidative action of melatonin. Considering the potential importance of the findings of Urata and co-workers (1999), it is in need of confirmation particularly *in vivo* and in a variety of cell types.

Synergistic actions of melatonin with classic antioxidants

According to Gitto and co-workers (2001a), under *in vitro* conditions and using end products of lipid peroxidation as an indices of free radical damage, melatonin augments the protective actions of vitamin E, vitamin C and GSH against free radical-mediated oxidation of polyunsaturated fatty fats. The clear implication, and the conclusion reached by the authors, is that combinations of melatonin with other antioxidants clearly increase their efficacy. The mechanism of the synergy remains unknown and confirmation of these findings, particularly *in vivo*, is important. When compared under conditions of high oxidative stress *in vivo*, melatonin has proven superior to vitamins C and E in reducing oxidative damage (Tan *et al.*, 2002).

Actions of melatonin at the level of the mitochondria

Mitochondria are a major source of free radicals and as a consequence these subcellular organelles are exposed to extensive oxidative abuse. The inner mitochondrial membrane is the site of the ETC (Fig. 4), a system of

oxido-reductant protein complexes (complexes I, II, III and IV). In aerobic cells, mitochondrial oxidative phosphorylation (OXPHOS) is responsible for an estimated 90–95% of the total ATP generated by cells. Deficiencies in the ETC can lead to the leakage of electrons which thereafter form free radicals and other toxic reactants which results in molecular damage in mitochondria; this damage culminates in and contributes to what are referred to as mitochondria-related diseases (Acuña-Castroviejo *et al.*, 2002).

That melatonin has important actions at the level of mitochondria is suggested by a number of observations: a), melatonin is an efficient scavenger of ROS/RNS which are abundantly produced in mitochondria; b), although mitochondria are incapable of GSH synthesis (they take it up from the cytosol), they do possess GPx and GRd for GSH cycling, both enzymes of which are stimulated by melatonin; c), melatonin has been shown to have antiapoptotic effects, with the apoptotic signals originating in mitochondria; d), melatonin may be in higher concentrations in mitochondria than elsewhere in the cell and higher than serum concentrations of melatonin (Acuña-Castroviejo *et al.*, 2002).

Long term melatonin administration has been reported to increase the number of mitochondria in cells (Decker & Quay, 1982) while experiments with radioactive melatonin suggests mitochondrial binding sites for the indole (Poon & Pang, 1992). Additionally, melatonin was shown to inhibit NADPH-dependent lipid peroxidation in human placental mitochondria (Milczarek *et al.*, 2000), to protect the fetal rat brain against oxidant-mediated mitochondrial damage (Wakatsuki *et al.*, 2001) and to stimulate mitochondrial respiration in the brain and liver of senescence-accelerated mice (Okatani *et al.*, 2002a; 2002b; 2003a; 2003b).

The first tests of melatonin in reference to mitochondrial physiology were performed *in vivo*. In these studies, melatonin was shown to significantly increase hepatic and brain complex I and complex IV activities of the mitochondrial ETC (Martin *et al.*, 2000a). Fur-

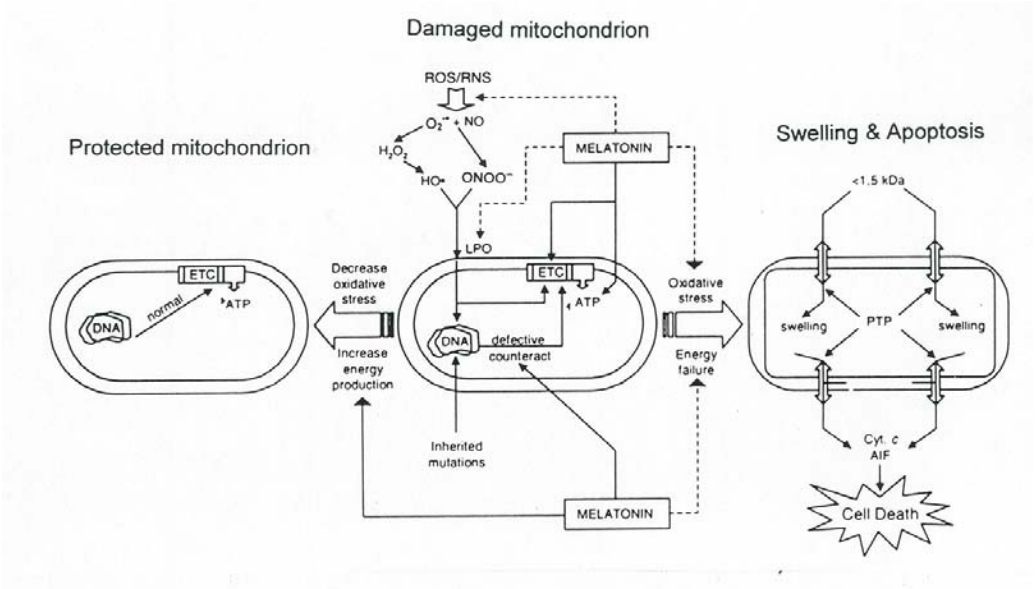

Figure 4. Actions of melatonin at the mitochondrial level increase its efficiency as an antioxidant by reducing free radical generation and increasing ATP production.

These actions of melatonin are summarized in the text. Cyt c, cytochrome *c*; RNS, reactive nitrogen species; ROS, reactive oxygen species; ETC, electron transport chain.

thermore, it was shown that melatonin reverses the inhibitory effect of 1-methyl-4-phenylpyridinium (MPTP) (Absi *et al.*, 2000) and ruthenium red (Martin *et al.*, 2000a) on the activities of these complexes.

In *in vitro* experiments, Martin *et al.* (2000b) documented that when oxidative damage was induced in mitochondria by incubating them with *t*-butyl hydroperoxide (t-BHP), the effect was prevented with a 100 nM concentration of melatonin; conversely, neither the addition of *N*-acetylcysteine, vitamin E nor vitamin C protected mitochondria against t-BHP toxicity. Melatonin's stimulation of complexes I and IV activities were dose-dependent. Finally, melatonin reduces cyanide toxicity at the level of mitochondria as well (Yamamoto & Yang, 1996) These actions of melatonin would be very important physiologically considering ETC efficiency is coupled to OXPHOS. Subsequent studies, in fact, reported that ATP production is also elevated when mitochondria are treated with melatonin (Martin *et al.*, 2001). As an energy source, ATP is critical to the cell for virtually all functions including the repair of oxidatively-damaged molecules. Thus, besides protecting molecules from damage due to ROS and RNS, once molecules are damaged melatonin may indirectly hasten their repair. This is an area of melatonin research that awaits further experimentation. Also, increasing the efficiency of the ETC theoretically, at least, may reduce electron leakage and free radical production. The actions of melatonin at the mitochondrial are summarized in Fig. 4.

PATHOPHYSIOLOGICAL EVIDENCE OF THE ANTIOXIDANT FUNCTIONS OF MELATONIN IN HUMANS

Antioxidants have attracted a great deal of attention as potential agents for forestalling age-related free radical-based diseases (Halliwell, 2001; Harman, 2002; Reiter *et al.*, 2003). The information on the scavenging actions of antioxidants may be particularly important for the aged where free radical-mediated diseases are numerous and, furthermore, the aging process itself is believed, in part, to be a result of the persistent accumulation of molecular debris resulting from the unending mutilation by free radicals (Harman, 1998; 1999; Fossel, 2002).

In the last 10 years, a vast amount of published literature has been amassed that provides unequivocal documentation that, *in vivo*, melatonin has the capability of diminishing destruction of DNA, proteins and lipids that are a result of their reactions with ROS and RNS. The number of publications regarding these actions in non-human mammals is so massive that it is not possible to discuss these findings in the current report and the reader is referred to other sources for this information (Reiter *et al.*, 2000a; 2002a; 2003; Cardinali *et al.*, 2003; Cheung, 2003). Rather, summarized below are some of the studies in which melatonin has been used to combat free radical damage in humans.

Based on a substantial amount of data documenting melatonin's ability to reduce neural damage in models of Alzheimer's disease (AD) (Pappolla *et al.*, 2000; 2003), several groups have administered melatonin to individuals diagnosed with this neurodegenerative condition in attempt to ameliorate disease symptoms. The first of these reports appeared in 1998 when Brusco *et al.* (1998a) showed that giving one of a pair of monozygotic twins (both with AD) 6 mg melatonin daily for 36 months significantly delayed the progression of the disease and reduced the degree of brain atrophy (as estimated by nuclear magnetic resonance imaging of the CNS). Melatonin is known to readily cross the blood-brain barrier and neuronal loss in AD is believed to be a consequence of free radical-mediated apoptosis. Thus, in this study melatonin's antioxidant functions presumably helped preserve neurons from mutilation and death. Using be-

havioral endpoints, several studies have also shown that melatonin may also improve locomotor activity and affect in AD patients (Brusco *et al.*, 1998b; Cohen-Mansfield *et al.*, 2000). In addition to the report that recently appeared (Asayama *et al.*, 2003), more extensive investigations that are double-blind and placebo-controlled are needed. There is certainly ample experimental evidence to justify treating AD patients and elderly with related degenerative diseases of the CNS with melatonin (Reiter *et al.*, 2000a; Cheung, 2003). Also, there is virtually no acute or chronic toxicity of melatonin (Jahnke *et al.*, 1999; Jan *et al.*, 2000; Seabra *et al.*, 2000) which should encourage its long-term use in individuals with age-related neurodegenerative diseases.

Melatonin has proven useful as a treatment for septic shock in premature newborns as well (Gitto *et al.*, 2001b). Sepsis is a serious condition and occurs in 1–10 cases per 1000 births with even higher rates than this in low-birth-weight neonates. The mortality rates in newborns who become septic can be as high as 50%. Sepsis culminates in multiple organ failure and death with the tissue damaged generally believed to be due, at least in part, to excessive free radical generation. Since melatonin had been shown to be an effective treatment for bacterial lipopolysaccharide (Sewerynek *et al.*, 1995), which causes multiple organ failure in rats, Gitto and co-workers (2001b) tested its efficacy as a treatment for sepsis in human premature newborns.

Twenty newborns judged to be septic were randomly divided into 2 groups of 10 infants each; all were given conventional antibiotic therapy but 10 were also supplemented with 2 doses of 10 mg oral melatonin (separated by a 1 h interval). Within 1 h and also at 4 h after melatonin administration, the levels of lipid peroxidation products in the serum were already depressed relative to those in non-melatonin treated children. Additionally, over the next 72 h all clinical parameters improved significantly more quickly in the children given melatonin. As is usual, 3 or 10 neonates not treated with melatonin died; conversely, due to their more rapid recovery all of the melatonin treated children survived. Gitto *et al.* (2001b) attributed the beneficial actions of melatonin in this study to the antioxidant properties of the indoleamine although there may have been other yet to be defined actions which permitted the children to exhibit a more rapid recovery from sepsis. Regardless of the mechanisms of protection by melatonin, the outcome of this study seemingly justifies the use of melatonin in the treatment of this serious condition not only in children but in adults as well.

A variety of conditions in neonates is associated with elevated oxidative stress (Gitto *et al.*, 2002). Considering the high efficacy and low toxicity of melatonin in these conditions and stimulated by the successful use of melatonin as described above (Gitto *et al.*, 2001b), Fulia and co-workers (2001) used the indoleamine to treat newborns who were suffering from transient asphyxia; free radical damage has been implicated in the pathophysiology of neonatal asphyxia. Twenty newborns with perinatal asphyxia diagnosed within the first 6 h of life were studied, 10 of who were given 80 mg melatonin orally. In the asphyxiated neonates given melatonin, serum levels of malondialdehyde (a lipid peroxidation product) and nitrite/nitrate concentrations were significantly reduced relative to those in the non-melatonin treated, asphyxiated children. Likewise, the clinical improvement was faster in the neonates given melatonin and all of these individuals survived; conversely, 3 of 10 non-melatonin-treated, asphyxiated children died (Fulia *et al.*, 2001).

Reactive oxygen species have also been implicated in the pathogenesis of respiratory distress syndrome (RDS) and its complications (Gitto *et al.*, 2001c). Given this, the rationale for treating children with RDS (grade III or IV) with melatonin is clear. In this case, 40 RDS newborns were given melatonin (100

mg given intravenously over a 2 day period) and 34 were provided conventional therapy only. At 24 and 72 h and at 7 days after melatonin administration, serum interleukin (IL)-6, IL-8, tumor necrosis factor alpha (TNF-α) and nitrite/nitrate levels were significantly lower in the melatonin-treated RDS neonates relative to the newborns suffering with RDS but given conventional treatment (Gitto *et al.*, 2004). Clearly, melatonin improved the outcome of the RDS afflicted newborns by reducing oxidative and inflammatory parameters associated with this condition.

Melatonin has also been tested as an agent to reduce oxidative stress in adult humans subjected to cardiopulmonary bypass surgery (CPB) (Ochoa *et al.*, 2003). Melatonin when given in advance of surgery onset reduced the degree of lipid peroxidation products in erythrocyte membranes of blood collected at various intervals after the onset of the operation. Another index of the breakdown of membrane lipids also documented the protective effect of melatonin. Thus, the increase in red blood cell membrane rigidity (decreased membrane fluidity) was also attenuated in the CPB individuals treated with melatonin. An increased membrane rigidity correlates positively with augmented levels of products of lipid peroxidation (Garcia *et al.*, 1999).

CONCLUDING REMARKS

Since the discovery of melatonin as an antioxidant in 1993 (Tan *et al.*, 1993), there has been an burgeoning number of reports documenting this action under an almost unlimited number of conditions, many of which have direct clinical relevance. Thus, melatonin has been shown to reduce the toxicity of drugs and in some cases improve their efficacy (Reiter *et al.*, 2002c), to reduce the severity and degree of tissue damage following ischemia/reperfusion in the brain (Cheung, 2003) and other organs, to prevent degenerative changes in the CNS in models of Alzheimer's (Pappolla *et al.*, 2000) and Parkinson's disease (Antolin *et al.*, 2002), to reduce free radical damage to DNA which may lead to cancer (Reiter *et al.*, 1998), and many other situations too numerous to mention in this brief report.

A major unresolved issue, as already mentioned above, relates to the significance of the various actions of melatonin that function in reduction of oxidative stress. At this point, it is unknown which of the multiple actions of melatonin, i.e., whether free radical scavenging, stimulation of antioxidative enzymes, increasing the efficacy of mitochondrial ETC and reducing electron leakage, improving the efficiency of other antioxidants, etc., are most important in contributing to its high efficacy. It is also likely that both receptor-independent and receptor-dependent actions of melatonin participate in its function as an antioxidant (Tan *et al.*, 2003a). Its successful use in human conditions where excessive free radical generation occurs, however, should encourage its continued use in the treatment of other disease processes, and there seem to be many, where oxidative stress is a component.

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