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Mini-review

Suppression of oxygen toxicity by melatonin

Russel J REITER[1], TAN Dun-Xian, QI Wen-Bo
(*Department of Cellular and Structural Biology, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio TX 78284-7762, USA*)

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ABSTRACT Melatonin, the chief secretory product of the pineal gland, was recently found to be a free radical scavenger and antioxidant. While most studies to date have used pharmacological quantities of melatonin to limit oxidative damage, physiologic concentrations of the indole which are present in aerobic organisms have also been shown to resist molecular damage inflicted by free radicals. Melatonin has several functions in terms of its antioxidative ability. It readily scavenges the most highly toxic free radical, the hydroxyl radical, and it directly detoxifies the peroxynitrite anion, nitric oxide, singlet oxygen, and the peroxyl radical. Precisely how efficient melatonin is in neutralizing each of these toxic agents remains to be determined. Melatonin also may stimulate several antioxidative enzymes including superoxide dismutase, glutathione peroxidase, and glutathione reductase as well as inhibiting the pro-oxidative enzyme, nitric-oxide synthase. Finally, melatonin chelates transition metal ions and prevents the deterioration of cellular membranes. This combination of actions may all contribute to melatonin's ability to reduce oxidative damage. Melatonin is highly effective in reducing nuclear DNA damage and membrane lipid destruction due to toxic free radicals *in vivo*. These findings have implications for disease processes, eg, neurodegenerative and cardiovascular diseases, which involve free radicals and for aging itself, which also is believed to be related to accumulated oxidative damage.

INTRODUCTION

Oxygen (O_2) is toxic because of its high electron (e^-) affinity which causes it to degrade into a number of reactive and damaging intermediates. The reduction of O_2 produces a number of products which are either free radicals or products that form free radicals as follows:

$$O_2 \xrightarrow{e^-} O_2^{\cdot-} \quad (1)$$

$$O_2^{\cdot-} \xrightarrow[SOD]{e^-} H_2O_2 \quad (2)$$

$$H_2O_2 \xrightarrow[Fe^{2+}]{e^-} \cdot OH \quad (3)$$

Additionally, O_2 can add to an already existing radical ($R\cdot$) to yield a peroxyl radical:

$$R\cdot \xrightarrow{O_2} ROO\cdot \quad (4)$$

Furthermore, O_2 forms a high-energy form, ie, singlet oxygen ($^1O_2$):

$$O_2 \xrightarrow{h\nu} {}^1O_2 \quad (5)$$

A major mediator of oxygen toxicity is $O_2^{\cdot-}$ which is formed *in vivo*[1]. A primary site of $O_2^{\cdot-}$ generation is the mitochondrion. During oxidative phosphorylation a small percentage of the e^- leak onto O_2. Since O_2 accepts one e^- at a time, the first product is $O_2^{\cdot-}$ (equation 1). A family of enzymes, the superoxide dismutases (SOD), which are present in cells of all aerobic organisms, reduce $O_2^{\cdot-}$ to H_2O_2 (equation 2). While H_2O_2 is not a radical and, unless in high concentrations within cells, it *per se* is generally considered relatively unreactive. However, H_2O_2 does have a long half-life and easily passes through cell membranes; in so doing it can spread oxidative damage throughout the cell or even between cells. Its secondary toxicity derives from the fact that when H_2O_2 comes in contact with a transition metal ion, eg, Fe^{2+}, Cu^{1+}, it catalytically forms the $\cdot OH$ (equation 3). $\cdot OH$ is generally considered to be the most damaging radical generated from oxygen.

$^1O_2$ is also not a free radical but it is capable of damaging tissues. This reactive species is sometimes used in the treatment of cancer in pho-

[1] Correspondence to Prof Russel J REITER.
Phn 1-210-576-3859. Fax 1-210-567-6948. E-mail Reiter@uthscsa.edu
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tosensitization (photodynamic therapy). This treatment involves the administration of certain porphyrin derivatives to cancer patients; these derivatives preferentially localize in the tumor cells which are then exposed to visible light to generate $^1O_2$ which in turn damage the microvasculature of the tumor thereby leading to cell death.

Besides its dismutation to H_2O_2, O_2^- can also combine with the gaseous neurotransmitter nitric oxide (NO·) to form the peroxynitrite anion ($ONOO^-$).

$$O_2^- + NO\cdot \longrightarrow ONOO^- \quad (6)$$

$ONOO^-$, another nonradical product, is also highly toxic and furthermore it can degrade into the ·OH or a ·OH-like radical[2].

$$ONOO^- + H^+ \rightarrow HONOO \rightarrow NO_2^+ + \cdot OH \quad (7)$$

Combatting reactive oxygen species and free radicals are the function of antioxidants. Antioxidants can be either direct free radical scavengers, enzymes that metabolize reactive species to nontoxic products (eg, SOD) or they may function in the prevention of free radical generation (eg, transition metal ion chelators).

The most recently discovered molecule with antioxidant properties is melatonin. Melatonin is produced in several organs but blood levels of the indole are derived primarily from the pineal body in the brain[3]. Unlike most other antioxidants, melatonin easily crosses all morphophysiologic barries, eg, placenta and blood-brain-barrier, and distributes to all parts of the cell where it provides protection against free radicals[4]. While melatonin is generally known for its high lipid solubility[5], it is also quite soluble in aqueous media[6]. This review briefly summarizes the antioxidant activities of melatonin.

Melatonin as a direct free radical scavenger

The discovery that melatonin is a free radical scavenger was revolutionary and opened an area of investigation that has been aggressively pursued. While the initial suggestion that melatonin may detoxify free radicals appeared in 1991[7], it was not until 1993 when the first definitive evidence was provided[8]. In this study the authors used electron spin resonance spectroscopy (ESR) to show that melatonin scavenged the most toxic of the radicals, namely, the ·OH. In the cell-free system melatonin successfully competed with the spin trap DMPO (5, 5-dimethylpyrroline-*N*-oxide) thereby reducing the adduct formed by the spin trapping agent and the ·OH; ·OH were generated by exposing H_2O_2 to 254 nm ultraviolet light[8].

Since this discovery, melatonin's ability to neutralize the ·OH has been repeatedly confirmed using ESR and other technologies. Many verified the high ·OH scavenging activity of melatonin *in vitro*[9-11]. Melatonin *in vivo* also detoxifies the ·OH[12]. These workers used microdialysis to collect extracellular fluid from the brain of rats treated with salicylate (which forms an adduct with the ·OH) and successfully showed that melatonin, just as in the *in vitro* studies, scavenges the ·OH.

The ROO· is highly damaging and propagates the chain reaction of lipid peroxidation. Melatonin is a more effective scavenger of the ROO· than vitamin E (tocopherol)[13]. This is a remarkable claim considering the great efficacy of vitamin E in detoxifying the ROO·. While melatonin is very effective in reducing the peroxidation of lipids, whether it functions as a chain breaking antioxidant by scavenging the ROO· is still uncertain. We[14] and others[15] have tested melatonin's ability to scavenge the ROO· and the conclusion from these studies is that it is not particularly efficient in scavenging the ROO·. As will be noted below, however, melatonin is highly effective in resisting the oxidative breakdown of membrane lipids, especially *in vivo*[16].

The high energy form of O_2, ie, $^1O_2$, is toxic within cells and can cause extensive damage. It is usually generated by treating an animal with a photosensitizing agent and then exposing specific tissues to high intensity light. This method was used to determine the ability of melatonin to quench $^1O_2$[17]. While the evidence presented is indirect in that melatonin prevented lipid peroxidation in the brain of rats treated with a photosensitizer and then exposed to light, the findings are consistent with melatonin quenching $^1O_2$. This finding has yet to be confirmed.

$ONOO^-$ is a highly toxic agent formed by the combination of O_2^- and NO·. Besides its inherent toxicity, $ONOO^-$ also degrades into

other toxic agents including perhaps the ·OH[2]. Melatonin may also scavenges $ONOO^-$[18] as well a greatly reducing its toxicity *in vivo*[19]. Considering the important role of $ONOO^-$ in promoting tissue damage in severe inflammatory states, melatonin's potential as an anti-inflammatory agent deserves additional study.

In a recent preliminary report it was also shown that melatonin directly scavenges NO·[20]. NO· functions as an important gaseous neurotransmitter but it can also be highly toxic under certain circumstances, for example, when it is excessively released during ischemia-reperfusion injury. The observation that melatonin scavenges NO· could potentially explain in part melatonin's protection against ischemia-reperfusion injury[21].

While melatonin appears to be a rather ubiquitous free radical scavenger, it seems to lack the ability to directly detoxify $O_2^{\cdot-}$. On the other hand, it scavenges hypochlorous acid (HOCl)[14]. Both $O_2^{\cdot-}$ and HOCl are generated by activated monocytes and macrophages when they engulf and kill bacteria. Melatonin actually promotes $O_2^{\cdot-}$ formation by activated human monocytes[22,23].

The products formed when melatonin scavenges a free radical are only beginning to be uncovered. Melatonin may work via e^- donation during which the indolyl cation radical is formed[24]. This radical product was then proposed to combine with $O_2^{\cdot-}$ to produce N^1-acetyl-N^2-formyl-5-methoxykynuramine.

Whereas the possibility has not been discounted that melatonin may function via e^- donation in the scavenging of the ·OH, three theoretical products may result from an alternative interaction[25]. With the use of proton nuclear magnetic resonance (^{1}H-NMR), COSY ^{1}H-NMR, mass spectrometry, and thermodynamic stability analysis we have identified one product of the interaction of melatonin with the ·OH to be cyclic 3-hydroxymelatonin (unpublished observations by DX Tan, LC Manchester, RJ Reiter).

Melatonin + 2·OH ⟶ cyclic 3-hydroxymelatonin (8)

This product results after melatonin scavenges two ·OH and we have shown that it appears in the urine of humans and other mammals. Furthermore, its urinary concentrations are increased when animals are exposed to ionizing radiation which is known to generate the ·OH.

Melatonin's actions on anti- and pro-oxidative enzymes

A variety of enzymes play vital functions in the removal of reactive oxygen species from cells. As noted above, a family of SOD plays a central role in converting $O_2^{\cdot-}$ to H_2O_2, a nontoxic product. mRNA levels are stimulated following the exogenous administration of melatonin suggesting a possible rise in activity of SOD, although this was not actually documented[26].

The over expression of SOD without a commensurate rise in the H_2O_2 detoxifying enzyme, glutathione peroxidase (GPx), however, increases oxidative damage as seen in Down syndrome, amyotrophic lateral sclerosis, *etc*. Thus, the observations that melatonin also stimulates both mRNA for and the activity of GPx[27] was important since it assured the removal of the excess H_2O_2 that would result from the over stimulation of SOD. Furthermore, GPx also functions as an $ONOO^-$ reductase[28], thereby removing this toxic agent from cells as well.

In the process of metabolizing H_2O_2 to nontoxic products, GPx oxidizes glutathione (GSH) to its disulfide form (GSSG) (Fig 1). So that GSH is not depleted from cells, GSSG is reduced to GSH in a reaction catalyzed by glutathione reductase (GRd). This replenishes GSH and allows for further metabolism of H_2O_2 (and other hydroperoxides) by GPx. Melatonin also stimulates the activity of GRd, thereby ensuring the replenishment of GSH[29].

By virtue of its ability of generate NO·, nitric-oxide synthase (NOS) can be viewed as a pro-oxidative enzyme. The discovery of the inhibition of NOS by melatonin would directly reduce oxidative damage caused by NO·, as well as its secondary toxicity after it combines with $O_2^{\cdot-}$ to form $ONOO^-$[30]. Additionally 5-lipoxygenase activity is reduced by melatonin[31].

Antioxidative actions of melatonin

Free radicals and reactive oxygen intermediates indiscriminately damage molecules, often in the immediate vicinity of where they are generated, leading to cellular death via either

apoptosis or necrosis (Fig 2). The destruction inflicted by these agents is usually measured in terms of damaged macromolecules including nuclear and mitochondrial DNA, polyunsaturated fatty acids (PUFA) and proteins.

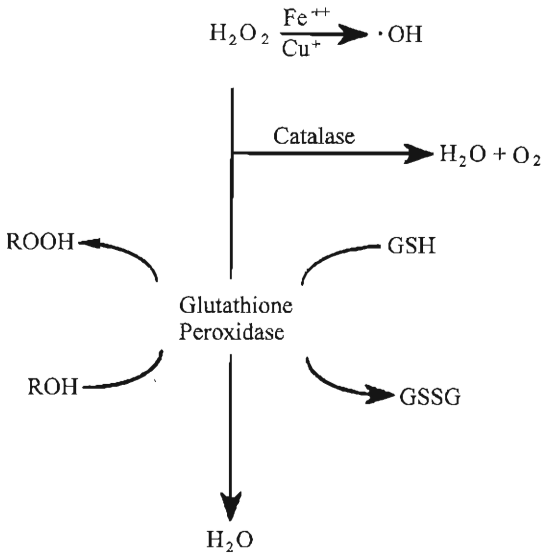

Fig 1. Major enzymes involved in removal of hydrogen peroxide (H_2O_2) from cells include catalase and glutathione peroxidase (GPx), both of which metabolize H_2O_2 and hydroperoxides (ROOH) to nontoxic products. In metabolizing these molecules, glutathione (GSH) is converted to its disulfide form (GSSG) which is enzymatically metabolized back to GSH by the enzyme glutathione reductase (GRd) (not shown). Both GPx and GRd are stimulated by melatonin. If H_2O_2 is not removed enzymatically, it can be converted, via the Fenton reaction, to the highly toxic hydroxyl radical (·OH).

Either endogenously-derived or exogenously-administered melatonin affords nuclear DNA considerable protection from oxygen toxicity[32]. In 1993, shortly after melatonin's ability to neutralize the ·OH was uncovered, the indole was found to protect hepatic DNA from damage induced by the administration of the chemical carcinogen safrole[33]. While in this study exogenously administered melatonin was provided, the same group also found that pinealectomized rats (which are deficient in endogenously produced melatonin) accumulate oxidatively damaged DNA more rapidly than intact rats when given safrole, thus illustrating that melatonin is not only pharmacologically relevant as an antioxidant but physiologically as well. Certainly, the ability of melatonin to reduce the oxidation of macromolecules in the nucleus is consistent with the seemingly high concentrations of the indoleamine in nucleoplasm[34].

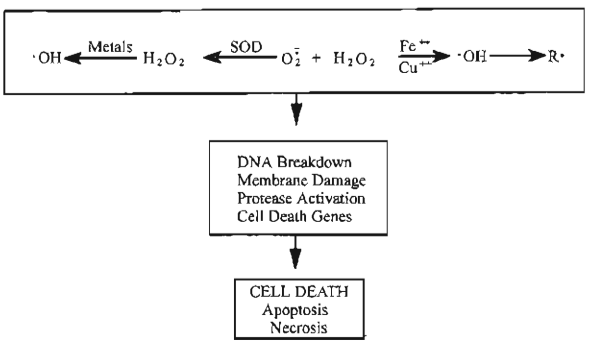

Fig 2. Molecular debris that accumulates within cells as a result of persistent bludgeoning of macromolecules by free radicals and reactive oxygen intermediates eventually leads to cell death due to necrotic processes or apoptosis. While the most toxic agent is generally considered to be the hydroxyl radical (·OH), other agents also participate in this destructive onslaught.

Since these early observations, the ability of melatonin to abate nuclear DNA damage normally induced by free radicals has been repeatedly confirmed. Ionizing radiation, a physical agent known to cause the homolytic scission of H_2O to generate the ·OH, is highly carcinogenic because of its destructive actions at the level of the genome. Since free radicals are involved in ionizing radiation-induced damage, we used this model to test the protective actions of melatonin. Using a variety of cytogenetic procedures to estimate chromosomal damage, giving melatonin in advance of exposure to ionizing radiation protects the cells from genetic damage; this was shown in both *in vivo* and *in vitro* studies and in human and nonhuman cells[35,36].

In the last 3 years numerous experiments have been reported illustrating that melatonin readily enters the nucleus, situates itself near DNA and shelters the genetic material from oxidative attack[37]. Considering that, for example, the ·OH, once generated, is estimated to travel no more than 0.3 – 0.5 nm before it interacts with another molecule such as DNA, the melatonin in the nucleus must be in very close proximity of the DNA, ie, within the reacting distance of the ·OH.

By far the largest number of publications related to the protective effects of melatonin against free radicals have measured products of lipid peroxidation[38]. There are several reasons for this. Firstly, lipids are easily oxidized so their damaged products often appear in relatively high concentrations. Secondly, there are a number of simple and reliable assays to measure the products of lipid peroxidation. And thirdly, the mechanisms of oxidative damage to lipids are generally well understood. While melatonin has clearly been shown capable of reducing oxidative damage to membrane lipids in a large number of experimental situations[39-41], there seems to be some disagreement as to how this beneficial effect is achieved. Whereas early claims indicated that melatonin was a powerful chain breaking antioxidant (like vitamin E)[13], later studies have not always come to the same conclusion[14,15]. Thus, rather that preventing excessive lipid damage by interrupting the chain reaction of events that occur during the breakdown of lipids by scavenging the ROO·, the primary means by which melatonin counteract oxidative attack to PUFA may be by reducing the initiating events in the process; this would be accomplished when melatonin scavenges reactive species such as the ·OH and $ONOO^-$. It may be that melatonin inhibits both the initiation as well as the propagation of lipid peroxidation and that the relative importance of each of these processes in protecting PUFA from the destructive effects of reactive species varies under different circumstances.

Because of melatonin's ability to pass through all membranes and apparently be sequestered in all cells and organs, its protective effects against lipid peroxidation are widespread. This contrasts with some other lipid antioxidants, eg, vitamin E, which because of their inability to efficiently penetrate the blood-brain-barrier, are not especially effective in reducing lipid breakdown in the central nervous system (CNS). By contrast, in a variety of experimental settings melatonin, when administered acutely, has been shown to retard damage to the CNS induced by free radical generating agents or toxins[42-45].

Methods to measure oxidative damage to proteins are less numerous and more difficult to reliably perform compared to the techniques for measuring lipid and DNA damage. Because this, there are generally fewer reports related the damaging effects of reactive species protein. Thus, it is not surprising th melatonin has been less frequently tested for i ability to protect against free radical-induce protein damage. It has been inferred from number of studies, however, that melatoni reduces protein damage caused by fre radicals[46]. Furthermore, in cell-free *in vitr* studies we have recently shown melatonin t protect purified proteins from the damaging effect of the ·OH (unpublished observations by S. Kim, J Cabrera, R J Reiter).

Final Commentary

The unexpected discovery that the chief secretory product of the pineal body, melatonin, functions as a free radical scavenger has led to a vast number of studies in recent years which have clarified some of the multiple actions by which melatonin protects macromolecules from oxidative damage. While melatonin has been shown to have both direct and indirect means for neutralizing free radicals and ROI, there may be other processes by which melatonin assists cells in resisting oxidative damage. As an example, melatonin chelates metals[47] and acts at the level of the cell membrane to maintain its optimal fluidity[48,49]. This latter action would ensure the proper functioning of the numerous critical activities of cellular membranes thereby helping them to resist toxic challenges. It is likely that other actions of melatonin will be discovered that permit it to function as an efficient antioxidant.

The multiple actions of melatonin would help to explain how a molecule, which is normally in such low concentrations in the cell (perhaps in the low to medium nanomolar range when it is at its highest levels) is so effective as an antioxidant. The fact that melatonin is normally replenished (it is synthesized perhaps in all aerobic organisms), is also an important feature of its ability to provide protection against oxidants. While melatonin is endogenously produced in young mammals, increasing age is associated with a marked reduction in the generation of this important antioxidant[50,51]. Since oxidative damage accumulates more rapidly

age-associated drop in melatonin may be causative in the acceleration of oxidatively damaged products[52,53]. This has clear implications for a variety of age-related debilitating conditions, eg, cardiovascular[54] and neurodegenerative diseases[55-57] as well as for aging itself[58] since each of these processes involves free radical damage[59]. Of particular interest in this regard is Alzheimer disease and associated dementias. Alzheimer disease is believed to be related in part to oxidative destruction of critical neurons[56]. Recent studies have shown that melatonin may be beneficial in preventing the loss of these cells[42] and delaying the progression of the disease[42]. This contention is supported by the recent observation that pinealectomy (which creates a relative melatonin deficiency throughout life) in young rats leads to an accelerated accumulation of oxidatively damaged lipid and DNA especially in the brain (unpublished observations by RJ Reiter, DX Tan, W Qi, SJ Kim). In view of the findings summarized herein, investigations into melatonin's antioxidative actions will likely continue unabated in the foreseeable future.

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褪黑激素抑制氧毒性

Russel J REITER[1], 谭敦宪, 齐文波

关键词 褪黑激素; 自由基; 抗氧化剂; 氧化应激; 衰老