

Melatonin: Lowering the High Price of Free Radicals

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The endogenous antioxidative defense system reduces molecular toxicity of oxygen and nitrogen-based reactive species. Melatonin is an efficient direct and indirect antioxidant. It detoxifies the highly reactive hydroxyl radical and neutralizes other toxic species, including singlet oxygen, hydrogen peroxide, nitric oxide, and peroxynitrite anion, and stimulates several antioxidative enzymes.

Eight years ago I wrote an article for *News in Physiological Sciences* entitled "Melatonin: That Ubiquitously Acting Pineal Hormone" (*News Physiol Sci* 6: 223–227, 1991) in which the then-known functions of this secretory product were summarized. Little did I realize at the time that knowledge concerning the scope of melatonin's actions was about to change dramatically and that these discoveries would further broaden the already wide-ranging activities of this agent. These newly uncovered functions of melatonin are summarized here. What makes these, as well as other discoveries related to melatonin, so remarkable is that one of the organs of melatonin production, the pineal gland, was thought to be totally without function until 40 years ago. It now appears that its major secretory product, melatonin, may influence the physiology of every cell in the organism. Recent studies have shown that melatonin is phylogenetically a very old molecule and possibly exists in all animals, from algae to human. Melatonin is estimated to have evolved 2.5–3.0 billion years ago, coincident with the development of oxygen-based metabolism.

Direct free radical scavenging by melatonin

The pineal gland and melatonin were initially identified as the interface between the prevailing environmental photoperiod and seasonal reproductive capability in photoperiodic mammals. Subsequently, melatonin was linked to phenomena such as sleep and circadian rhythms. More recently, functions of melatonin include effects on the immune system and as an oncostatic agent. Finally, in 1993, we reported melatonin to be a free radical scavenger and antioxidant (13).

Free radicals are molecular marauders that have an unpaired valence electron. As such, these species are highly reactive and often destructive to other molecules in the vicinity of their production. Many free radicals that are generated in organisms derive from molecular oxygen (O_2). By far the largest portion (<95%) of O_2 taken in by aerobes is converted to energy in the form of ATP by mitochondrial oxidative phosphorylation. The remaining small percentage of inspired O_2 , however, forms semireduced species and reactive oxygen intermediates (Fig. 1). Many of these partially reduced brigands plunder, mutilate, and destroy

essential macromolecules within cells, resulting in physiological inefficiency and eventually molecular pathology and even death of the cells.

The most reactive, short-lived, and toxic of the free radicals is the hydroxyl radical ($\cdot OH$) (Fig. 1). Once produced, its estimated half-life within cells is on the order of 1×10^{-9} s, and it travels no more than a few Ångströms before it interacts with and damages another molecule. It is estimated that 50% of all free radical damage that occurs in aerobic organisms is attributable to the highly toxic $\cdot OH$.

This being the case, molecules that neutralize, detoxify, or scavenge the $\cdot OH$ play an important role in protecting against what is known as oxidative stress. Molecules that have the capability of detoxifying radical species are referred to as antioxidants, and their role allows them to prevent macromolecular damage and the overt dysfunction that results from that mutilation.

The ability of melatonin to neutralize the $\cdot OH$ was almost a serendipitous discovery and followed a hypothesis that some of melatonin's effects were receptor independent. In 1993, all of melatonin's known actions were believed to involve well-characterized, and subsequently cloned, cellular membrane receptors (9). The ability of melatonin to directly scavenge the $\cdot OH$ was proven using a combination of spin-trapping methodologies and electron spin resonance (ESR) spectroscopy (3, 13). This basic discovery was followed by a series of confirmatory observations that documented melatonin's ability to directly scavenge the highly toxic $\cdot OH$ at a near diffusion-controlled rate (3, 10, 12). Besides ESR, pulse radiolysis and additional indirect methods showed that melatonin neutralized the $\cdot OH$ with a high degree of efficiency (2, 10, 11). The rate constant for the scavenging of the $\cdot OH$ by melatonin is calculated to be on the order of $2.7 \times 10^{10} M^{-1} \cdot s^{-1}$ (3, 5). Additionally, melatonin may also act synergistically with other antioxidants in the detoxification of the $\cdot OH$ and other reactive intermediates (2, 5).

Most recently, we have identified one product of the interaction of melatonin with two $\cdot OH$; this product is a novel metabolite, cyclic 3-hydroxymelatonin (3-OHM) (Fig. 3) (14). Importantly, cyclic 3-OHM is excreted in the urine of mammals, with the quantity excreted varying with the amount of melatonin endogenously produced or exogenously administered and with the oxidative status of the organism (14). Since the amount of cyclic 3-OHM in the urine varies with the level of oxidative stress the animal has recently experienced, the metabolite may be a valuable biomarker of in vivo $\cdot OH$ generation, and it may be useful as a clinical index of the oxidant

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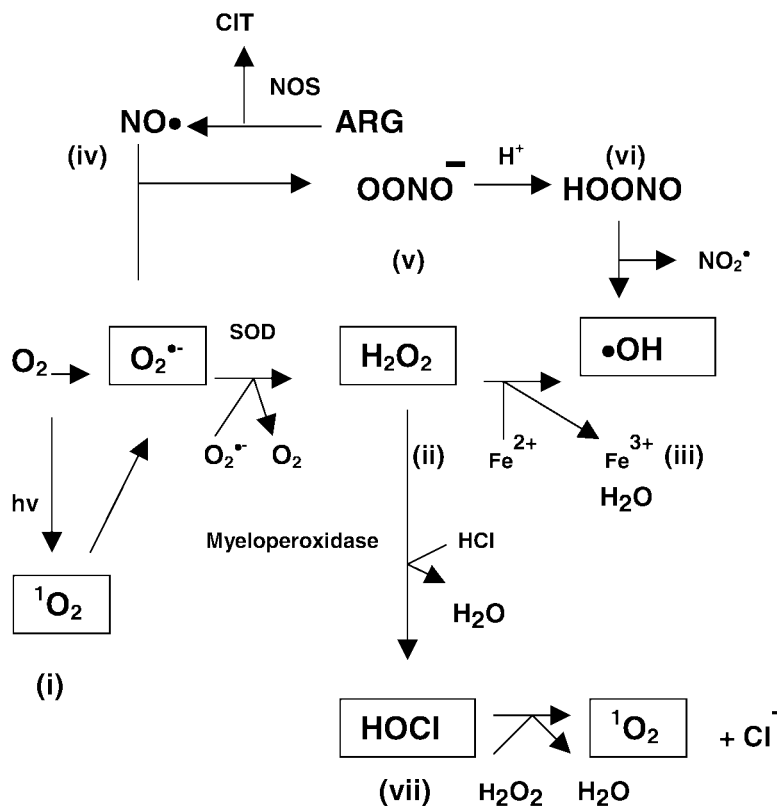


FIGURE 1. The dark side of oxygen (O_2). A small percentage (<5%) of the O_2 that aerobes inhale is converted to free radicals (molecules with an unpaired valence electron) and reactive oxygen intermediates. Because of the inherent toxicity of these molecules, when aerobic organisms based their metabolism on O_2 it proved to be evolutionarily risky; however, they have developed a complex antioxidative defense system to combat the destructive effects of O_2 by-products. Unfortunately, this defense system is not perfect and some molecular damage always occurs, leading to diseases and aging. O_2 initially undergoes a single electron reduction to produce the superoxide anion radical ($O_2^{\bullet-}$), which is either dismutated to hydrogen peroxide (H_2O_2) or combines with nitric oxide ($NO\cdot$) to form the peroxy-nitrite anion ($ONOO^-$). H_2O_2 is converted to the hydroxyl radical ($\cdot OH$) in the presence of a transition metal such as iron; this is identified as the Fenton reaction. $ONOO^-$ can also degrade into the $\cdot OH$ or a similarly toxic metabolite. The sites at which melatonin reportedly acts to directly detoxify free radicals are indicated by the numbers *i-vi*. Thus melatonin directly scavenges or quenches a number of oxygen and nitrogen-based reactive species that have the capability of destroying essential molecules within cells. NOS, nitric oxide synthase; SOD; superoxide dismutase; CIT, citrulline; ARG, arginine; hv, light energy.

status of an individual or the presence of a free radical-related disease. Cyclic 3-OHM is the signature molecule that results when melatonin scavenges two $\cdot OH$.

Although the detoxification of the $\cdot OH$ is, in itself, an important antioxidative accomplishment, melatonin's direct scavenging actions do not end with this action. Melatonin also reportedly neutralizes its precursor, hydrogen peroxide (H_2O_2) and other oxidants including singlet oxygen (1O_2), nitric oxide ($NO\cdot$), and the product of the interaction of the superoxide anion radical ($O_2^{\bullet-}$) and $NO\cdot$, namely, peroxy-nitrite anion ($ONOO^-$) and/or its metabolite. This latter agent exhibits very high toxicity and also degrades (Fig. 1) into a product that is equivalent in reactivity to the $\cdot OH$ (6). Overall, melatonin is certainly capable of directly neutralizing a variety of free radicals and/or their reactive intermediates and thereby reducing macromolecular destruction.

Indirect antioxidative actions of melatonin

Antioxidants can function as direct scavengers of free radicals and reactive oxygen species or they can act indirectly to metabolize free radicals or their intermediates into harmless products. A number of indirect antioxidants are enzymes that

remove toxic molecules either before they damage the cell or prevent more toxic agents from being formed. Classic antioxidative enzymes include the superoxide dismutases (SOD), subspecies of which are located throughout the cell, and the glutathione (GSH) peroxidases (GPx), GSH reductase (GRd), and catalase (CAT). The GPxs and CAT catalytically remove H_2O_2 and lipid hydroperoxides from the cell, thereby reducing the generation of the $\cdot OH$ (Fig. 2). In the process of doing so, GPx converts GSH to its oxidized product, GSH disulfide (GSSG). Since GRd reduces GSSG to GSH, it has an important role in the recycling of GSH and thereby reducing free radical damage. Besides functioning in the removal of H_2O_2 from cells, GPx also reduces $ONOO^-$, thus having an additional catalytic function to lower oxidative stress.

Melatonin, at least in pharmacological concentrations, has the capability of increasing either mRNA levels or the activities of these major antioxidative enzymes. There is also evidence that the physiological night time increase in melatonin is associated with a rise in the activities of these key antioxidative catalysts. Furthermore, the pro-oxidative enzyme nitric oxide synthase (NOS), at least in the cerebellum and hypothalamus, is inhibited by melatonin (7). This affords antioxidant protection by reducing levels of $NO\cdot$,

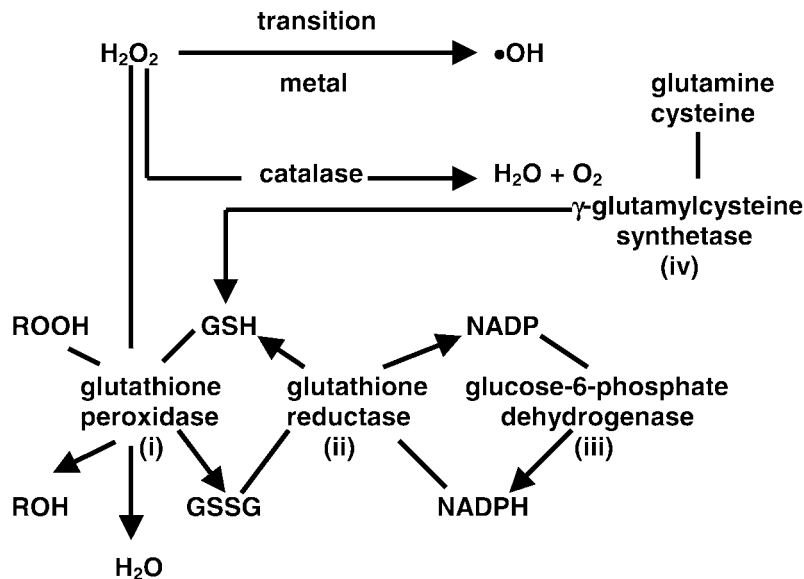


FIGURE 2. Enzymatic removal of oxygen by-products is an important aspect of the total antioxidative defense system of an organism. Hydrogen peroxide (H_2O_2), the immediate precursor of the highly toxic hydroxyl radical ($\bullet OH$), is metabolized to nontoxic products via the action of two enzymes, catalase and glutathione (GSH) peroxidase (GPx). Besides H_2O_2 , GPx also metabolizes reactive hydroperoxides (ROOH). In the process of detoxifying H_2O_2 and ROOH, GPx also oxidizes GSH to its disulfide form (GSSG), which is recycled back to GSH by the action of glutathione reductase (GRd). A cofactor for GRd is NADPH, which is supplied by the action of glucose-6-phosphate dehydrogenase. These enzymes work in concert to remove H_2O_2 and ROOH from cells, thereby protecting other macromolecules from the damaging effects of reactive oxygen metabolites. Melatonin reportedly stimulates several important enzymes as a manifestation of its indirect antioxidative actions (*i-iii*). Additionally, melatonin increases GSH production by stimulating the rate-limiting enzyme γ -glutamylcysteine synthetase (*iv*) in its synthesis. Also shown in this figure is the conversion of H_2O_2 to the $\bullet OH$ in the presence of a transition metal, i.e., either iron (Fe^{2+}) or copper (Cu^+). This conversion is known as the Fenton reaction.

which under conditions such as neural ischemia-reperfusion injury is highly toxic, and by lowering the generation of $ONOO^-$. The relative importance of these indirect antioxidant actions in terms of the total antioxidative capacity of melatonin remains unknown. It seems likely, however, that the benefits of each of these actions of melatonin vary with the nature of the oxidative stress and with the organ involved. Although the mechanisms whereby melatonin stimulates the activities of antioxidative enzymes remain unknown, these actions may be mediated via specific receptors for the indoleamine. In reference to inhibition of NOS activity, melatonin binds calmodulin, thereby reducing the activity of this calmodulin-dependent enzyme.

Role of melatonin in reducing oxidative damage

Other indirect methods used to estimate the efficiency of an antioxidant include the measurement of oxidatively damaged products. Of the major categories of macromolecules that are damaged by free radicals, i.e., lipids, proteins, and DNA, the bulk of the studies have examined the role of melatonin in protecting membrane lipids from free radical destruction (8). Without exception, in both in vitro and in vivo studies melatonin has been shown to reduce the accumulation of the major products of lipid peroxidation (usually measured as malondialdehyde and 4-hydroxyalkenals) when membranes are exposed to radical-generating agents. Thus lipid destruction induced by paraquat, amyloid β -peptide of Alzheimer's disease, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, chromium, lipopolysaccharide,

carbon tetrachloride, porphyrins, kainic acid, ischemia-reperfusion, hyperbaric hyperoxia, ibuprofen, aspirin, alcohol, and many other agents are reduced in the presence of melatonin. The mechanisms of inhibition of lipid peroxidation by melatonin probably involve the direct scavenging of the initiating radicals, especially $\bullet OH$ and $ONOO^-$. Independent of or associated with the inhibition of lipid peroxidation, melatonin also functions in maintaining the stability of cellular membranes, i.e., preventing changes in membrane fluidity. In so doing, melatonin may act to prevent membrane lipids from being oxidized by free radicals.

Much less is known concerning melatonin's ability to protect proteins from oxidative challenges. Available in vitro and in vivo data, however, do show that proteins are protected from the destructive actions of free radicals by melatonin (8, 15). This is consistent with the observation that melatonin also prevents the decline in the activities of antioxidative enzymes at sites of massive oxidant damage.

Nuclear DNA has been repeatedly shown to be protected by melatonin in situations in which free radical generation is augmented. Besides its ability to reduce the accumulation of DNA adducts induced by carcinogens, the premiere example of melatonin's ability to neutralize free radicals comes from observations in which ionizing radiation and/or radiomimetic agents were utilized as a DNA-damaging agent (14). High-energy ionizing radiation is known to cause the homolytic scission of H_2O to generate the $\bullet OH$, which in turn attacks DNA. Nuclear DNA damage sustained as a consequence of ionizing radiation has been shown in numerous studies to be blunted when melatonin

is present. The radioprotective effect of melatonin is also indicated by the increased survival rates of mice exposed to otherwise lethal doses of ionizing radiation.

As noted, DNA, like other macromolecules, is readily damaged by free radicals. The oxidative damage to any base molecule in DNA is usually transferred to guanine, since its oxidation potential is low relative to that of other DNA bases. Melatonin has been found capable of repairing the guanine radical ($G\cdot$), presumably via electron transfer, and it does so with a high degree of efficiency (6). This important finding has obvious physiological implications, since the $G\cdot$, which is long lived, could be repaired when melatonin is present in the nucleus. For example, DNA damaged during the day could undergo repair at night when physiological levels of melatonin rise. Several studies have already measured high nuclear concentrations of melatonin relative to levels found in other subcellular compartments or in the blood (7). The mere fact that melatonin is such an efficient protector of nuclear DNA from ionizing radiation suggests a close physical association of melatonin with DNA molecules.

The efficacy of any antioxidant, including melatonin, is multiplied if it is recycled. Recycling has been amply demonstrated for some other important antioxidants, e.g., ascorbate recycling of α -tocopherol. When examined, melatonin was found to be recycled by both ascorbate and urate (2). This observation further emphasizes the importance of melatonin as a free radical scavenger and antioxidant.

One problem with redox cycling compounds is that they may function, under certain circumstances, as pro-oxidants. A classic example is ascorbate. This nutritional antioxidant, in the presence of free iron (Fe^{2+}), becomes a powerful $\cdot OH$ -generating agent. Most other so-called antioxidants exhibit a similar pro-oxidant behavior under some circumstances. To date, however, melatonin has not been shown to function in the pathological generation of free radicals. If continued investigation shows this property of melatonin to be valid, it would provide the indole a major advantage over other antioxidants, which scavenge but also generate free radicals.

The vast majority of known physiological antioxidants are compartmentalized due to their unique solubilities. Thus α -tocopherol is found in cell membranes due to its exclusive lipid solubility, whereas ascorbate is soluble in aqueous environments, thus excluding its presence in lipid-rich membranes. Although melatonin is highly lipid soluble, it also displays some aqueous solubility. Being amphiphilic, it can protect against free radical damage throughout the cell and, indeed, has been shown to limit breakdown of membrane lipids, proteins in the cytosol, and DNA in the nucleus (8).

Besides being distributed, possibly unevenly, throughout the cell, melatonin has another beneficial feature as an antioxidant. As demonstrated in a variety of experimental studies, there are no morphophysiological barriers to melatonin. The indole readily crosses the blood-brain barrier, the placenta, and all other potential cellular impediments (4). Again, this contrasts with α -tocopherol, which only passes through the blood-brain barrier and placenta with difficulty. Even when given shortly before an insult, melatonin protects the brain and the fetus from dam-

age induced by free radical generators and toxins. When melatonin is given peripherally, it presumably distributes to all organs, to all cells, and to all subcellular compartments.

The low concentration of melatonin in the blood of mammals has been used as an argument against melatonin being a physiologically relevant antioxidant. This caution is based on the assumption that similar low concentrations of melatonin exist in cells, since it has been presumed that melatonin levels throughout the organism are in equilibrium. Preliminary evidence, however, has shown that intracellular concentrations of melatonin in some cells (e.g., brain and bone marrow cells) and body fluids (e.g., bile cerebrospinal fluid) may exceed those in the blood by several orders of magnitude (7). These high concentrations of melatonin may be maintained by specific proteins that bind melatonin. Assuming a widespread but differential distribution of such melatonin-binding molecules, melatonin concentrations may be organ and fluid specific.

Although the direct free radical scavenging or quenching activity is receptor independent, the indirect antioxidative actions (e.g., stimulation of antioxidative enzymes) of melatonin may be receptor mediated. The concentrations of melatonin required to activate membrane melatonin receptors (20–160 pM) are in the range of blood melatonin levels, and, because of receptor mediation, the efficacy of the indoleamine as an indirect antioxidant could be greatly enhanced. Several varieties of melatonin receptors have been characterized and cloned (9).

Implications and potential benefits

Numerous disease processes are believed to have a free radical component. Some of these include cancer initiation and growth, ulcerative colitis, senile cataracts, macular degeneration, ischemia-reperfusion injury (e.g., stroke and heart attack), emphysema, multiple organ failure due to lipopolysaccharide, heavy metal toxicity, drug toxicity, asbestosis, inflammation, Alzheimer's disease, and Parkinsonism. Pharmacologically, melatonin has been tested as to its protective effects in models of each of these conditions,

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and it has never failed to reduce the severity of the damage (7, 8). Similarly, surgical removal of the pineal gland, one source of melatonin in vertebrates, lowers circulating melatonin levels and exaggerates oxidative damage, attesting to the relevance of melatonin as a physiological antioxidant (7).

Besides their involvement in disease processes, free radicals are believed to be the culprit in some of the degenerative physiological changes associated with aging (7). Melatonin is the only antioxidant known to wane with age. Thus, after middle age, circulating levels of melatonin gradually diminish in all species, which contrasts with other antioxidants. This age-related reduction in melatonin is correlated with a parallel depression in the total antioxidative capacity of

human serum (1). Thus the adage that the older one gets the faster one ages may in part be a consequence of the gradual, albeit persistent, loss of melatonin during aging.

Conclusions

Endogenously produced melatonin may have a significant role in deferring a number free radical-related diseases and some pathophysiological changes associated with aging. This indoleamine is a widely acting free radical scavenger and antioxidant that has the capability of penetrating all morphophysiological barriers and entering all subcellular compartments. Melatonin's antioxidant capacity involves the direct, receptor-independent scavenging of toxic free radicals and reactive oxygen intermediates in addition to indirect antioxidative actions that may depend on cellular receptors for this action. A number of clinical studies are currently underway to more accurately define melatonin's efficacy in averting diseases that have as major causative agents toxic oxygen and nitrogen by-products. The generation of reactive oxygen species by aerobic organisms comes with a high physiological price, which can be lowered by antioxidants such as melatonin.

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