Review

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Actions of Melatonin in the Reduction of Oxidative Stress

A Review

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Key Words

Antioxidant · Free radical scavenger · Hydroxyl radical · Melatonin · Oxidative damage · Peroxynitrite anion · Reactive oxygen species

Abstract

Melatonin was discovered to be a direct free radical scavenger less than 10 years ago. Besides its ability to directly neutralize a number of free radicals and reactive oxygen and nitrogen species, it stimulates several antioxidative enzymes which increase its efficiency as an antioxidant. In terms of direct free radical scavenging, melatonin interacts with the highly toxic hydroxyl radical with a rate constant equivalent to that of other highly efficient hydroxyl radical scavengers. Additionally, melatonin reportedly neutralizes hydrogen peroxide, singlet oxygen, peroxynitrite anion, nitric oxide and hypochlorous acid. The following antioxidative enzymes are also stimulated by melatonin: superoxide dismutase, glutathione peroxidase and glutathione reductase. Melatonin has been widely used as a protective agent against a wide variety of processes and agents that damage tissues via free radical mechanisms.

Despite its discovery over 40 years ago, melatonin was not recognized as a free radical scavenger and antioxidant until the last decade. Prior to that time, the circadian rhythm of melatonin in the blood of mammals was known to be functionally linked to the adjustment of 24-hour cycles and to circannual rhythm regulation. Additionally, however, melatonin's actions include modulation of immune function, tumor growth inhibition and influences on retinal physiology.

Melatonin is a derivative of the amino acid tryptophan and it is best known as being produced in the pineal gland. This, however, is not the only organ capable of synthesizing melatonin. Certainly, its production in the retina [71] is well documented and there is evidence for its formation in the ovary, lens, gastrointestinal tract, etc. Some cells and body fluids contain exceptionally high levels of melatonin. For example, bone marrow cells [21, 108] have melatonin concentration orders of magnitude greater than those in the serum; this is also true for bile [106] and cerebrospinal fluid [100] where the levels of melatonin are very much higher than in blood [71, 85]. Within individual cells, melatonin may not be evenly distributed within subcellular compartments [71, 85] and generally rather little is known about the concentrations of melatonin within cells.

That melatonin is a free radical scavenger was first suggested by Ianas et al. [42]. While the language in this report made the methodologies used somewhat difficult to decipher, the clear implication of the study is that melatonin had the capability of detoxifying highly reactive spe-

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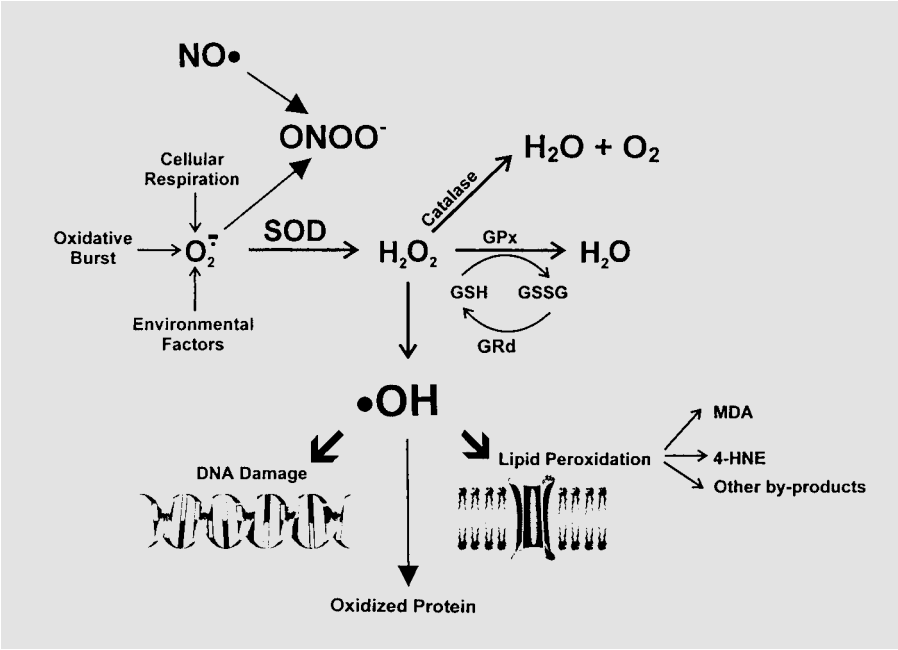

Fig. 1. Byproducts of oxygen metabolism, as summarized in this figure, can be highly destructive to macromolecules, subcellular organelles and to cells. The damage these semi-reduced oxygen metabolites produce is believed to be responsible for a variety of diseases and to the degenerative signs of aging. Oxygen is readily reduced to the superoxide anion radical ($O_2^{\bullet-}$) as a result of normal cellular respiration and under a wide variety of pathophysiological conditions. The $O_2^{\bullet-}$ can couple with nitric oxide ($NO^{\bullet}$) to generate the peroxynitrite anion ($ONOO^-$), a non-radical nitrogen-based metabolite with high reactivity. Besides its inherent toxicity, $ONOO^-$ degrades into other reactive species, some or which may be more toxic than $ONOO^-$ itself. $O_2^{\bullet-}$ is also dismutated by a family of enzymes, the superoxide dismutases (SOD), to produce the non-radical oxygen metabolite, hydrogen peroxide (H_2O_2). This molecule, because of its relatively long half-life and its ability to penetrate cellular membranes, has the potential capability of spreading the damage associated with free radical generation. Via the Fenton reaction, which requires a transition metal, H_2O_2 is converted to the devastatingly reactive hydroxyl radical ($^{\bullet}OH$). This product indiscriminately mutilates any molecule in the immediate vicinity (within a few Ångstroms) of where it is produced. While the damage inflicted by the $^{\bullet}OH$ is usually measured in terms of mutilated DNA, lipids and proteins, $^{\bullet}OH$ are promiscuous in terms of their molecular interactions. The exclusive fate of H_2O_2 is not, however, its conversion to the $^{\bullet}OH$. It can also be enzymatically removed by antioxidative enzyme catalase and the glutathione peroxidases (GPx). During the metabolism of reduced glutathione (GSH) to its disulfide (GSSG) by GPx, H_2O_2 and lipid hydroperoxides are used as substrates. GSSG is recycled back to GSH by another antioxidative enzyme, glutathione reductase (GRd). MDA = Malondialdehyde; 4-HNE = 4-hydroxynonenals.

cies derived from oxygen. Using a combination of spin trapping and electron resonance spectroscopy (ESR), 2 years later we showed that in fact melatonin was capable of directly scavenging the highly reactive hydroxyl radical [104]. Since then, there have been literally hundreds of publications which demonstrate the free radical scavenging [36, 93, 107] and antioxidant actions [73, 87, 89] of melatonin both in in vitro and in vivo settings.

Oxygen Toxicity

The use of molecular oxygen (O_2) as a basis of metabolism is both a benefit and a bane for aerobic organisms. While O_2 provides for efficient mitochondrial energy production via oxidative phosphorylation, its by-products are clearly detrimental to the optimal functioning of cells. Thus, O_2 could be considered a major environmental pollutant. O_2 by-products, known as free radicals and reactive species, mutilate and destroy essential macromolecules in cells thereby reducing their physiological efficiency. With continued molecular destruction by these destructive agents, the livelihood of organelles and the cells themselves are compromised eventually leading to death of cells either via apoptosis or necrosis.

Greater than 95% of the O_2 inhaled by aerobes is utilized for the production of ATP in mitochondria. The remainder is chemically reduced leading to the generation of the superoxide anion radial ($O_2^{\bullet-}$) (fig. 1). $O_2^{\bullet-}$ is produced as a consequence of normal cellular respiration,

during the oxidative burst in macrophages, as a result of drug metabolism, and as a consequence of environmental factors such as psychological and physical stress and radiation [22]. When one considers the quantity of $O_2^{\bullet}$ generated as a result of all these processes, it is estimated that a 70-kg individual generates in the order of 2 kg $O_2^{\bullet}$ each year.

While the toxicity of the $O_2^{\bullet}$ is considered to be rather limited, it is converted to a variety of more reactive and destructive species. For example, $O_2^{\bullet}$ couples with nitric oxide ($NO^{\bullet}$) to produce the peroxynitrite anion ($ONOO^-$), a reactive nitrogen-based species that is capable of inflicting extensive molecular destruction [84].

Besides its coupling with $NO^{\bullet}$, $O_2^{\bullet}$ can also be spontaneously and enzymatically dismutated to hydrogen peroxide (H_2O_2). The enzymes involved in this reaction are the superoxide dismutases (SOD) which, depending on their chemical composition, are differentially distributed intracellularly [38]. H_2O_2 is not a free radical and has rather low toxicity; however, because of its relatively long half-life (in seconds) and its ability to pass through cell membranes it has the capability of distributing the damage induced by free radical generating processes over progressively larger areas [14].

While H_2O_2 can be enzymatically degraded to O_2, some of its escapes this fate and, in the presence of a transition metal (most often iron), H_2O_2 to metabolized to the hydroxyl radical ($^{\bullet}OH$). Known as the Fenton reaction, the generation of the $^{\bullet}OH$ by this means can have devastating consequences for the cell because of the very high toxicity of the $^{\bullet}OH$ [48]. It is estimated that of the total molecular damage sustained as a consequence of free radicals, in excess of 50% is attributable to the $^{\bullet}OH$ [40]. This highly reactive species has an estimated half-life within organisms on the order of 10^{-9} s and it travels only a few Ångstroms before it interacts with another molecule. Thus, the molecular mutilation carried out by the $^{\bullet}OH$ is very near its site of generation and the damage, as a consequence, is often referred to as being site specific. The area encompassed by the distance traveled by the $^{\bullet}OH$ has been referred to as its 'reaction cage' [12].

The attack of the $^{\bullet}OH$ is totally random. This indiscriminate plundering leads to the molecular disfiguring of lipids, DNA, proteins, carbohydrates, etc. It is, however, the mutilated products of lipids, DNA and proteins that are typically used as an index of free radical damage in general and $^{\bullet}OH$ damage in particular. Unlike its precursors, i.e. $O_2^{\bullet}$ and H_2O_2, $^{\bullet}OH$ is not enzymatically detoxified within cells. Thus, it can only be neutralized by direct free radical scavengers.

Protection against Oxygen Toxicity

Thwarting the damage inflicted by free radicals and reactive species is the function of a complex antioxidative defense system [13, 19, 46]. Indeed, to ensure survival it was necessary for aerobic organisms to develop mechanisms to combat the cytotoxic changes associated with oxygen availability and dependence. Cells and organisms have evolved a host of methods to repel oxidative damage; these agents are collectively known as antioxidants. Typically, when the term antioxidant is mentioned most often the low molecular mass molecules and/or the so-called chain-breaking antioxidants come to mind. These constitute only a few of the many molecules and processes that aerobes use to neutralize free radicals.

The most commonly used and thoroughly experimentally studied antioxidants are the vitamins, i.e. ascorbic acid, tocopherol and β-carotene. These diet-derived agents are key elements in reducing molecular damage due to reactive oxygen and nitrogen species and there is an extensive literature, which describes their multiple actions [45]. Besides serving in the direct detoxification of free radicals, they also interact in recycling processes to generate reduced forms of the vitamins. For example, α-tocopherol is reconstituted when ascorbate recycles the tocopherol radical; likewise, dehydroascorbate, which is generated in that reaction, is recycled by glutathione (GSH). While vitamins are generally considered to function as antioxidants, they also exhibit toxicity and pro-oxidative actions under some conditions [97].

Besides the vitamins, there is a long list of other molecules that function as direct free radical scavengers or indirect antioxidants. In virtually all aerobes, the redox state of cells is highly dependent on the maintenance of glutathione (γ-*L*-glutamyl-*L*-cysteinyl-glycine) in its reduced form, i.e. as GSH. GSH is the most abundant intracellular thiol and its concentration ranges up to 10 m*M* [58]. Besides functioning as a direct free radical scavenger, GSH serves as a co-factor for many enzymes and it forms conjugates with endo- and exobiotics/xenobiotics. Furthermore, GSH functions as a co-substrate for glutathione peroxidase (fig. 1), the antioxidative enzyme which catalyzes the reduction of H_2O_2 and lipid hydroperoxides during which it is oxidized to its disulfide form, GSSG. The normal ratio of GSH:GSSG is 99+:<1%. If this ratio changes only slightly in favor of GSSG, the effect on cells is highly deleterious. The cytosolic enzyme glutathione reductase (GRd) is crucial in the regeneration of GSH from GSSG.

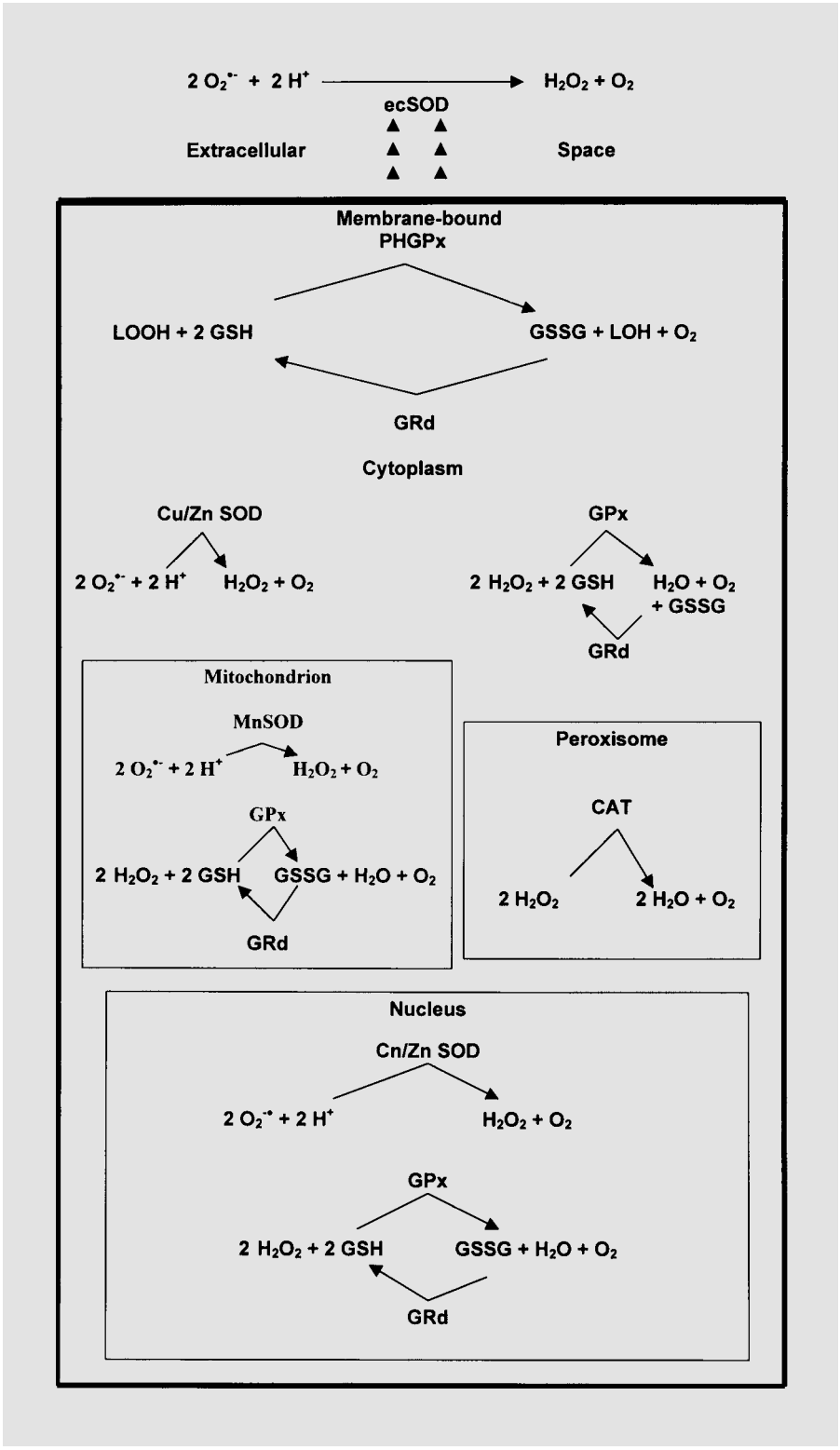

Fig. 2. A summary of the major antioxidant enzymes which play a role in controlling free radical damage. The glutathione peroxidases (GPx) and glutathione reductase (GRd) are widely distributed within cells and are found in the cytoplasm, mitochondria and nucleus. Catalase (CAT) which, like the GPx, removes H_2O_2 is located in a subcellular organelle, the peroxisome. The superoxide dismutases (SOD) are also located at various sites within cells. Copper/zinc (CuZn)SOD is found in both the cytoplasm and the nucleus while manganese (Mn)SOD is confined to the mitochondria. SOD is also released into the extracellular space (ecSOD) where it also functions in the dismutation of the $O_2^{\bullet-}$.

Examples of lesser known antioxidants include uric acid, ubiquinone (also called coenzyme Q_{10}), phenols, polyphenols and flavonoids, phytoestrogens, α-ketoacids and a variety of thiols (in addition to GSH) including taurine, homocysteine, α-lipoic acid, etc. This list could obviously be expanded to include a large number of other minor free radical scavengers and antioxidants.

Enzymes also play a major role in ridding subcellular compartments of toxic reactants. This category of antioxidative catalytic agents includes SOD, catalase (CAT), GPx, GRd and glucose-6-phosphate dehydrogenase (G6PD) among others. The intracellular distribution of these catalytic agents is summarized in figure 2.

SOD protects against oxidative damage by catalyzing the dismutation of the $O_2^{\cdot-}$ to H_2O_2. In vertebrates, manganese-containing (MnSOD) and copper/zinc-containing SOD (CuZnSOD) are the predominant isoforms. The biological relevance of SOD in reducing oxidative damage has been demonstrated under many experimental conditions. In some exceptional cases, e.g. Down syndrome patients, overexpression of SOD activity leads to increased oxidative damage, a likely result of increased generation of the $^{\cdot}OH$ [53].

Many cells are also equipped with the antioxidative enzymes GPx and CAT which metabolize H_2O_2 and hydroperoxides to innocuous products thereby reducing the quantity of molecular destruction inflicted by oxygen by-products [98]. Among tissues, CAT activity varies widely with some cells, for example those in the central nervous system [54], having little CAT activity. Hence, this antioxidative enzyme is believed to have limited importance in the brain. GPx activity is due to the expression of several isoforms. Both GPx and CAT remove H_2O_2 from cells but their relative importance in doing so is both species and organ specific.

As noted above, GPx oxidizes GSH to its disulfide, GSSG. The recycling of GSSG back to GSH is the function of another antioxidative enzyme, GRd. A co-factor in this reaction, i.e. NADPH, is supplied by G6PH; because of this, G6PD is also considered an antioxidative enzyme since it contributes indirectly to the regeneration of GSH.

Melatonin as a Direct Free Radical Scavenger

While the redox properties of indoles have been known for several decades, the chemical reactivity of melatonin with free radicals is a more recent development [37, 77, 104]. There are, of course, certain thermodynamic and kinetic requirements that a molecule must possess if it is to act as an efficient antioxidant. For example, although thermodynamically possible, not all interactions are kinetically feasible because the rate constant is so low that it has little biological significance. Additionally, when a scavenger detoxifies a reactant, the radical formed by the scavenger molecule must be essentially harmless (neither a strong oxidizing nor a strong reducing agent). The kinetics of the interaction must require it to work at low concentrations and, finally, it should be recycled so as to have the capability of repeatedly scavenging toxic agents. To varying degrees, these requirements seem to have been met for melatonin, although more extensive work is required to establish its definitive physiological role in the antioxidative defense system.

The following paragraphs briefly summarize what is known of the interaction of melatonin with reactive species. While this brief review cannot possibly consider the detailed interactions of melatonin with each reactive species and free radical which it is believed to neutralize, it does summarize much of the data and the original papers as well as several reviews can be consulted for additional information [37, 88, 93, 107].

Hydroxyl Radical ($^{\cdot}OH$)

The $^{\cdot}OH$ is generally considered destructive to cells because of its very high reactivity with any molecule it encounters. As noted above, Tan et al. [104] were the first to document that melatonin detoxifies the $^{\cdot}OH$. The approaches use to demonstrate this interaction included the direct scavenging of $^{\cdot}OH$ by melatonin after the photolysis of H_2O_2 with 254 nm ultraviolet light and studies in which melatonin competed with the spin trap, 5,5-dimethylpyrroline-N-oxide (DMPO), for the $^{\cdot}OH$. In this competition study melatonin, in increasing concentrations (from 1 to 100 μ*M*) dose-dependently reduced the formation of DMPO-$^{\cdot}OH$ adducts which were estimated and identified by high performance liquid chromatography with electrochemical detection (HPLC-EC) and electron spin resonance spectroscopy (ESR). ESR is considered the most definitive test to identify spin trap-$^{\cdot}OH$ adducts (fig. 3). The dose-response study showed that the concentration of melatonin required neutralizing 50% of the $^{\cdot}OH$ generated, i.e. the IC_{50}, was 21 μ*M*. This value proved to be roughly 5 and 10 times lower than that for two known $^{\cdot}OH$ scavengers, glutathione and mannitol, respectively.

We followed these studies with a kinetic competition experiment in which the specific radical trapping reagent 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)

(ABTS) was used as a probe for the •OH generated by Fenton reactants (H_2O_2 plus $FeSO_4$) [78, 79]. When melatonin was co-incubated in this system, it reacted rapidly with the •OH thereby reducing the oxidation of ABTS to its stable thiazoline radical. From this study, we calculated the rate constant of melatonin's interaction with the •OH to be $0.6 \times 10^{11}\ M^{-1}\ s^{-1}$.

Also using the experimental protocol employed by Tan et al. [104], Matuszek et al. [57] confirmed the ability of melatonin to reduce the formation of the DMPO-•OH adduct in competition studies. They also used ESR to identify the DMPO-•OH adduct and its reduction in the presence of melatonin when •OH were generated using H_2O_2 and $FeSO_4$. Their calculated rate constant for the scavenging of the •OH by melatonin was $2.7 \times 10^{10}\ M^{-1}\ s^{-1}$.

Since these reports, there have been a number of confirmatory studies using a wide variety of methodologies. Without exception, the investigations have shown melatonin to be an efficient •OH scavenger (table 1). While most of these studies have been conducted in pure chemical, cell-free systems, animal studies have also shown melatonin to scavenge the •OH in vivo [47, 105]. The average calculated rate constant for the scavenging of the •OH by melatonin is similar to that of other known efficient •OH scavengers [93].

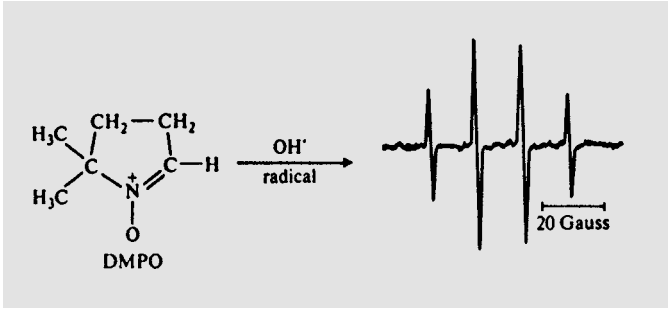

Fig. 3. Electron spin resonance (ESR) spectroscopic identification of the DMPO-•OH adduct as used in the study of Tan et al. [104]. DMPO in the presence of the •OH (generated by the ultraviolet photolysis of H_2O_2) forms a specific adduct with a known spectrum on ESR examination. In this case, the adduct yields a 1:2:2:1 spectrum as shown on the left. When melatonin was added to this mixture, the ESR signal was increasingly quenched with progressively higher doses of melatonin indicating a reduction of the number of DMPO-•OH due to the scavenging of the •OH by melatonin.

The product of the interaction of melatonin with the •OH has recently been identified. Using a combination of mass spectrometry (MS), proton nuclear magnetic resonance (^{1}H NMR), COSY ^{1}H NMR, and calculations of the relative thermodynamic stability, we identified the molecule that is formed when melatonin neutralizes two

Table 1. A tabulation of some of the studies that have investigated the •OH scavenging activity of melatonin

Research group	Source of •OH	Method of measurement	Rate constant $M^{-1}\ s^{-1}$
Studies where the rate constant was determined			
Poeggeler et al. [79]	Fenton reagents	kinetic competition with ABTS	0.6×10^{11}
Matuszek et al. [57]	Fenton reagents	spin trapping and ESR	2.7×10^{10}
Stascia et al. [102]	pulse radiolysis of water	absorption spectra of indolyl radical	1.2×10^{10}
Chyan et al. [20]	ultraviolet photolysis of H_2O_2	kinetic competition with ABTS or DMPO	4.0×10^{10}
Mahal et al. [52]	pulse radiolysis of water	absorption spectra of indolyl radical	1.25×10^{10}
Studies where the rate constant was not determined			
Tan et al. [104]	photolysis of H_2O_2	spin trapping and ESR	
Poeggeler et al. [78]	Fenton reagents	reduction in melatonin fluorescence	
Li et al. [47]	ischemia/reperfusion	production of 2,3-dihydroxybenzoate	
Susa et al. [103]	chromium and H_2O_2	spin trapping and ESR	
Pähkla et al. [70]	Fenton reagents	kinetic competition with terephthalic acid	
Stascia et al. [101]	pulse radiolysis of water	absorption spectra of indolyl radical	
Tan et al. [105]	ionizing radiation	formation of cyclic 3-hydroxymelatonin	
Bandyopadhyay et al. [7]	Cu^{2+}/ascorbate system	kinetic study with methanesulfonic acid	
Brömme et al. [15]	glutathione/alloxan/Fe^{2+} system	spin trapping and ESR	
Khaldy et al. [44]	dopamine autoxidation	production of 2,3-dihydroxy-benzoate	

ABTS = 2,2′-Azino-bis(c-ethylbenz-thiazoline-6-sulfonic acid); ESR = electron spin resonance spectroscopy; DMPO = 5,5-dimethyl-pyroline-N-oxide.

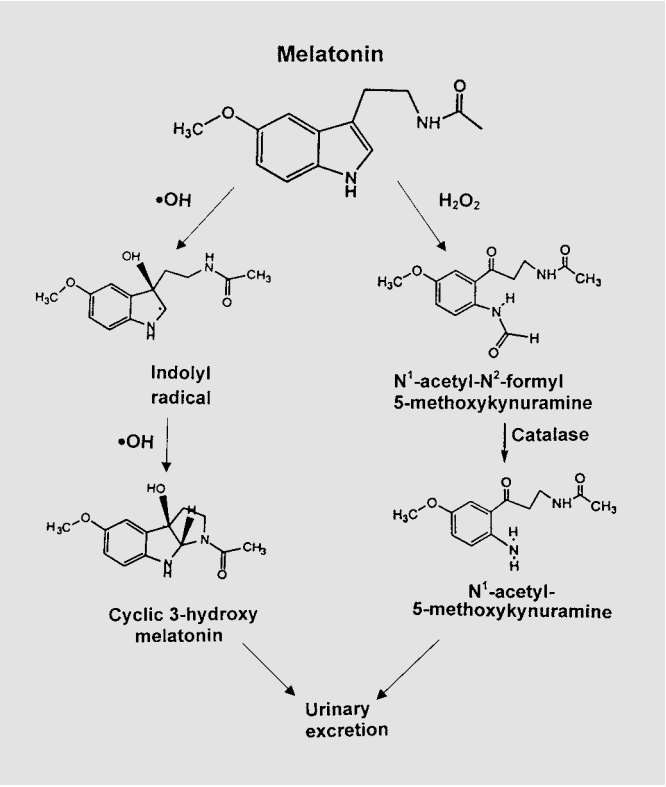

Fig. 4. Proposed pathways for the scavenging of the •OH (left) and H_2O_2 (right) by melatonin. Scavenging of a single •OH by melatonin generates the indolyl radical, which then detoxifies a second •OH to produce cyclic 3-hydroxymelatonin. The quantity of this product formed in vivo represents the number of •OH scavenged in vivo. After its formation in vivo, cyclic 3-hydroxymelatonin is excreted in the urine and is used as a biomarker of •OH generation. There are a number of radical intermediates between the indolyl radical and cyclic 3-hydroxymelatonin; the details of the complete pathway are available in the original paper by Tan et al. [104]. Mahal et al. [52] claim that melatonin may be regenerated from the indolyl radical by ascorbate or urate. Melatonin may also scavenge $^1O_2$ and H_2O_2 with the production of N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) which is then enzymatically converted, by catalase, to N^1-acetyl-5-methoxykynuramine. These are also potential urinary excretion products. AFMK in particular may retain free radical scavenging activity.

•OH [105] (fig. 4). The product formed has a molecular mass of 248 and was characterized using HPLC-EC as cyclic 3-hydroxymelatonin (3-OHM). This product was also identified by MS, ^{1}H NMR and COSY ^{1}H NMR. 3-OHM has been found in the urine of both rats and humans and its quantity in the urine increases, expectedly, when melatonin is injected into rats. Furthermore, when rats were subjected to whole body ionizing radiation, which generates large numbers of •OH, and exogenously given melatonin, the urinary excretion of 3-OHM is markedly augmented. These findings are consistent with melatonin scavenging the •OH in vivo and the organism ridding itself of the resultant product, i.e. 3-OHM, by excreting it in the urine. It could, however, be eliminated from the body by other means as well, e.g. in the feces.

While any molecule that effectively scavenges the •OH is potentially important in protecting organisms from the devastating effect of the toxicant, it is essential to note that, as described above, the half-life of the •OH and the distance it travels before it destroys an adjacent molecule are both miniscule [12]. Thus, for any scavenger to protect against the resulting damage, it must be virtually at the site where the •OH is formed. So far as is known, melatonin enters every cell in the organism and passes into every subcellular compartment. Thus, it is known to protect polyunsaturated fatty acids in cellular membranes, organelles in the cytosol and nuclear and mitochondrial DNA from free radical damage [91, 94, 95]. Its subcellular distribution is more extensive than that of some other classical antioxidants, e.g. vitamin E which is confined to the lipid environments of cells. Likewise, there are no known morphophysiological barriers to melatonin. It readily transverses the blood-brain barrier [87] and the placenta [63], both of which are significant impediments to vitamin E. Melatonin's ability to distribute widely in organisms and cells is believed to be a significant aspect of its efficiency in quelling free radicals and limiting their damage.

As mentioned above, tocopherol is regenerated from the tocopheryl radical by ascorbate. This allows tocopherol to repeatedly function as free radical scavenger and greatly increases its efficiency as an antioxidant. There is also some evidence that the indolyl radical is recycled back to melatonin. Whereas Mahal et al. [52] suggested that either ascorbate or urate is capable of recycling the indolyl radical, the details of these potential interactions are only scanty and as yet unconfirmed. If in fact melatonin is regenerated, it could explain the synergistic actions of ascorbate and melatonin as described by Poeggeler et al. [77].

Hydrogen Peroxide (H_2O_2)

Obviously, molecules that are capable of scavenging the •OH are very important in restricting molecular damage. Anytime the •OH is generated, however, it has the potential of escaping scavengers and damaging a molecule. Thus, preventing the formation of the •OH would even be a more efficient means to reduce •OH-induced damage. This can be accomplished by several means: che-

lating the transition metals (e.g. Fe^{2+}) which catalyze the Fenton reaction, enzymatically removing H_2O_2, and directly scavenging H_2O_2 by an antioxidant. There are two reports claiming that melatonin has this latter capability [107, 112] but an equal number of publications have claimed that the indole is incapable of directly detoxifying H_2O_2 [18, 79].

According to Zang et al. [112], melatonin interacts with H_2O_2 as indicated by the dose-response reduction in its concentration in a mixture to which increasing amounts of melatonin were added. Details of the interaction mechanisms of melatonin with H_2O_2 were, however, not provided. According to Tan et al. [107], melatonin scavenges H_2O_2 with the formation of N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK); this molecule may also possess significant scavenging activity. When AFMK was incubated with CAT, the enzyme catalytically converted AFMK to N^1-acetyl-5-methoxykynuramine (AMK). This proposed scheme is summarized in figure 4 and suggests a cascade which could increase the efficiency of melatonin as an antioxidant since the products formed, i.e. AFMK and AMK, may also be free radical scavengers.

If melatonin does indeed remove H_2O_2 in vivo, it would be important because it would proportionally reduce the formation of the highly reactive $^{\bullet}OH$. While two in vitro studies indicate such an interaction [107, 112], as mentioned above, others do not [18, 79] and whether melatonin scavenges H_2O_2 within cells remains unknown.

Superoxide Anion Radical ($O_2^{\bullet-}$)

According to Marshall et al. [55] and Chan and Tang [18], melatonin is incapable of directly scavenging the $O_2^{\bullet-}$. In the former study, $O_2^{\bullet-}$ was generated by the hypoxanthine-xanthine oxidase system in the presence of either cytochrome C or nitroblue tetrazolium and with/without melatonin. In this system, there was no evidence that $O_2^{\bullet-}$ levels were diminished when melatonin was present. Likewise, in a competition study in which the auto-oxidation of *DL*-epinephrine was stimulated by the xanthine-xanthine oxidase system, melatonin did not retard the conversion of epinephrine to adrenochrome indicating no reduction in the number $O_2^{\bullet-}$ [39]. Using the quenching of the ESR signal of the DMPO-$O_2^{\bullet-}$ adduct as an index of melatonin's ability of scavenge the $O_2^{\bullet-}$, Zang et al. [112] reported that melatonin may have such an action but the effect was minor. One report has proposed a theoretical scheme by which the indolyl radical may neutralize the $O_2^{\bullet-}$ [37].

Singlet Oxygen ($^1O_2$)

$^1O_2$ is a high-energy form of O_2 with considerable potential for damaging macromolecules. Most often $^1O_2$ is generated in photosensitization reactions of substrates including dyes and biological pigments. It has been shown that riboflavin, upon exposure to bright white light, catalyzes the formation of $^1O_2$, which is suppressed by melatonin [79]. Also, melatonin was shown to reduce $^1O_2$-dependent 2,2,6,6-tetramethylpiperidine oxide radical generation during rose bengal photoexcitation [112]. These findings are consistent with the observations that melatonin reduces the cytotoxicity of $^1O_2$ to cerebellar granule cells incubated with the photosensitive dye, rose bengal, and exposed to light [16].

Hypochlorous Acid (HOCl)

HOCl is produced by activated macrophages to kill bacteria; however, it is also capable of damaging other adjacent molecules. In a competition experiment, which assessed HOCl-induced oxidation of 5-thio-2-nitrobenzoic acid (TNB), melatonin was found to reduce the oxidation of TNB [55]. This observation is consistent with melatonin scavenging HOCl. Like Marshall et al. [55], Chan and Tang [18] also provided evidence that melatonin neutralizes HOCl. In their studies they used the absorbance quenching of β-carotene by HOCl as an index of melatonin's ability to detoxify HOCl. The most extensive studies of the interaction of melatonin with HOCl were provided by Dellegar et al. [25]. From their results the authors calculated the rate constant for the deactivation of HOCl by melatonin to be $7.0 \times 10^3\ M^{-1}\ s^{-1}$.

Peroxyl Radicals

Unraveling any interaction of melatonin with the peroxyl radical has been difficult and the outcomes of attempts to do so have been variable. In the first reports which investigated these interactions, Pieri et al. [74, 75] concluded that melatonin may be a more efficient scavenger of the peroxyl radical than is trolox (water-soluble vitamin E). To make their assessments, this group used a fluorescent assay in which β-phycoerythrin was the indicator protein and the peroxyl radicals were generated by the thermal decomposition of water-soluble 2,2′-azo-bis(2-amidino-propane) dichloride.

The findings of Pieri et al. [74, 75] are in contrast to the results obtained by others where melatonin was found to be significantly less efficient in scavenging the lipoperoxyl radical. Marshall et al. [55] utilized ox brain phospholipid liposomes incubated with $FeCl_3$ and vitamin C; in this system melatonin was found to have an IC_{50} of 210 μ*M*.

This seemingly limited ability of melatonin to scavenge lipoperoxyl radicals is consistent with the findings of Livrea et al. [49] who carried out a number of thorough studies. On the basis of their results they concluded that melatonin's ability to scavenger the peroxyl radical was moderate compared to that of vitamin E. The results of Longoni et al. [50] also led them to the conclusion that melatonin is certainly less effective than vitamin E in the systems they used to neutralize the lipoperoxyl radical. In possibly the most thorough study published to date, Antunes et al. [6] claimed that melatonin's ability as a lipoperoxyl radical scavenger is weak.

Overall, the results of the in vitro experiments published to date do not support melatonin as being a potent peroxyl radical scavenger. When vitamin E, a premier peroxyl radical scavenger, was compared with melatonin in terms of their relative abilities to reduce the formation of lipid peroxidation products induced by nitric oxide in brain homogenates, tocopherol was found to be significantly better than melatonin [28]. In vivo, however, melatonin effectively reduces the oxidative breakdown of lipids induced by a variety of means [86, 92]. Melatonin could accomplish this by scavenging the initiating radicals, e.g. the $^{\bullet}OH$, or by means that have yet to be identified. Whether melatonin has the ability to scavenge the peroxyl radical in vivo has not been determined.

Nitric Oxide ($NO^{\bullet}$)

Nitric oxide ($NO^{\bullet}$), a nitrogen-based radical, is believed to cause significant macromolecular destruction under certain experimental circumstances, e.g. cerebral ischemia/reperfusion injury. In the one study where it has been tested, melatonin was found to scavenge $NO^{\bullet}$ [61]. In this cell-free system, 1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene was used to generate $NO^{\bullet}$. Besides melatonin, serotonin as well as N-acetyl-5-hydroxytryptamine and 5-hydroxytryptophan were compared and all were found to reduce $NO^{\bullet}$ in a dose-dependent manner with melatonin being the most effective. Whether melatonin possesses this same capability in vivo has not been tested.

Peroxynitrite Anion ($ONOO^-$) and Peroxynitrous Acid (ONOOH)

The coupling of $NO^{\bullet}$ with $O_2^{\bullet-}$, a reaction that occurs at a diffusion-controlled rate, results in the generation of the peroxynitrite anion ($ONOO^-$). Although not a free radical, this species is highly destructive to nearby macromolecules and has been implicated as an agent contributing to the loss of neurons in amyotrophic lateral sclerosis [9].

That melatonin neutralizes the $ONOO^-$ was originally demonstrated in a cell-free system which depended on the $ONOO^-$-induced oxidation of dihydrorhodamine 123 to rhodamine, a reaction that was reduced in a concentration-dependent manner when melatonin was included in the mixture [32]. In an ancillary in vitro study, this group also reported that melatonin prevented DNA strand breaks normally caused by $ONOO^-$. These in vitro observations have been exploited in whole animal studies by the group of Cuzzocrea et al. [22–24, 27] who have repeatedly shown melatonin to reduce immunocytochemically-detectable nitrotyrosine, a molecule which represents the nitration of tyrosine by $ONOO^-$, in models of inflammation. These studies are consistent with melatonin scavenging of the $ONOO^-$ and are supported by the recent observations of Blanchard et al. [11] who described the in vitro nitrosation of melatonin by peroxynitrite.

The most complete reports related to melatonin's potential interaction with nitrogen-based reactants are those of Zhang et al. [113, 114]. While they found that melatonin is capable of reacting with $ONOO^-$, they also reported that at physiological pH melatonin is only reactive toward peroxynitrous acid (ONOOH) or its activated form, ONOOH*. In this reaction the initial product that is formed is the melatonyl radical. They also showed that one final product resulting from the interaction of melatonin with ONOOH* is 6-hydroxymelatonin. This is the same metabolite that is formed when melatonin is metabolized enzymatically in the liver; this being the case, Zhang et al. [114] mention that urinary 6-hydroxymelatonin is probably not a useful marker of melatonin's in vivo antioxidant activity.

When these studies are considered collectively, it seems apparent that in vivo melatonin does serve as a buffer against molecular damage associated with nitrogen-based toxicants; however, the means by which it does so are not definitively established. Melatonin's protective actions may, in fact, involve direct scavenging of nitrogen-related species and reducing their generation.

Influence of Melatonin on Antioxidative and Pro-oxidative Enzymes

There are several enzymes that are important in metabolizing reactive oxygen species to harmless products thereby reducing oxidative damage. As summarized above, major antioxidative enzymes include SOD, CAT and GPx (fig. 5). At least at pharmacological, but possibly also physiological, levels melatonin has been shown to

either stimulate gene expression for these enzymes or to increase their activity [4]. In this study the mRNA levels of both isoforms of SOD, i.e. MnSOD and CuZuSOD, were increased by 35 and 51%, respectively, after melatonin administration.

SOD is considered a critical antioxidative enzyme inasmuch as it dismutates $O_2^{\bullet}$ to H_2O_2 and, in addition to removing a radical species, i.e. $O_2^{\bullet}$, it also reduces the likelihood of $O_2^{\bullet}$ coupling with $NO^{\bullet}$ to form the highly reactive $ONOO^-$. The overexpression of SOD in the absence of a commensurate increase in either CAT or GPx (or both) may, however, be detrimental, as seems to be the case in individuals with Down syndrome or trisomy 21 [53].

When male rats were treated either acutely (one injection of 500 µg/kg body weight) or chronically (50 or 500 µg/kg body weight daily for 30 days) with melatonin, gene expression for MnSOD and Cu/ZnSOD were both increased in whole brain [5]. Chronic melatonin treatment, however, led to a larger increase in mRNA levels. The authors feel that this action of melatonin probably requires genomic activation and it seems likely these responses were receptor-mediated. In a study in which melatonin was found to protect the brain from oxidative damage, Antolin et al. [5] also reported SOD levels to be elevated suggesting to the authors that, besides protecting neural tissues from damage due to its direct free radical scavenging activity, melatonin may be indirectly protective as well due to its ability to enhance the dismutation of $O_2^{\bullet}$. The activity of this metalloenzyme has been shown to be stimulated in the brain of rat fetuses after the injection of melatonin into the mother [63]. This finding not only supports the stimulatory effect of melatonin on SOD activity but also reaffirms the ability of melatonin to cross from the maternal to the fetal circulation in sufficient quantities to produce physiological changes.

As mentioned above, once formed, H_2O_2 has several fates including its conversion to $^{\bullet}OH$ via the Fenton reaction. Importantly, much of the H_2O_2 generated is utilized as a substrate in other catalytically-supported reactions that convert it to essentially harmless products. The two major enzymes that function in the removal of H_2O_2 are GPx and CAT [38].

The first evidence that melatonin may promote GPx activity utilized pharmacological doses of the indole [8]. In this study, when melatonin was given at a dose of 500 µg/kg body weight, neural GPx activity increased 2-fold in the female rat within 30 min. That melatonin, even at physiological levels may enhance GPx activity was suggested by the observations that when the activity

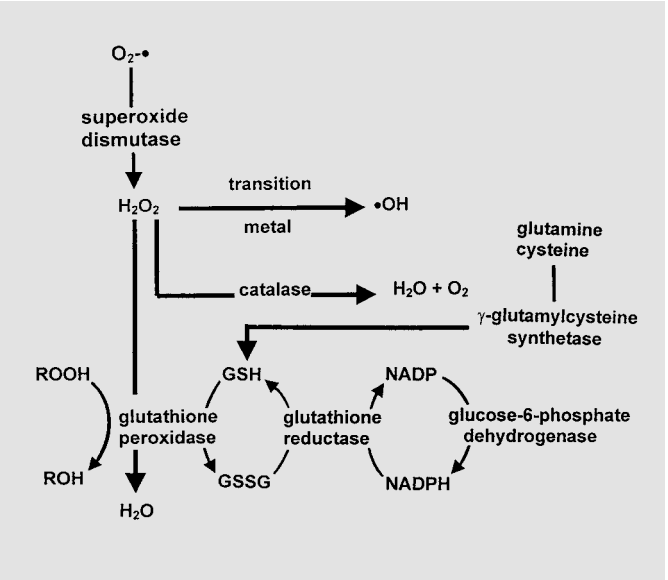

Fig. 5. Actions of melatonin on gene expression and activities of antioxidative enzymes as summarized in the text. Reduced glutathione (GSH) is the major antioxidative thiol in cells. Melatonin promotes glutathione homeostasis by stimulating the enzymes (glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase) involved in its metabolism and recycling. By augmenting glutathione peroxidase, melatonin acts to remove hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH) which are substrates for the enzyme. Additionally, melatonin has been shown to stimulate the rate-limiting enzyme in GSH synthesis, i.e. γ-glutamylcysteine synthetase. Catalase also enzymatically removes H_2O_2 from cells. The effects of melatonin on this enzyme have been infrequently investigated, but some studies have shown that its depressed activity in situations of high oxidative stress is prevented by melatonin. Finally, gene expression for superoxide dismutase has been shown to be stimulated by melatonin.

of this enzyme was compared in the brain of rats killed during the day and night, nighttime levels were higher. This correlated positively with the nocturnal elevation in circulating melatonin concentrations. There are a couple of potential mechanisms by which melatonin may have stimulated GPx activity. It could have preserved the protein from degradation by free radicals or seemingly, more likely, it promoted the activity of the enzyme via a receptor-dependent mechanism. The stimulatory effect of melatonin of GPx activity of the brain may be of particular antioxidative relevance since in this tissue this selenoenzyme plays a much greater role in the detoxification of H_2O_2 than does CAT [43].

Melatonin's ability to enhance GPx activity is not limited to the brain or to mammalian tissues. Pablos et al. [67] found that within 90 min after the injection of a phar-

macological dose (500 μg/kg body weight) of melatonin into chicks, GPx activity increase significantly within 90 min in the lung, gut, kidney, pineal gland, liver, brain, heart, and erythrocytes with the percentage increases ranging from 22 to 138%. This action of melatonin was presumed to involve a nuclear receptor for the indole since the administration of a nuclear melatonin receptor agonist, CGP 52608, also promoted GPx activity to the same level and with the same lag time as melatonin itself [68]. An enhancement of fetal brain GPx activity has also been demonstrated after giving melatonin to the mother's [64].

As in the study utilizing rats [8], Pablos et al. [67] reported that GPx activity in several chick brain areas increased at night coincident with the nocturnal elevation in melatonin. Furthermore, when chicks were kept in light at night to suppress the melatonin rhythm, the nocturnal increase in neural GPx activity was likewise eliminated [67, 69].

In the metabolism of H_2O_2 and hydroperoxides catalytically supported by GPx, GSH is oxidized to its disulfide (GSSG). As mentioned above, more than 99% of the total glutathione within cells normally exists as GSH. Thus, when formed GSSG is quickly recycled back to GSH by the enzyme GRd. Because of this action, GRd is also considered an important antioxidative enzyme [38]. Studies in chicks and mice have shown that, pharmacologically, melatonin enhances GRd activity via mechanisms possibly involving a nuclear receptor [68]. Also, as with GPx, brain GRd activity increases at night in chicks exposed to a light:dark cycle of 14:10 h and, furthermore, this rhythm is suppressed when the birds are exposed to light at night [69]. This implies that the nocturnal increase in GRd activity may be impelled by the endogenous melatonin rhythm.

GRd requires the co-factor NADPH which is generated by the enzyme glucose-6-phosphate dehydrogenase (G6PD). An inadequate supply of NADPH could thereby limit GRd activity and the recycling of glutathione. On this basis, G6PD is classified as an antioxidative enzyme. In general, the evidence indicating a role for melatonin in influencing G6PD is meager, although the one experiment in which a potential association was investigated showed that the indole increased the activity of G6PD [76]. Assuming this finding is valid, collectively the data suggest melatonin may be important in determining glutathione homeostasis within cells. Additionally, Urata et al. [109] recently reported that the total amount of glutathione within cells might also be influenced by melatonin. When human endothelial cells were incubated in the presence of melatonin, the rate-limiting enzyme in glutathione synthesis, i.e. γ-glutamylcysteine synthethase (fig. 5), was stimulated, as were total cellular glutathione levels. This study also revealed that the stimulation of this enzyme by melatonin involved activator protein-1 (AP-1) providing a window into the mechanism of action with regard to glutathione synthesis.

Collectively, the data related to the effects of melatonin on glutathione recycling and synthesis are convincing and imply an important role of melatonin in the maintenance of this crucial antioxidant. While many of the studies utilized pharmacological concentrations of melatonin, the relevance to normal physiology is emphasized by the fact that changes in at least two glutathione-related antioxidative enzymes, i.e. GPx and GRd, correlate with fluctuations in endogenous melatonin levels.

Relatively less is known concerning the effect of melatonin on the other enzyme that catalytically detoxifies H_2O_2 within cells. CAT has broad phylogenetic occurrence; it is found in possibly all aerobic organisms and is normally located in peroxisomes (fig. 2). In mammals, the brain levels of CAT are usually lower than in other tissues [38]. The activity of CAT, like other antioxidative enzymes, is sometimes diminished under conditions of intensive oxidative stress. This drop in CAT activity under such conditions is attenuated when melatonin is present [59]. This may be due to the general protective effect of melatonin on macromolecules including proteins.

Whereas several antioxidative enzymes are enhanced by either pharmacological or physiological levels of melatonin, one potentially pro-oxidative enzyme has been shown to be inhibited by melatonin. Nitric oxide synthase (NOS) generates $NO^{\bullet}$ from arginine. $NO^{\bullet}$, when produced in excess, may be toxic and, furthermore, it combines with $O_2^{\bullet}$ to form a highly toxic agent, $ONOO^-$. In this context, NOS can be considered a pro-oxidative enzyme. Pozo et al. [80] and Bettahi et al. [10] were the first to show that physiological concentrations of melatonin in both in vitro and in vivo conditions inhibit NOS activity. Mechanistically how this is achieved was uncovered by Pozo et al. [81] in a later report. Melatonin reduces NOS activity by binding calmodulin which in turn limits the rise in NOS, a calmodulin-dependent enzyme. The functional relevance of melatonin's inhibition of NOS activity has been documented in an experimental model of ischemia/reperfusion injury [33].

Other Actions of Melatonin Which May Contribute to Its Antioxidative Effects

When melatonin is taken into cellular membranes, it mainly localizes in a superficial position in lipid bilayers near the polar heads of membrane phospholipids [17]. While in this position it obviously is capable of functioning as a free radical scavenger and it may also provide an indirect means by which the membranes can resist oxidative damage. Several studies have shown that melatonin stabilizes cell membrane fluidity thereby preserving their functional efficiency [30, 31]. While this effect may relate exclusively to melatonin's ability to detoxify radicals before they damage membrane proteins and lipids, it is feasible that its position in the membrane may aid in allowing the molecules to interact in such a way that membrane fluidity is optimized. This possibility deserves further examination.

Recent studies also show that melatonin influences the function of mitochondrial complex I and IV [1, 56]; these are involved in oxidative phosphorylation and the mechanisms of action of melatonin in this case are unknown. The implication of the finding is, however, that melatonin increases the efficiency of oxidative phosphorylation. This could be important in reducing oxidative stress by attenuating the electron leakage and the resultant generation of the $O_2^{\cdot}$. For example, inhibition of complex 1 stimulates the production of $O_2^{\cdot}$ [49], H_2O_2 and the $^{\cdot}OH$ [2] and may trigger the death of cells [66]. Thus, stimulation of complex 1 by melatonin may be another indirect means whereby melatonin limits the molecular destruction of essential molecules by reactive oxygen species.

Concluding Remarks

This review briefly summarizes the known processes by which melatonin protects against oxidative damage. It points out that melatonin has several options which it employs to reduce the damage normally inflicted by free radicals. Unfortunately, a very wide variety of processes, e.g. xenobiotic metabolism, environmental pollutants, ultraviolet and ionizing radiation, chemical toxins, and drugs, promote free radical generation. The efficiency of any agent that destroys macromolecules by processes involving free radicals should be decreased if antioxidants, including melatonin, are also present. This has been shown to be the case with melatonin; it has been effectively utilized in vitro and in vivo to combat an incredibly wide number of toxicants including peroxynitrite [22–24, 65], indomethacin [3], alloxan [26], cisplatin [51], glutamate [29], carbon tetrachloride [62], adriamycin [60], hydrogen peroxide [96], amyloid β protein [72, 73], carrageenan [22–24], cerulein [83], nitrilotriacetate [82] and many others [35, 87, 93, 99]. Melatonin has proven equally effective in reducing oxidative damage in conditions where free radical involvement has been established; such situations include ischemia/reperfusion injury [22, 111], biliary obstruction [59], ionizing radiation [110], etc. The antioxidative protection afforded by melatonin has also been observed in organisms as diverse as unicells [4] and mammals [34, 93]. Thus, it would appear that resisting free radical damage is a feature of melatonin in many species, in all organs and against many radical and reactive species.

Despite the wealth of information that has accumulated relative to the antioxidative properties of melatonin, there are a number of critical deficiencies in the knowledge as to some of the resulting products that are formed when melatonin scavengers reactive species, the physiological relevance of melatonin as an antioxidant, and its relative importance compared to other better known endogenous and exogenous free radical scavengers. Whereas some antioxidants are produced outside the organism and must be consumed, e.g. the vitamin antioxidants, and others are endogenously synthesized, e.g. glutathione, melatonin derives from both sources. Thus, its synthesis in all vertebrates is well documented and it also occurs in foodstuffs, sometimes in very high concentrations [41, 90]. Furthermore, when consumed it is readily absorbed [51]. Future research will surely help fill the gaps in the current state of knowledge concerning this novel protector of macromolecules.

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