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AGING AND OXYGEN TOXICITY: RELATION TO CHANGES IN MELATONIN

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ABSTRACT

Melatonin (N-acetyl-5-methoxytryptamine) is a chemical mediator produced in the pineal gland and other sites in the body. The melatonin found in the blood is derived almost exclusively from the pineal gland. Since the pineal synthesizes melatonin primarily at night, blood levels of the indole are also higher at night (5-15 fold) than during the day. Some individuals on a nightly basis produce twice as much melatonin as others of the same age. Throughout life, the melatonin rhythm gradually wanes such that, in advanced age, melatonin production is usually at a minimum. Melatonin was recently found to be a free radical scavenger and antioxidant. It has been shown, in the experimental setting, to protect against both free radical induced DNA damage and oxidative stress-mediated lipid peroxidation. Pharmacologically, melatonin has been shown to reduce oxidative damage caused by such toxins as the chemical carcinogen safrole, carbon tetrachloride, paraquat, bacterial lipopolysaccharide, kainic acid, δ-aminolevulinic and amyloid β peptide of Alzheimer's disease as well as a model of Parkinson's disease involving the drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Additionally, the oxidative damage caused by agents such as ionizing radiation and excessive exercise is reduced by melatonin. Since free radical-induced molecular injury may play a significant role in aging, melatonin's ability to protect against it suggests a potential function of melatonin in deferring aging and age-related, free radical-based diseases. Besides its ability to abate oxidative damage, other beneficial features of melatonin may be important in combating the signs of aging; these include melatonin's immune-stimulating function, its sleep-promoting ability, its function as an anti-viral agent, and general protective actions at the cellular level. Definitive tests of the specific functions of physiological levels of melatonin in processes of aging are currently being conducted.

INTRODUCTION

Free radicals are molecules which have an electron deficit in their outer orbital; the presence of an unpaired electron makes these species highly reactive and often destructive to other molecules within cells. The destructive consequences of free radicals are usually estimated in terms of damage to macromolecules, most notably, lipids, proteins and DNA. The gradual accumulation of the resulting damaged products throughout a lifetime has been proposed to be consequential in the processes of aging and age-related diseases (1,2). This theory, known as the free radical theory of aging, has significant experimental support, although there are other viable theories that also attempt to explain aging processes (3,4). Besides the association of free radicals with aging, they are often considered responsible for a number of age-related diseases as well (5-10).

Interestingly, many of the free radicals of concern are derived from an essential environmental constituent, i.e., oxygen. For this and other reasons, oxygen metabolism has been extensively scrutinized over many decades with the toxic reactions of the molecule generating especially great interest during the last three decades. It is obvious from these studies that, while oxygen is unquestionably far more beneficial than it is detrimental, its toxicity is also substantial. Thus, an entire subdiscipline has arisen to investigate the mechanisms whereby oxygen toxicity can be combated.

OXYGEN TOXICITY

The seemingly paradoxical consequences of the beneficial and harmful effects of oxygen (O_2, dioxygen) have been known for several decades (11). While more than 95% of the O_2 taken in by aerobic organisms is fully reduced to water (H_2O) during the process of mitochondrial respiration, a small percentage (<5%) of the oxygen consumed is converted to semi-reduced species, i.e., the superoxide anion radical, (O_2–•), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) as well as to an activated form of oxygen referred to as singlet oxygen ($^1O_2$) (Fig.1) (12). These species, collectively referred to as reactive oxygen intermediates (ROI), can be highly toxic and they initiate a number of destructive reactions, often with macromolecules, which result in oxidative damage or oxidative stress (13). The most toxic of the ROI is the •OH which is often formed when O_2–• and H_2O_2 are exposed to the trace transition metals iron or copper. Because of their unpaired electron, O_2–• and •OH are referred to as free radicals.

The free radical O_2–• is formed when O_2, itself a diradical (it has two unpaired electrons), accepts a

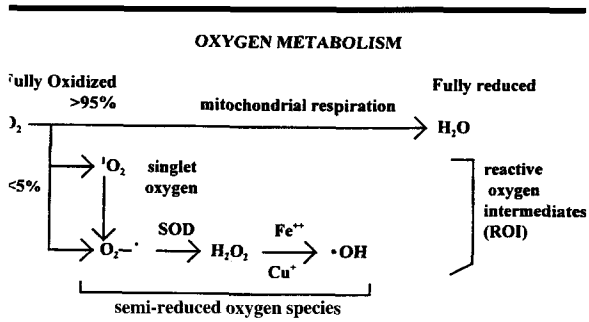

Fig. 1: Semi-reduced reactive oxygen species are produced by the successive one electron reductions of molecular oxygen (O_2). Two of these species are free radicals (O_2–• and •OH), with the •OH exhibiting the greatest toxicity. The semi-reduced oxygen species in combination with an activated form of O_2, i.e., singlet oxygen ($^1O_2$), and H_2O_2 are referred to as reactive oxygen intermediates (ROI). Five percent or less of the oxygen taken into cells is converted to ROI. Whereas usually considered because of their negative effects in cells, ROI can also serve as essential second messengers in certain signal transduction pathways and in the killing of engulfed bacteria by cells that carry out this important function.

single electron. O_2–• formation has given rise to the idea that this free radical is a major reactant in oxygen toxicity (14). While the experimental evidence supporting the O_2–• theory of O_2 toxicity is quite substantial, the precise mechanisms by which this toxicity is exerted is not always apparent. The O_2–• itself has limited reactivity although it has the capability of directly inactivating some enzymes, e.g., mammalian creatine kinase.

O_2–• is formed within cells in a variety of ways. Under usual conditions, perhaps the major source of O_2–• is due to the activity of electron transport chains in mitochondria and endoplasmic reticulum; in this process some of the electrons "leak" directly from the intermediate electron carriers to O_2; as noted in Fig. 1 the percentage of electrons that "leak" from this process is generally agreed to be less than 5% but under increased O_2 concentrations more electrons may escape from the total electron flow through the chains described above (15). In this case, the toxicity may be a result of the excessive production of O_2–• due to the faster electron leakage and the increased autooxidation of molecules. The estimated concentrations of O_2–• is very small, perhaps no more than 10^{-12}-10^{-11}M(16).

Not all O_2–•, however, induce molecular changes that have a negative outcome. Activated phagocytic cells generate O_2–• which are an essential mechanism whereby engulfed bacteria are killed (17). Some examples of cells that utilize the toxicity of O_2–• for organismal benefit include monocytes, neutrophils, eosinophils and a variety of macrophages, including the microglial elements of the central nervous system (18). Also, free radicals function as second messengers in a number of signal transduction pathways (19). Thus, free radicals, although often considered in light of their destructive reactions, also can play important beneficial roles within cells.

The major mechanism for removal of O_2–• from within cells is via its dismutation by a family of enzymes referred to as superoxide dismutase (SOD) (20). These important antioxidant enzymes are ubiquitously distributed and are generally considered as an essential component of the antioxidative defense system of cells (21) although an increase in the activity of these enzymes alone without a concommitant rise in the activities of other antioxidative enzymes may be detrimental, e.g., in Down's syndrome (Trisomy 21) (22), resulting in exaggerated oxidative stress. Conversely, a reduction in the activity of SOD due to a defect in the gene encoding for the enzyme leads to the familial dominant form of amyotrophic lateral sclerosis (23).

The product resulting from the single electron reduction of O_2–• during its dismutation by SOD to H_2O_2, is summarized in the following reaction.

$$2O_2\text{–}\bullet + 2H^+ \rightarrow H_2O_2 + O_2 \qquad [1]$$

Besides this major pathway for the generation of H_2O_2, other enzymes also produce H_2O_2; some of these enzymes are L-amino acid oxidase, glycollate oxidase and monoamine oxidase (MAO). MAO oxidatively deaminates dopamine, the chief catabolic pathway for dopamine in nerve terminals, and may in part be responsible for the free radical destruction of dopaminergic neurons in Parkinson's disease (24).

Under some conditions H_2O_2, a non-radical species, can act as an oxidizing agent. In contrast to O_2–•, however, H_2O_2 also has the capability of readily passing through cell membranes. Thus, the damage eventually inflicted by the product of H_2O_2 metabolism can be at a site distance from where H_2O_2 was produced. The H_2O_2 concentrations within cells varies according to cell type. In hepatic cells it has been calculated that free H_2O_2 generation is on the order of 90 nmol min^{-1} per gm wet tissue weight (25) while the steady state level of H_2O_2 in tissue is on the order of 10^{-7}-10^{-9} M (26). H_2O_2 concentrations are held in check by two enzymes, i.e., glutathione peroxidase (GPx) and catalase (CAT), which metabolize it to non-harmful products. These enzymes are unequally distributed in the organism with the brain containing significantly higher GPx activity than it does CAT (27).

H_2O_2 in the presence of transition metals (most frequently Fe^{2+} but also Cu^{1+}) is quickly converted to the •OH in the following reaction, referred to as the Fenton reaction.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\text{–} + \bullet OH \qquad [2]$$

There are also other schemes in which •OH are generated including during exposure to ionizing radiation. Very high energy electromagnetic radiation causes the homolytic scission of H_2O into a series of products, one of which is the •OH (28). Regardless of how they are induced, •OH react at virtually a diffusion controlled rate with any molecule in the vicinity of where they are generated. It is estimated that the half-life of a •OH *in vivo* at 37° is on the order of 1×10^{-9} sec and that it travels only a few molecular diameters before it terminates in an

interaction with a radical or non-radical species (7). The •OH is not enzymatically destroyed and can only be detoxified by direct radical scavengers.

Since iron in biological systems is almost always bound to a ligand, the generation of •OH via the Fenton reaction is, therefore, site-specific with the molecule most frequently damaged being the ligand itself. This is not true during the radiolysis of H_2O by high energy radiation. As a rule, Fenton chemistry reactions produce •OH with the resulting damage being highly specific, while the molecular destruction produced by •OH generated by the radiolysis of water is more general, or non-specific. As a result, knowing the location of iron (or Cu^{1+}) at the subcellular level helps explain the damage that results from the •OH. Not all radicals produced are destined to inflict damage within cells; some undergo termination (recombination) reactions with other radical species and, therefore, they cause no damage.

Because of the very low concentrations of O_2–•, H_2O_2 and •OH within cells and due to their very short half-lives, these species are difficult to directly measure *in vivo* and their flux is virtually always estimated by using indirect methods. Two general indirect approaches are characteristically used to measure free radical generation. Thus, the administration of exogenous radical traps which form stable and unique products with the radicals which accumulate and can be measured has yielded information on free radical generation in a variety of experimental settings. A second, and perhaps the most common method to indirectly estimate free radical generation, is to quantify the oxidation products that result from free radical interactions with biological targets. Common endpoints include the measurement of oxidized proteins and nucleic acids, and lipid peroxidation products. Additionally, there are some enzymes whose activity is very sensitive to oxidative damage.

A free radical that has stimulated the interest of oxygen biologists is nitric oxide (NO•); the formation of this product is catalyzed by nitric oxide synthase (NOS). NO• is produce especially in vascular endothelial cells and in phagocytes (29) but in other cells as well; it reacts with O_2–• at physiological pH to produce the non-radical product, peroxynitrite ($ONOO^-$), as follows.

$$NO\bullet + O_2\text{–}\bullet \rightarrow ONOO^- \quad [3]$$

$ONOO^-$ is presumed to be directly damaging to cells via the oxidation of thiol groups (30) but, additionally, $ONOO^-$ undergoes decomposition into a variety of products including the •OH (31). While the interactions of NO• with O_2–• are often considered detrimental, there may be circumstances under which O_2–• actually antagonizes the neurotoxic actions of NO• (32).

As already mentioned, free radicals are often considered part and parcel of the process of aging itself and of age-related diseases. While the free radical theory is not the exclusive theory of aging, it does seem to be the most widely accepted. Certainly, the number of age-related diseases which have incriminated free radicals as being at least partially causative is high (6-10,33-35). Perhaps nowhere is this more obvious than in the brain which is not only particularly vulnerable to free radical attack, but the number of neurodegenerative conditions that have been theoretically linked to oxygen toxicity is extensive (10,24,36,37).

THE ROLE OF MELATONIN IN COMBATING OXYGEN TOXICITY

Prevention of the damaging actions of ROI has been an interest of many experimentalists and clinicians in recent decades. The hope is that by reducing the damage that is a consequence of this molecular bludgeoning, diseases related to their damaging effects and the processes of aging itself will be delayed (38,39). The postponement of age-related, free-radical dependent changes could lead to a better quality of life as well as potentially increasing longevity. To date, the only experimental procedure that has been reliably shown to prolong the maximal life span and to reduce oxidative damage in experimental rodents is dietary restriction (40,41). Imposing calorie limits on animals has been effective in keeping them in better health for a greater portion of their life (41). While the mechanisms whereby dietary restriction defer aging processes seem to be highly complex, a reduction in free radical damage in these animals has been suggested (37-39). The applicability of calorie restriction, because of its degree (25-40% reduced food intake), to the human condition, however, has never been considered to be particularly acceptable.

Besides dietary restriction, a second area of interest that has emerged as a potential means to defer aging and its associated diseases is to neutralize free radicals thereby preventing them from damaging macromolecules (42,43). Molecules and or processes that neutralize free radicals or prevent their formation are referred to as antioxidants (44). Antioxidants and antioxidative processes are numerous in most organisms. Antioxidants are either endogenously produced or exogenously generated and then eaten by the organism. The best known antioxidants that are normally consumed by humans are vitamins E (tocopherol), C (ascorbic acid) and β-carotene (45) while endogenously produced antioxidants include glutathione and enzymes such as SOD, GPx and CAT (46). Besides these, there are many other molecules which serve to prevent free radicals from being formed or incapacitate them once they are generated.

Melatonin as a Free Radical Scavenger

A newly-discovered free radical scavenger and antioxidant is the secretory product of the pineal gland melatonin (N-acetyl-5-methoxytryptamine) (Fig. 2) (47). This was first suggested by Ianas and colleagues (48) who investigated melatonin's ability to scavenge free radicals using what is known as the oxygen radical absorbing capacity assay. Soon thereafter, Tan et al (49) extended the finding by showing that melatonin efficiently neutralized the highly toxic•OH. In these studies

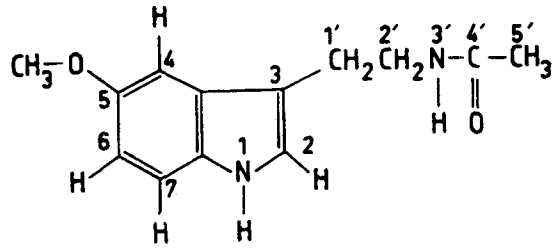

Fig. 2: Melatonin (N-acetyl-5-methoxytryptamine) is a product of tryptophan metabolism. The melatonin measured in the blood of mammals is derived primarily from the pineal gland. Melatonin, in all animals, is produced in a circadian manner with highest production and secretion occurring at night. The acetyl group on the side chain and the methoxy group at position 5 of the indole nucleus are required for maximal free radical scavenging activity of this molecule.

the authors generated •OH by exposing a solution of H_2O_2 to 254nm ultraviolet light. In this system the radicals were prevented from undergoing termination reactions by adding the spin trap 5, 5-dimethyl-pyrroline-N-oxide(DMPO) resulting in the formation of DMPO-•OH adducts; these were quantified by HPLC with electrochemical detection and verified with electron spin resonance spectroscopy. The •OH scavenging capacity of melatonin was then measured by adding it to the reaction mixture; comparisons were made with other known scavengers, i.e., reduced glutathione and mannitol. In this cell free, *in vitro* system melatonin proved to be a better •OH scavenger than either glutathione or mannitol (49).

These observations were followed by a series of studies which investigated melatonin's ability to neutralize another damaging radical, i.e., the peroxyl radical (LOO•) which is formed during the process of lipid peroxidation (Fig. 3) (26). This radical is of major significance to oxidative stress since once produced during the degradation of membrane lipids, it is sufficiently reactive to re-initiate (propagate) the process by oxidizing another lipid molecule in the vicinity of where it is formed. Thus, lipid peroxidation can become a

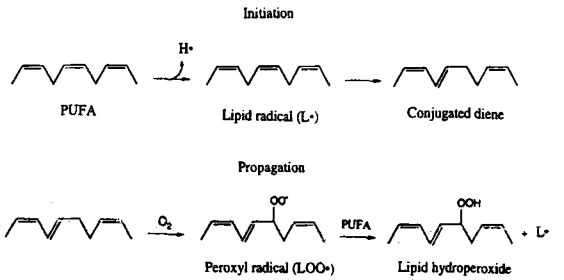

Fig. 3: Lipid peroxidation, summarized in this figure, is devastating to normal cell physiology since the integrity of cellular membranes is disrupted by this oxidative process. Initiating radicals, e.g., the •OH, oxidizes a polysaturated fatty acid (PUFA) resulting eventually in the generation of the LOO• which re-initiates (propagates) and thereby continues the degenerative process of lipid peroxidation. This chain reaction continues until it is interrupted by a chain breaking antioxidant.

progressively damaging chain reaction which is normally interrupted by chain breaking antioxidants, the best of which is reportedly vitamin E (α-tocopherol) (50). According to the studies of Pieri and co-workers (51,52), melatonin is a superior LOO• scavenger than vitamin E; indeed, they estimated melatonin to be twice as effective as tocopherol.

Other authors question the claim by Pieri and collaborators (51, 52) in reference to the efficacy of melatonin as a LOO• scavenger. While Scaiano (53) did no direct measurements, based on his studies he surmised that melatonin would be a good scavenger of the LOO• but he made no specific estimates of its efficacy relative to vitamin E. Somewhat in contrast to Pieri et al (51,52), Marshall and co-workers (54) presumed melatonin was not as effective as vitamin E in scavenging the LOO•. They did, however, as did Chan and Tang (55), find that melatonin does neutralize hypochlorous acid.

On the basis of these studies, there is obviously disagreement as to how effective melatonin is under *in vitro* conditions in detoxifying the LOO•. The indole, however, has been shown to be very effective in reducing lipid peroxidation induced by a variety of toxicants (10, 56, 57) and whether this is due to the direct or indirect antioxidative actions of melatonin remains to be resolved. The protection melatonin affords because it functions as a free radical scavenger and antioxidant has been speculated to relate to processes and diseases of aging (9,36,58).

Melatonin as an Antioxidant

Within a very short time, melatonin has been extensively tested for its ability to resist oxidative processes both *in vitro* and *in vivo*. In this capacity melatonin has been shown to combat oxidative stress to both DNA (59-62) and lipids (63-66) and there is suggestive evidence that it limits the oxidation of protein in the cytosol as well (67). This implies that melatonin concentrations are sufficiently high in lipid-rich membranes as well as in the aqueous cytosol and nucleoplasm to afford significant antioxidative protection. Although not frequently studied, both radioimmunoassay as well as immunocytochemical findings show high levels of melatonin in the nucleus and they generally indicate that intracellular concentrations of melatonin exceed those measured in the blood (68). The reported subcellular distribution is consistent with its high lipid solubility (69) as well as with its ability to dissolve in aqueous media (70).

Many different free radical-generating toxicants have been used to induce oxidative damage and in each case melatonin has proven effective in attenuating the molecular destruction. Some of the most toxic agents which have been utilized and shown to be counteracted by melatonin include the excitatory neurotransmitter analogue kainic acid (71-73), H_2O_2 (74,75), potassium cyanide (76), lipopolysaccharide (60,77,78), hydrophobic bile acids (79), carbon tetrachloride (61), and the carcinogen safrole (63,64). Also, the damage caused by a physical agent, i.e., ionizing radiation (65,80), and

a highly destructive experimental procedure known to generate free radicals, i.e., induced ischemia followed by reperfusion (81-83), has been shown to be reduced by concurrent melatonin administration. In most of these reports, pharmacological doses of melatonin were used to reduce the massive tissue destruction which normally results as a consequence of these devastating insults. A number of reports have, however, shown that even physiological levels of melatonin derived from the pineal gland are capable of protecting against oxidative stress (64,83).

Perhaps of more specific interest to the current review is the ability of melatonin to reduce the signs of neural destruction induced by experimental agents which are used to induce models of human disease. Thus, the Parkinson-like signs induced in animals by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been shown to be significantly reduced by melatonin (84,85). Likewise, amyloid β (Aβ) peptide-induced neuronal death and oxidative damage is overcome by the addition of melatonin to the culture medium (86). Aβ peptide is the agent believed responsible for the loss of some neurons in individuals suffering with Alzheimer's disease (AD), with the destruction of the cells occurring because the 25-35 amino acid residue of the Aβ protein gives rise to free radicals (87). Obviously, both Parkinson's disease (PD) as well as AD are common neurodegenerative conditions in the aging population; these will be discussed more extensively later in this report relative to melatonin. That melatonin may have utility in deferring the onset of these conditions is of widespread interest and because of the ease with which melatonin crosses the blood-brain barrier and its efficacy as an antioxidant, there is a considerable amount of investigation directed at its neuroprotective potential (10,88).

Oxygen-derived free radicals are clearly detrimental to successful aging. These destructive renegades incessantly destroy molecules leading to reduced function and eventually death of cells. These degenerative changes accumulate over time and thereby eventually seriously compromise organismal physiology. The reduction in function induced by oxygen radicals is believed to be a major component of the aging process (2,5). Since melatonin effectively combats oxidative damage, it has been proposed that its availability, or lack thereof, may relate to aging and age-related diseases (9,10,36,58,89).

CHANGES IN MELATONIN LEVELS DURING AGING AND POTENTIAL CONSEQUENCES

The Melatonin Rhythm Throughout Life

Considering the capacity of melatonin to counteract free radicals and the damage they inflict, the loss of melatonin during aging has attracted the attention of a number of biological gerontologists. In all species where it has been investigated, melatonin production by the pineal gland seems to fall steadily once adulthood is reached (90). This was reported for the human in 1981 (91), an observation that has been frequently confirmed (92-94), and at about the same time in the rat (95, 96) Syrian hamster (97-99) and Mongolian gerbil (97). Since then, this age-related decline in melatonin production by the pineal gland has been found in so many species that it is now presumed to be true for all mammals (Fig. 4).

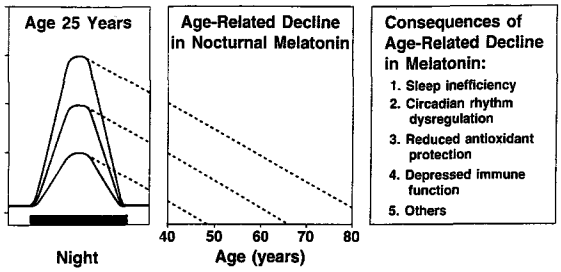

Fig. 4: Age-related changes in the circadian melatonin rhythm. Represented here are the changes believed to occur in humans. In young adults, the amplitude of the melatonin rhythm (which is genetically determined), varies widely between individuals. Thus, some individuals produce more than twice as much melatonin on a nightly basis than other individuals of the same age. Assuming the age-related decline in melatonin proceeds at the same rate in all individuals there are some who, by the time they reach 50 years of age, are essentially devoid of melatonin whereas others, even as octagenarians, still have a low amplitude melatonin rhythm. In the right panel are potential consequences of the loss of melatonin with age. If melatonin is related to aging or onset of age-related diseases, in this scheme it is obvious that some individuals may suffer from signs of aging much earlier than others.

There may be a feature of the melatonin rhythm that makes it particularly relevant to aging in some individuals. There are some individuals who produce, on a nightly basis, twice as much melatonin as other individuals of the same age. The amplitude of the nocturnal melatonin peak is genetically determined (100) and is believed to be stable from one night to the next. Those individuals with a more robust nocturnal increase, over the course of a life time, produce considerably more melatonin than those who have an attentuated melatonin cycle. Whether this is consequential in terms of the total antioxidative defense capacity of these individuals remains unknown.

Beginning with the onset of puberty in humans, melatonin levels begin to wane. According to some authors, the nightly rise in melatonin falls substantially during the 3-4 year period encompassing puberty and, in fact, these workers feel that gonadal maturation may be related to the drop in total melatonin production (101). Once adulthood is reached nocturnal melatonin levels continue to decline gradually such that in elderly individuals there may be a total absence of a nocturnal increase in pineal melatonin production and serum melatonin levels (92). Assuming a similar rate of melatonin decline in all individuals, it is possible that those subjects who had only a weak melatonin rhythm initially are devoid of a circadian melatonin cycle by the time

they are middle-aged while those with initially a very robust cycle still retain a low amplitude melatonin rhythm in advanced age (Fig. 4). If the total quantity of melatonin produced, because of its antioxidant (56, 57) and/or immune-stimulating (102) effects, is relevant in terms of the total antioxidant capacity of individuals, and if free radicals are indeed related to the aging process as suggested (1, 2, 5), then loss of the melatonin rhythm at an early age could impact the rate at which age-related deterioration occurs.

Preservation of the Melatonin Rhythm

There in one experimental paradigm that has been routinely successfully used to prolong the life of rats. This procedure is caloric restriction which requires that the animals have their food intake reduced by up to 40% (103). Not only do these animals live 15-20% longer, but they remain in better health for a greater portion of their life as well (41). Recently, Stokkan et al (104) compared nocturnal pineal melatonin synthesis and serum melatonin levels in ad libitum fed and food-restricted Fisher 344 rats when they were 28 months of age; this is near the maximal life span of ad libitum fed animals. Although not as high as in young animals, the calorie-restricted rats exhibited a significant preservation of their pineal biosynthetic activity based on all parameters measured. Hence, the animals that were restricted in terms of their food intake had higher nocturnal pineal N-acetyltransferase (NAT) activity (the rate limiting enzyme in melatonin production), as well as higher pineal and serum melatonin levels (104). The preservation of pineal biosynthetic activity in these animals was presumed to be a consequence of the higher number of β-adrenergic receptors on the pinealocytes of the underfed rats (105); these receptors are involved in mediating the nighttime rise in melatonin production (106). While the partially preserved melatonin cycle in underfed rats could be used as an argument for the better health and increased longevity of these animals, there is no justification for this conclusion at this time. While the higher melatonin levels could have assisted in deferring aging and decreasing the likelihood of free radical related-diseases in these animals, it is just as likely that the preservation of the melatonin cycle was an effect of underfeeding and unrelated to the fact that these animals survived longer. Until the appropriate studies are done, these observations exist only as correlations with no evidence of a causal association.

Age-related Diseases in which Melatonin has been Studied

There are specific diseases in which free radical production is believed to be elevated. As noted above, individuals with Down's syndrome (Trisomy 21) suffer from excessive oxidative stress because of the increased production of free radicals due to over-expression of the enzyme superoxide dismutase (SOD (22). Humans with Down's syndrome are generally considered to age prematurely and they exhibit a number of diseases, e.g., Alzheimer's disease which may have a free radical component, at an unusually early age (22). They do, however, have no attentuation of their melatonin cycle judging from the urinary excretion of 6-hydroxymelatonin sulfate (107), the chief hepatic metabolite of melatonin (108).

In another disease where free radicals may be involved, however, a relative melatonin deficiency may be factor in the progress of the disease. Acute intermittent porphyria (AIP) is a dominant inherited condition in which there are defects in the heme biosynthetic pathway, which leads to the accumulation of a heme precursor, δ-aminolevulinic acid, (ALA); ALA undergoes enolization at pH <7.0 and subsequently autooxidizes with the generation of both oxygen and carbon-based free radicals (109).

The increase in free radical generation results in damage to a number of organs and this is the basis for some of the pathophysiology of AIP. Recently, Puy and colleagues (110, 111) reported that individuals with AIP have an attenuated nighttime increase in plasma melatonin levels associated with an increase in circulating tryptophan levels; tryptophan is a precursor of melatonin (106). Puy et al (111) also showed that the lower than normal nighttime rise in melatonin in people with AIP is probably due to an inhibition of pineal melatonin synthesis by ALA. This conclusion was based on studies using the rat pineal gland wherein they showed that ALA, like γ-aminobutyric acid, inhibited the activity of the rate limiting enzyme in melatonin synthesis, i.e., N-acetyltransferase. Since the activity of this enzyme closely correlates with blood melatonin concentrations, the mechanism whereby ALA reduces melatonin may well be as Puy and co-workers have predicted.

Regardless of the mechanism, the reduction of melatonin in people with AIP may exaggerate the severity of the condition since melatonin could serve to neutralize the free radicals and thereby reduce the tissue damage that is so common in these individuals. Experimentally, the exposure of rat tissues of ALA led to large increased in oxidatively damaged lipid products (malondialdehyde and 4-hydroxyalkenals). The damage, which was measured in kidney, liver and brain, was significantly reduced when melatonin was also given (R.C.G. Carneiro and R.J. Reiter unpublished). These studies were conducted both *in vitro* and *in vivo* and suggest that reduced melatonin synthesis in AIP patients may contribute to organ deterioration and dysfunction since they have a compromised antioxidative defense system. The severity of this condition may get progressively worse during aging as endogenous melatonin levels additionally drop.

Age-related Disease Models in which Melatonin has been Tested

Two age-associated neurodegenerative diseases, i.e., AD and PD, have been of special interest to free radical biologists since they have both been speculated to involve oxidative damage to neurons (87, 112-115).

While these diseases undoubtedly involve highly complex pathophysiological processes which have not rapidly yielded to intensive investigative effort, it seems likely free radical-induced cell loss is a process related to these conditions. In AD this seems to be a consequence of amyloid β (Aβ) protein while in PD it is the autooxidation of dopamine which leads to cell death.

As already briefly summarized above, Pappolla and colleagues (86, 116) recently scrutinized the potential utility of melatonin in preventing the cyctotoxic actions of Aβ protein in an *in vitro* preparation. In this case, murine neuroblastoma (N2a) cells were grown in the presence of the 25-35 amino acid residue of the Aβ protein; this constituent is known to be the active portion of the molecule in terms of free radical generation. Control N2a neurons were incubated in the same amino acids constituting the toxic Aβ fragment but the amino acids were scrambled, thereby rendering them essentially inactive in terms of free radical generation. At the end of the 24h incubation period, 50mM Aβ protein had killed, by apoptosis, roughly 80% of the neurons while in the controls, where the scrambled peptide was used, only a small percentage (roughly 10%) of the cells had died. If 10mM melatonin had been added to the medium containing cells that were incubated with the cytotoxic Aβ fragment neuron death was reduced to levels seen in the controls. To further support the idea that free radicals were probably accounting for the neuronal death in this study, Pappolla et al (86, 116) included neurons that were incubated in the presence of adriamyrin, a known free radical generator. This drug, like the Aβ fragment, also killed a large percentage of the neurons.

This is not the first study in which inhibition of apoptosis and been found to be a feature of melatonin treatment. *In vivo* as well, albeit in non-neuronal tissues, both Cagnoli and co-workers (66) and Sainz and colleagues (117) have reported that melatonin reduced steroid-induced apoptosis in the thymus of maturing rats and $^1O_2$-induced apoptotic cell death in the rat brain, respectively. The specific molecular mechanisms whereby melatonin alters the intracellular events leading to apoptosis are of great interest and are currently under investigation.

In the experiments of Pappolla on co-workers (86, 116) neuronal death due to apoptosis was not the only parameter measured that suggested the involvement of free radicals. They also measured lipid peroxidation products in cells treated with Aβ without or with melatonin and found oxidatively-damaged lipids were in much higher concentrations when melatonin was absent from the incubation medium, i.e., melatonin had also inhibited the peroxidation of lipids in the cell membranes of N2a cells. A spin-trapping agent, α-phenylnitrone, like melatonin, also reduced the damaged lipid products. Alpha-phenylnitrone, like melatonin, neutralizes free radicals.

Finally, Pappolla et al (86) showed that the large increases in $[Ca^{2+}]_i$ induced by Aβ-protein were also essentially eliminated when melatonin was present in the incubation medium. High $[Ca^{2+}]_i$ levels are toxic to neurons and, in this case, were believed to be a consequence of damage to calcium channel proteins induced by free radicals generated as a consequence of the Aβ protein; melatonin's protective action against this response was assumed to be because it maintained the integrity of the calcium channels.

Collectively, the finding of Pappolla and colleagues (86, 116) are of great interest and potential importance since, as already noted, melatonin is an effective free radical scavenger and, importantly, it crosses the blood-brain barrier and enters neurons with ease. Thus, it may be capable of reducing some of the neuronal damage that accompanies AD. Of particular note is that, in the aged, where AD obviously prevails, melatonin levels are known to be substantially depressed relative to young individuals. In a rather poorly controlled study, it may be noteworthy that melatonin levels were found to be very low in individuals who died of AD (118). Whether chronically low melatonin levels, however, are associated with AD cannot be assumed from this study.

The loss of nigrostriatal dopaminergic neurons due to cytotoxic free radicals is believed to account, in part, for the neurodegenerative movement disorder of PD (24, 114). Assuming that melatonin, because of its anti-oxidative actions, may assist in reducing neuronal damage due to the autooxidation of dopamine, Miller et al (119) tested this possibility using what has come to be known as the oxygen radical absorbance capacity (ORAC) assay; this method measures the oxidation of the fluorescent protein porphyridium cruentum β-phycoerythrin (β-PE) in the presence of oxidizing agents such as dopamine. When melatonin was added to the ORAC assay it significantly decreased the oxidation of β-PE induced by the presence of dopamine. Miller and colleagues (119), therefore, surmised that, in view of melatonin's free radical scavenging ability it could potentially play a role in deferring dopaminergic cell loss due to catecholamine autooxidation such as occurs in PD.

One recently published report also suggests a similar action of melatonin *in vivo* in protecting against MPTP (1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine) neurotoxicity. The administration of MPTP induces Parkinson-like signs in rodents and is an often used model of PD (120). When given to mice, Acuña-Castroviejo et al (84) reported a loss of immunoreactive tyrosine hydroxylase activity in the striatum, evidence of neural damage in several brain areas, and increased lipid peroxidation in the hippocampus and striatum within 4 hours. In mice treated with melatonin (10 mg/kg) 30 min prior to MPTP administration, the signs of MPTP-neurotoxicity were prevented. MPTP, after being taken up by brain cells, presumably glial elements, is metabolized to MPP+ which eventually enters neurons where it generates free radicals which cause the PD-like neurodegenerative changes (Fig. 5) (121). Absent from this study was a measure of nigrostriatal dopamine levels so whether

melatonin would have prevented the drop of this catecholamine, as suggested by the study of Miller et al (119), under *in vivo* conditions remains to be investigated.

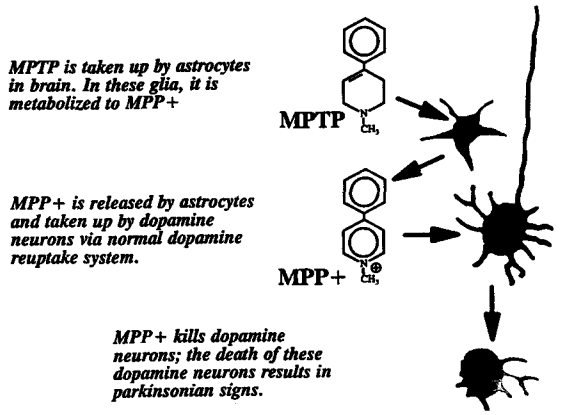

Fig. 5: The means by which MPTP, when injected into animals or ingested by humans, is believed to induce loss of dopaminergic neurons is summarized here. The MPTP metabolite, MPP^+, when finally taken up by dopaminergic neurons, is believed to kill these cells via free radical mechanisms. Antioxidants that effectively pass the blood-brain barrier and enter neurons could potentially protect against dopaminergic degeneration and, thus, the signs of Parkinsonism.

Because of the age-associated drop in pineal and blood melatonin concentrations, old individuals in the population are generally considered as being relatively melatonin-deficient (58, 88, 90). Since melatonin is generally accepted as being an effective neural antioxidant (9, 10, 47), it would be expected that a melatonin-deficiency may be associated with an increased susceptibility of the brain to free radical damage. To test this possibility was the goal of a recent study by Manev and colleagues (83). Pinealectomized rats, which have low circulating melatonin levels and no nocturnal rise in blood melatonin concentrations, and intact controls were subjected to models of induced neurodegeneration which are known to involve free radical cytotoxicity. Using a model of transient ischemia and reperfusion which involved temporary middle cerebral artery occlusion, Manev et al (83) showed that the degree of neural damage was more extensive in pinealectomized than in control rats. Likewise, the same result was observed when rats were treated with KA which induced seizures and neuronal cell death; the neuropathological changes were again more extensive in the melatonin-deficient rats. The implication of these findings, as emphasized by Manev et al (83), is that the nervous system of elderly individuals may exhibit an increased vulnerability to stroke and excitotoxic cell death because they essentially lack a neuroprotective agent, i.e., melatonin. The findings are also consistent with the idea that endogenous, physiological levels of melatonin play a significant role in protecting against oxidative damage to the brain and possibly to other organs as well.

FINAL COMMENT

While definitive evidence linking the age-associated reduction in melatonin with either aging, longevity or age-related diseases has yet to be provided, there is suggestive evidence for these assumptions. Melatonin's potentially beneficial effects against the degenerative signs of aging relate not only to its free radical and antioxidant activities (9, 36, 47), but also to its ability to synchronize circadian rhythms (122) promote sleep (123), stimulate immune function (102), and to its general protective effect in cells (124) (Fig. 4). It does seem likely that melatonin has beneficial effects in all cells to help them cope with the devastating actions of toxins (60, 62, 64), to resist viral infections (125, 126), and to overcome debilitating changes associated with aging (84, 86, 116). How effective physiological or pharmacological levels of melatonin will eventually prove to be in terms of protecting against the inevitable and insidious changes of aging is currently being intensely investigated.

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