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MINI REVIEW

One molecule, many derivatives: A never-ending interaction of melatonin with reactive oxygen and nitrogen species?

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Abstract: Melatonin is a highly conserved molecule. Its presence can be traced back to ancient photosynthetic prokaryotes. A primitive and primary function of melatonin is that it acts as a receptor-independent free radical scavenger and a broad-spectrum antioxidant. The receptor-dependent functions of melatonin were subsequently acquired during evolution. In the current review, we focus on melatonin metabolism which includes the synthetic rate-limiting enzymes, synthetic sites, potential regulatory mechanisms, bioavailability in humans, mechanisms of breakdown and functions of its metabolites. Recent evidence indicates that the original melatonin metabolite may be N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) rather than its commonly measured urinary excretory product 6-hydroxymelatonin sulfate. Numerous pathways for AFMK formation have been identified both in vitro and in vivo. These include enzymatic and pseudo-enzymatic pathways, interactions with reactive oxygen species (ROS)/reactive nitrogen species (RNS) and with ultraviolet irradiation. AFMK is present in mammals including humans, and is the only detectable melatonin metabolite in unicellular organisms and metazoans. 6-Hydroxymelatonin sulfate has not been observed in these low evolutionary-ranked organisms. This implies that AFMK evolved earlier in evolution than 6-hydroxymelatonin sulfate as a melatonin metabolite. Via the AFMK pathway, a single melatonin molecule is reported to scavenge up to 10 ROS/RNS. That the free radical scavenging capacity of melatonin extends to its secondary, tertiary and quaternary metabolites is now documented. It appears that melatonin's interaction with ROS/RNS is a prolonged process that involves many of its derivatives. The process by which melatonin and its metabolites successively scavenge ROS/RNS is referred as the free radical scavenging cascade. This cascade reaction is a novel property of melatonin and explains how it differs from other conventional antioxidants. This cascade reaction makes melatonin highly effective, even at low concentrations, in protecting organisms from oxidative stress. In accordance with its protective function, substantial amounts of melatonin are found in tissues and organs which are frequently exposed to the hostile environmental insults such as the gut and skin or organs which have high oxygen consumption such as the brain. In addition, melatonin production may be upregulated by low intensity stressors such as dietary restriction in rats and exercise in humans. Intensive oxidative stress results in a rapid drop of circulating melatonin levels. This melatonin decline is not related to its reduced synthesis but to its rapid consumption, i.e. circulating melatonin is rapidly metabolized by interaction with ROS/RNS induced by stress. Rapid melatonin consumption during elevated stress may serve as a protective mechanism of organisms in which melatonin is used as a first-line defensive molecule against oxidative damage. The oxidative status of organisms modifies melatonin metabolism. It has been reported that the higher the oxidative state, the more AFMK is produced. The ratio of AFMK and another melatonin metabolite, cyclic 3-hydroxymelatonin, may serve as an indicator of the level of oxidative stress in organisms.

Key words: antioxidants, bioavailability, free radical, melatonin, metabolism, oxidative metabolites, synthetic regulation

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Introduction

Melatonin, a tryptophan derivative, was first isolated from bovine pineal glands and was structurally identified in 1958 [1]. Subsequently, melatonin was found to be a sleep promoter [2], a chemical signal of light and darkness (Zeitgeber) as well as a regulator of photoperiod-dependent seasonal reproduction in some vertebrates. Using the fluctuating endogenous melatonin signals, vertebrates synchronize both their circadian rhythms and their circannual reproductive activities [3, 4]. Thus, the daily and seasonally changing melatonin rhythms are involved in signaling 'time of day' and 'time of year' and, thus, they serve as a bioclock and a bio-calendar in vertebrates [5].

Melatonin is also a potent, endogenously produced and diet-derived free radical scavenger and broad-spectrum antioxidant [6, 7]. The pineal production of melatonin in vertebrates exhibits an unambiguous circadian rhythm with its peak near the middle of scotophase and basal levels during the photophase. The amount of melatonin produced by the pineal gland of mammals changes as animals age. The tendency is that pineal melatonin production wanes with advanced age. In humans, melatonin production not only diminishes in the aged but also is significantly lower in many age-related diseases including Alzheimer's disease [8, 9] and cardiovascular disease [10–13].

Understanding the metabolism of melatonin will help to explain the multiple functions of melatonin in organisms. Here, we discuss several important issues related to melatonin metabolism, which differ in their interpretation from some conventional opinions.

Melatonin biosynthesis

Pathways of melatonin synthesis

The initial precursor of melatonin biosynthesis is an amino acid, tryptophan. Via several enzymatic steps including tryptophan 5-hydroxylation, decarboxylation, *N*-acetylation and *O*-methylation, in that sequence, *N*-acetyl-5-methoxytryptamine (melatonin) is synthesized. Alternatively, but at lower flux rates, melatonin can be formed via *O*-methylation of serotonin and subsequent *N*-acetylation of 5-methoxytryptamine, or by *O*-methylation of tryptophan followed by decarboxylation and *N*-acetylation [14]. A detailed pathway of melatonin biosynthesis is illustrated in Fig. 1.

Rate-limiting enzymes?

During melatonin synthesis, two enzymes are believed to play important roles in its production; they are arylalkylamine *N*-acetyltransferase (AA-NAT) and hydroxyindole *O*-methyltransferase (HIOMT). AA-NAT is often stated to be the rate-limiting enzyme in melatonin biosynthesis. The reasons are that its activity exhibits the same circadian rhythm as does pineal melatonin level in many animals and both the enzyme activity and pineal melatonin levels are suppressed by light. Several excellent reviews have summarized these topics in detail [15–17]. However, recently, this predominant opinion has been challenged. Evidence directly and indirectly shows that rather than AA-NAT, HIOMT might be a rate-limiting enzyme for melatonin biosynthesis. Ribelayga et al. [18] observed that melatonin production in Siberian hamster pineal gland is not correlated with AA-NAT activity but rather was more directly related to the activity of HIOMT. In the follow-up study, this group confirmed, also in Djungarian hamsters (*Phodopus sungorus*), that elevated AA-NAT activity stimulated by both adrenergic α- and β-receptor agonists failed to promote melatonin production while the increased activity of HIOMT was positively related to pineal melatonin production [19]. Thus, they proposed that the rate of melatonin production, once initiated by AA-NAT activation, is limited by the level of HIOMT activity. Even at high AA-NAT activity, any change in HIOMT activity would result in a similar modification in melatonin production. A phenomenon of dissociation between the AA-NAT gene expression and melatonin production is also observed in the pineal gland of the sheep [20].

Recently, Liu and Borjigin [21] examined whether AA-NAT is a rate-limiting enzyme of melatonin biosynthesis using a genetic mutant rat model, i.e. a Long Evans cinnamon (LEC) rat. In the LEC rat, a point mutation in AA-NAT gene leads to a change in a conserved histidine residue to tyrosine at position 28 of the polypeptide chain. The H28Y mutation leads to a low AA-NAT expression

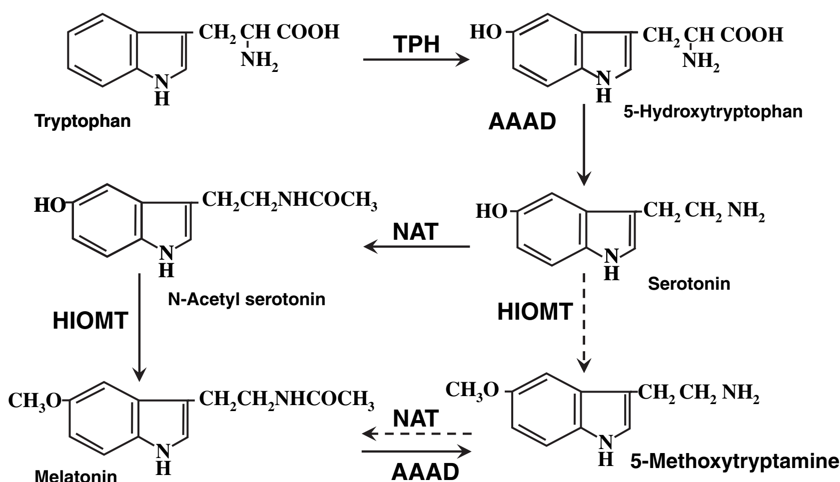

Fig. 1. Biosynthetic pathways of melatonin. TPH, tryptophan hydroxylase; AAAD, aromatic amino acid decarboxylase; AAA, aryl acylamidase; NAT, *N*-acetyltransferase; HIOMT, hydroxyindole *O*-methyltransferase.

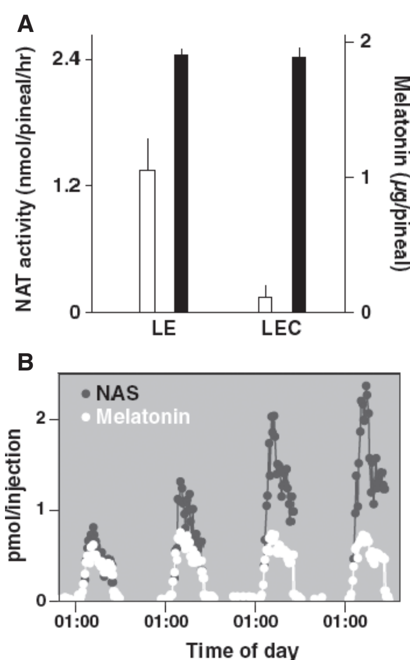

Fig. 2. (A) Melatonin levels in LEC rats. The open bars are *N*-acetyltransferase activity, while the black bars are the levels of melatonin. LE, Long Evans rats, n = 14; LEC, Long Evans cinnamon rats, n = 15; all rats were 2 months of age. (B) Simultaneous measurements of *N*-acetylserotonin (NAS) and melatonin in the initial days of long-term pineal microdialysis. Shown are four consecutive days of NAS and melatonin in a single rat pineal. The total amount of melatonin is much lower than NAS and does not correlate to the levels of NAS.

and also less stability of the AA-NAT protein. Compared with their wild-type counterparts (Long Evans rats, LE rats), pineal AA-NAT activity in LEC rat is reduced by 90%; however, melatonin synthesis in the LEC rat is not changed by this large reduction in AA-NAT activity. Pineal melatonin levels in LE and LEC rats are very similar (Fig. 2A). In the same study, with use of long-term pineal microdialysis, Liu and Borjigin [21] also observed that the levels of *N*-acetylserotonin (NAS), a product of AA-NAT, have little relation with pineal melatonin levels (Fig. 2B). Despite very high levels of NAS in the pineal gland, melatonin levels remain low. This strongly suggests that downstream mechanisms after AA-NAT are involved in the regulation of melatonin synthesis. Parenthetically, in some evolutionarily low-ranked organisms, such as insects, NAS concentrations exceed melatonin by three orders of magnitude; thus, *O*-methylation is a rate-limiting step in melatonin biosynthesis [22].

Based on these observations it appears that AA-NAT is not the exclusive rate-limiting enzyme for melatonin biosynthesis at night and HIOMT participates in controlling melatonin production. As a legitimate rate-limiting enzyme, HIOMT must satisfy the following criteria. Its gene expression and activity should have an obvious circadian rhythm which positively correlates with melatonin production and its level or activity should be subjected to light regulation, i.e. light exposure at night should inhibit its activity. Sparse information documents that HIOMT fulfills these criteria and more research is required to clarify which of the enzymes, AA-NAT or HIOMT, plays the key role in regulating the quantity of melatonin production.

Sites of melatonin synthesis

Originally, melatonin was believed to be synthesized exclusively in pineal gland of vertebrates. Studies have shown that many extrapineal tissues and organs have the capacity to synthesize melatonin. These include retina [23], ciliary body [24], lens [25], Harderian gland [26], brain [27], thymus [28], airway epithelium [29], bone marrow [30, 31], gut [32, 33], ovary [34], testicle [35], placenta [36] lymphocytes [37] and skin [38]. It is estimated that melatonin generated from gut is in excess of two orders of magnitude greater than that produced by the pineal gland [33]. Highly elevated levels of melatonin, compared with those in serum, have been documented in the bone marrow, bile and third ventricular fluid of rats and sheep [30, 39–41]. Recently, Fischer et al. [38] showed that the cultured HaCaT keratinocytes contain unexpectedly high levels of intracellular melatonin. It was calculated that a single keratinocyte contains about 34 pg melatonin (146 pmol/1000 cells). The melatonin levels in keratinocytes are much higher than those in pinealocytes of rat or hamsters during the scotophase, i.e. at their melatonin peaks. The peak levels of melatonin in the pineal gland are around 1500–2000 pg per pineal gland. The melatonin content of the rat/hamster pineal gland at their peak time is only equivalent to amount in 45–60 keratinocytes. A pineal gland contains thousands of pinealocytes. Assuming that the cell volume of a HaCaT keratinocyte is 1.43×10^{-6} μL [42, 43], the calculated intracellular melatonin concentration in this cell, based on the references mentioned above, should be approximately 102 mM. This calculated value for melatonin is much higher than that of the glutathione level in cells. Glutathione is usually believed to be in the highest concentration of the endogenously produced antioxidants. Extremely high levels of melatonin also have been reported in the shed exoskeleton (molted skin) of an insect (*Periostracum cicadae*). The measured melatonin level in the molted skin of cicadae is 3772 ng/g [44]. If the melatonin concentration of a keratinocyte is confirmed by other researchers, considering the size of the skin which actively participates in melatonin synthesis, the amount of melatonin produced by mammals would be orders of magnitude higher than previously expected.

An interesting phenomenon is that the potentially large quantity of extrapineal melatonin appears not to contribute significantly to the melatonin circadian rhythm in the circulation since surgical pinealectomy or chemical pinealectomy (constant light exposure), all markedly diminish the circulating melatonin levels in vertebrates [45, 46]. Thus, extrapineal melatonin, despite its large quantity, does not serve as a chemical signal of light/dark. It is logical that trace or small amounts of pineal melatonin, via receptor-mediated mechanisms, could enforce circadian changes of organisms. In contrast, extrapineal melatonin synthesis is not subjected to light/dark regulation. We speculate that the locally generated melatonin is consumed by the tissues in which it is produced as a protective mechanism of

oxidative stress. Especially in the gut and skin, both of which are continuously exposed to the hostile outside environments such as food pollutants, bacteria, parasites, toxins and ultraviolet (UV) or other irradiation. The function of locally produced high levels of melatonin may be to help them cope with these stressors as a paracoid, antioxidant, and anti-inflammatory agent [47].

In addition to its consumption, some extrapineal melatonin might be bound to melatonin-binding proteins as a storage mechanism. This binding mechanism may inhibit the free movement of melatonin from the cells in which it is produced into the circulation. If there is no local melatonin storage mechanism, it would be difficult to accept such high intracellular melatonin levels as present in HaCaT keratinocytes as the solubility of melatonin in the aqueous phase is low. Melatonin binding proteins have been tentatively identified in several tissues of rats [48].

Stress-induced and stress-consumed melatonin

As a protective mechanism for stress, organisms evolved to elevate their melatonin levels by stimulation of its biosynthesis. This was observed in the case of diet restriction (DR) in animals from rodents to the primates. DR is defined as a low-intensity stressor. It is widely accepted that the low-intensity stresses are beneficial factors for an organism's survival. They prepare organisms to more easily adapt to potentially catastrophic disasters. Stokkan et al. [49] observed that pineal and serum melatonin levels in long-term DR Fisher 334 rats were significantly higher than those of their age-matched, ad libitum-fed counterparts. In addition to melatonin levels, AA-NAT activity in pineal gland of these DR rats also significantly increased compared with their age-matched ad libitum-fed counterparts. The preserved serum melatonin circadian rhythms and levels have also been noted in long-term (12–15 yr) DR rhesus monkeys [50, 51]. Many other stressors including cold stress in a unicellular protist, plants and animals [52–54], swimming in rats [55] and moderate exercise in humans [56, 57] also cause increases in serum melatonin levels. Direct evidence that oxidative stress upregulates the gene expression of melatonin synthetic enzyme, AA-NAT, was provided by Jaworek et al. [58]. They observed that pancreatic oxidative stress induced by caerulein injection in rats resulted in a 2.5-fold rise of AA-NAT mRNA (Fig. 3).

To explore the relationship of melatonin production and oxidative stress, Afreen et al. [59] exposed the roots of a plant, *Glycyrrhiza uralensis*, to UV-B, a strong oxidative stressor because it induces the homolysis of hydrogen peroxide (H_2O_2) to form the hydroxyl radical ($^{\bullet}OH$); they reported that melatonin production in the roots which were exposed to UV-B irradiation increased several-fold over the controls. Melatonin levels in the UV-B-irradiated roots reached approximately 80 μg/g roots. The elevated melatonin levels were believed to be a response to the stress of UV-B exposure and a protective mechanism of plants against the generated free radicals [59].

It appears that stress-promoted melatonin production may be a universal phenomenon as it occurs in unicellular organisms, plants and animals including the human. The exact molecular mechanisms for this event are currently unknown. It likely involves the AP-1 pathway. Structural analysis of the human HIOMT gene promoter shows that it contains an AP-1 site at −166 position [60]. Computer sequence analysis also reveals a promoter-like region located within 530 bases upstream of the translational site consisting of TATA boxes, upstream promoter elements, regulatory elements and enhancer regions such as AP-1 transcription factor-binding site in the AA-NAT mouse gene [61]. AP-1 seems to promote both of these important enzymes to enhance melatonin synthesis. AP-1 is a transcription factor that can be regulated by oxidative stress in many cell types [62]. Stress uniformly stimulates glucocorticoid production in organisms [63]. Glucocorticoids upregulate the transcriptional activity of stress-responsive transcriptional factor AP1 and thus upregulate gene expression for melatonin synthesis. A reasonable explanation for upregulation of gene expression for melatonin and its enhanced production induced by stress is that the elevated melatonin would protect organisms against the potential damages caused by the stresses; alternatively, these responses may precondition organisms to readily cope with the subsequent more excessive or catastrophic stressors such as seen with gastric ulcers in water immersion-stressed rats [64] or ischemia/reperfusion heart injury [65–67].

If stress is intensive and if the consumption of melatonin is in excess of its synthesis, endogenous melatonin levels will rapidly fall. This has been documented in several reports. These reports reveal an unusual phenomenon of melatonin metabolism, i.e. an unaltered or elevated AA-NAT activity in pineal glands vs. reduced pineal and serum melatonin levels in animals subjected to different stresses. Wu et al. [68, 69], Ueck et al. [70] and Troiani et al. [71, 72] reported that high night-time melatonin levels in the pineal gland and in serum dropped precipitously when rats were forced to swim (15 min continuously swimming) without any

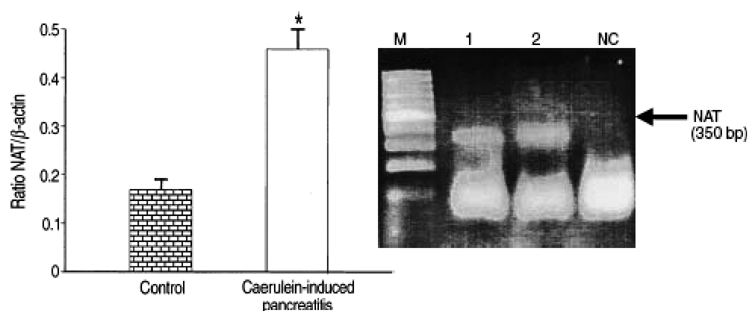

Fig. 3. The ratio of arylalkylamine *N*-acetyltransferase to β-actin (350 bp) mRNA in the pancreatic tissue of control rats (lane 1) and those subjected to caerulein-induced pancreatitis (lane 2). M: molecular weight marker (350 bp). Asterisk indicates significant ($P < 0.05$) change, when compared with the control. Mean ± S.E.M. of three separate experiments.

reduction in pineal AA-NAT activity. In some cases, under the forced swimming stress, AA-NAT activity in the pineal was doubled compared with that in control rats. In contrast to AA-NAT activity, pineal melatonin content in swimming rats rapidly dropped to less than half that in control rats [71]. It appears that the elevated oxidative stress caused by swimming results in a rapid discharge of pinealocytic melatonin into the circulation where it was quickly taken up by cells to defend against free radicals that were augmented because of swimming stress. The following evidence clearly shows that a rapid melatonin fall after stress is due to consumption rather than to its reduced synthesis. Wu et al. [68] observed that when isoproterenol was injected into rats during the daytime both the activity of AA-NAT and the melatonin production in pineal and in serum were significantly elevated. When these animals with highly augmented melatonin levels were subjected to a forced swimming stress, both elevated pineal and serum melatonin dropped in parallel; again this occurred without any reduction in pineal AA-NAT activity which remained elevated.

A similar phenomenon has been observed in rats exposed to toxic chemical stress. Pohjanvirta et al. [73] found that exposure of LE rats to an extremely toxic environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was accompanied by a rapid reduction in circulating melatonin levels both during day and at night. In the follow-up study [74], they confirmed, in another rat strain (Han/Wister rats), that TCDD administration also resulted in a similar rapid but persistent reduction in circulating melatonin.

To explore the potential mechanisms of altered melatonin metabolism after TCDD administration, this group analyzed the synthetic function of the pineal gland, the melatonin metabolic capacity of the liver (in which a substantial portion of melatonin is believed to be biotransformed to 6-hydroxylmelatonin sulfate) and the chief metabolites of melatonin excreted from urine in TCDD-sensitive and -resistant stains of rats under the stress of TCDD [75]. The results surprisingly indicated that pineal function and its melatonin content were not altered; the melatonin clearance rate in liver was not enhanced by TCDD, and more surprisingly, the major urinary metabolite of melatonin, 6-hydroxylmelatonin sulfate, was even reduced. From these results, it is obvious that the reduced circulating melatonin in TCDD-treated rats was not explained by conventional mechanisms of melatonin metabolism. Based on these observations they claimed that an accelerated, extrahepatic, peripheral melatonin breakdown must have occurred after treatment with TCDD.

At the time, an explanation for the reduction in melatonin after TCDD injection was not available. Currently, the apparent paradox of unaltered or high AA-NAT activity in the pineal gland and low level of melatonin in the circulation can be explained by the fact that melatonin functions as a free radical scavenger. Forced swimming, because it is stressful, results in transient and marked increase in reactive oxygen species (ROS)/reactive nitrogen species (RNS) generation including superoxide anion ($O_2^{\bullet-}$), H_2O_2, $^{\bullet}OH$, and nitric oxide ($NO^{\bullet}$). It is unavoidable that endogenously produced melatonin, as a free radical scavenger, would interact with the elevated ROS and be metabolized to oxidative melatonin derivatives (see below). TCDD metabolism in organisms produces profound and long-lasting oxidative stress with a dramatic rise in ROS/RNS [76, 77]. Thus, a rapid reduction and persistent lower serum melatonin levels would be expected after TCDD administration as observed by Pohjanvirta et al. [75].

More direct evidence that oxidative stress increases melatonin consumption in rats has been provided by Tan et al. [78]. They observed that rats loaded with melatonin and then exposed to ionized radiation (which generates $^{\bullet}OH$) significantly increased cyclic 3-hydroxymelatonin (C3-OHM) excretion in urine. The rapid drop in circulating melatonin under conditions of excessive stress can be considered a protective mechanism for organisms against highly damaging oxidants; in this sense, melatonin can be categorized as a first line of defensive molecule. In addition to direct prevention of tissue injury, the increased levels of melatonin metabolites such as N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and *N*-acetyl-5-methoxykynuramine (AMK) upregulate antioxidant enzymes and downregulate the pro-oxidative and proinflammatory enzymes (see below). In many disease states which reportedly involve free radicals and oxidative stress, e.g. Alzheimer's disease, cancer and coronary heart disease, melatonin levels in these patients are significantly lower than those in healthy counterparts. It is not clear whether the low melatonin levels in these patients are the result of consumption due to the elevated free radical production or due to the lower production of melatonin.

Based on evidence summarized here, it appears that many stressors including physical, chemical, environmental which are related to the elevated oxidative status upregulate melatonin synthesis while intensive stress in organisms cause melatonin to be rapidly consumed resulting in a reduction of endogenous melatonin levels. Thus, the apparent paradox of an elevated melatonin biosynthesis accompanied by reduced melatonin levels in organisms under stress is potentially explained.

Metabolism and metabolites of melatonin

The multiple pathways of melatonin metabolism are summarized in Fig. 4.

6-Hydroxymelatonin

Melatonin is a lipophilic molecule and for it to be readily excreted by the kidney it must be converted to a more hydrophilic species. For years it was thought that the majority of melatonin was catabolized to 6-hydroxymelatonin in liver by cytochrome P450 (CYP1A1, CYP1A2 and CYP2C19). Under an action of sulfotransferase, 6-hydroxymelatonin is conjugated to sulfate to form 6-hydroxymelatonin sulfate which is excreted in urine. Recent evidence indicates that some 6-hydroxylmelatonin sulfate may be formed at extrahepatic sites. The ubiquitously distributed, extrahepatic CYP1B1 can efficiently biotransform melatonin to 6-hydroxymelatonin [79]. High levels of 6-hydroxymelatonin sulfate have been identified in the cerebral cortex, kidney and heart of rats [80]. The amounts of

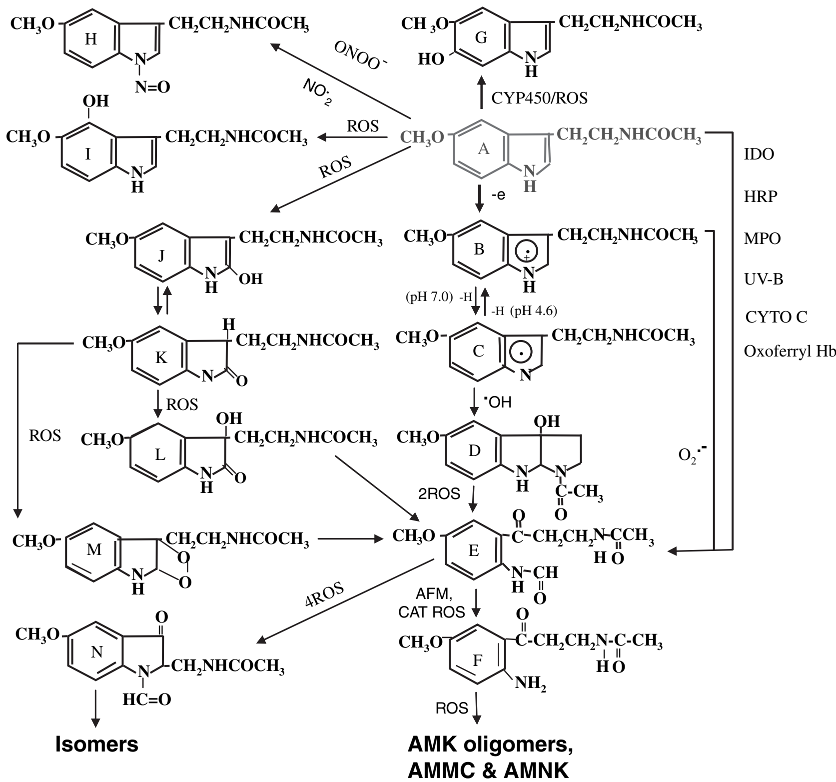

Fig. 4. Enzymatic and nonenzymatic pathways of melatonin metabolism with the resulting metabolites. A: melatonin, B: melatoninyl cation radical, C: melatoninyl neutral radical, D: cyclic 3-hydroxymelatonin, E: AFMK, F: *N*-acetyl-5-methoxykynuramine (AMK), G: 6-hydroxymelatonin, H: *N*-nitrosomelatonin, I: 4-hydroxymelatonin, J: 2-hydroxymelatonin, K: 3: melatonin 2-indolinone, L: 3-hydroxymelatonin 2-indolinone, M: melatonin dioxetane, N: *N*-(1-formyl-5-methoxy-3-oxo-2,3-dihydro-1*H*-indol-2-ylidenemethyl)-acetamide; AFM: arylamineformamidase, CAT: catalase, HRP: horseradish peroxidase, IDO: indoleamine dioxygenase, CYP450: cytochromone P450, CYTO C: cytochromone C, Oxoferryl HB: oxoferryl hemoglobin, ROS: reactive oxygen species, UV-B: ultraviolet B.

6-hydroxymelatonin sulfate in cerebral cortex are approximately 1000-fold greater than melatonin levels. In cell culture, the hormone-dependent MCF-7 and the hormone-independent MDA-MB231 (MDA) breast cancer cell lines [81] and in skin HaCaT keratinocytes [38] reportedly produce 6-hydroxymelatonin. Considering the high levels of melatonin in the intestine, cerebrospinal fluid and skin, with their high levels of CYP1B1, it seems likely that the liver is not the only site of formation of 6-hydroxymelatonin sulfate and large amounts of endogenous melatonin are catalyzed by extrahepatical organs.

In addition to enzymatic processes, 6-hydroxymelatonin can be generated by nonenzymatic means, i.e. via an interaction with ROS/RNS. Evidence shows that melatonin interacts with peroxynitrite ($ONOO^-$) [82, 83], •OH [84] or during UV-B irradiation [38] with the resultant formation of 6-hydroxymelatonin. Each of these relates to oxidative stress which organisms persistently experience; therefore, the persistent generation of 6-hydroxymelatonin via this pathway is expected.

2-Hydroxymelatonin

2-Hydroxymelatonin is also a melatonin-hydroxylated metabolite. Currently, there is little information regarding whether 2-hydroxymelatonin is catabolized enzymatically in vivo. All evidence indicates that 2-hydroxymelatonin is a resultant product of melatonin interaction with ROS/RNS. In an in vitro study, Dellegar et al. [85] first showed that a main product of melatonin interaction with hypochlorous acid is 2-hydroxymelatonin. Subsequently, 2-hydroxymelatonin and its keto tautomer, melatonin 2-indolinone, were identified to be the oxidative products of melatonin's interaction with oxoferryl hemoglobin [86] or •OH [84]. Interestingly, oxidation of cytochrome C by H_2O_2 in vitro also uses melatonin as a reductant to generate 2-hydroxymelatonin as an intermediate with subsequent formation of AFMK [87]. In cell culture, Fischer et al. [38] also observed that UV-B irradiation induced keratinoytes to use exogenously supplied or endogenously produced melatonin to form 2-hydroxymelatonin; this group believes that 2-hydroxymelatonin is subsequently transformed to AFMK by the cells. Tan et al. [78] have speculated that 2-hydroxymelatonin and its keto form can be generated in vivo as a tautomer of C3-OHM. Indeed, 2-hydroxymelatonin was identified in the urine of mice by Ma et al. [88]. It is estimated that 2-hydroxymelatonin excreted in urine comprises about 2% of the melatonin metabolized in mice. The detailed pathways of 2-hydroxymelatonin formation are illustrated in Fig. 4.

Cyclic 3-hydroxymelatonin

Cyclic 3-hydroxymelatonin was first isolated and structurally identified as an oxidative melatonin metabolite by Tan et al. [78]. They found that melatonin scavenges two •OH to form C3-OHM. When rats were exposed to ionizing radiation, which primarily generates •OH in vivo, urinary C3-OHM increased several fold. Thus, C3-OHM is a reliable biomarker of endogenous •OH levels. Subsequently, C3-OHM has been reported to be present after the interaction of melatonin with $ONOO^-$ [83, 89]. Tesoriere et al. [90] observed that hypervalent iron, i.e. oxoferryl hemoglobin, also oxidizes melatonin to form C3-OHM

and, as a result, the oxoferryl hemoglobin is reduced to its functional form, ferric hemoglobin. C3-OHM was also found in a reaction of melatonin with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) cation radicals [91]. In this reaction, C3-OHM scavenges two ABTS cation radicals to form AFMK. A method for the synthesis of C3-OHM from melatonin was recently published [92]. Both C3-OHM and AFMK have been identified in the same urine samples of mice [88]. The data prove that the coexistence of C3-OHM and AFMK is a common phenomenon in the metabolic pathway of melatonin both in vitro and in vivo.

AFMK and AMK

Research interest in AFMK has increased rapidly during the last few years. AFMK is now commercially available from Sigma Chemical Co. (St Louis, MO, USA) and this makes research easier. Published data indicate that AFMK is an intriguing molecule and it may be a pivotal molecule in melatonin metabolism. AFMK was found to be a melatonin metabolite in the brain of rats three decades ago [93]. Until recently, it was believed that AFMK was exclusively enzymatically formed by the catalytic enzyme, indoleamine 2,3-dioxygenase. In 1998, Tan et al. [94] reported a nonenzymatic process, i.e. an interaction of melatonin with H_2O_2, which generates AFMK. Subsequently, it was reportedly that enzymatic, pseudoenzymatic, UV irradiation and ROS processes result in AFMK formation. These AFMK forming pathways include melatonin being catalyzed by horseradish peroxidase, by myeloperoxidase [95–97], oxidized by oxoferryl hemoglobin [89], metabolized by cytochrome C [84], irradiated by UV-B [38], interacting with $O_2^{\bullet-}$ [98], H_2O_2 [93], singlet oxygen [99], carbonate radical and $ONOO^-$ [100] (Fig. 4). It was also found that neutrophils and macrophages activated by bacteria, virus or lipopolysaccharide (LPS) effectively oxidize melatonin to form AFMK [101–103] with these processes being myeloperoxidase and $O_2^{\bullet-}$ dependent. Human keratinocytes also generate AFMK from melatonin when these cells are exposed to oxidative stress, i.e. UV-B irradiation [38].

Poeggeler and Hardeland [104] reported in the evolutionarily low-ranked species including unicellular organisms (dinoflagellate *Lingulodinium polyedrum*) and small aquatic metazoans (chlorophycean *Chlorogonium elongatum*, ciliates *Paramecium caudatum* and *P. bursaria*, and the rotifer *Philodina acuticornis*, *L. polyedrum*) that when cultured with melatonin (500 μM) in darkness, the only melatonin metabolite detected was AFMK. In mammals, AFMK was detected in the retina and serum of rats by high performance liquid chromatography (HPLC), in cerebrospinal fluid (CSF) using in vivo microdialysis coupled with gas chromatography-mass spectrometry (GC-MS) [105], in blood by radioimmunoassay [106] and in the urine of mice by GC-MS [87]. Extremely high levels of AFMK are also found in the CSF of patients with meningitis [107]. Furthermore AFMK has been identified in an aquatic plant, the water hyacinth [*Eichhornia crassipes* (Mart) Solms] (Tan & Reiter, unpublished observation). All data indicate that organisms from unicellular alga, metazoans, plants, rodents to humans have the capacity to produce AFMK, probably via the nonenzymatic pathways.

Collectively, the data imply that evolutionarily, AFMK likely evolved much earlier than did 6-hydroxymelatonin sulfate and was the original metabolite of melatonin metabolism. AFMK may be a primary metabolite of melatonin in organisms under oxidative stress as, when tested under these conditions, melatonin undergoes oxidation with the resultant formation of AFMK. AFMK formation from melatonin has several different pathways with multiple steps and the details are illustrated in Fig. 4. It appears that melatonin dioxedane and C3-OHM are favorite intermediates in AFMK formation.

Recently, Rosen et al. [108] found that when AFMK interacts with the ABTS cation radical, this molecule has the ability to donate four electrons and cycles to form indolinones including Z-, E- isomers of *N*-(1-formyl-5-methoxy-3-oxo-2,3-dihydro-1*H*-indol-2-ylidenemethyl)-acetamide] and *N*-(1-formyl-2-hydroxy-5-methoxy-3-oxo-2,3-dihydro-1*H*-indol-2-ylmethyl)-acetamide. In addition, AFMK was further deformylated, either by arylamineformamidase, hemoperoxidases or interaction with ROS/RNS to form AMK. AMK has been found in cultured cells, in the serum of rats and in the urine of mice. AMK can be further metabolized by nonenzymatic processes. When AMK interacts with ABTS cation radical, the resulting products are its oligomers [109]. When it reacts with $ONOO^-$, 3-acetamidomethyl-6-methoxycinnolinone and N^1-acetyl-5-methoxy-3-nitrokynuramine are formed [110].

1-Nitromelatonin and nitrosomelatonin

Other minor melatonin metabolites found in vitro are 1-nitromelatonin and 1-nitrosomelatonin. Both are the products of melatonin's interaction with $ONOO^-$ [89, 111, 112] as well as with the nitrogen dioxide radical and $NO^{\bullet}$ [113]. Nothing is known as to whether they are formed in vivo.

AFMK and its derivatives may be primary metabolites of melatonin

AFMK and its derivatives are the only detectable melatonin metabolites in plants and in several low-ranked organisms including alga and metazoans [104]. Based on these data, it is reasonable to conclude that AFMK is a primitive and primary metabolite of melatonin. However, in vertebrates melatonin has long been thought to be almost exclusively metabolized by hepatic P450 mono-oxygenases and, following conjugation with sulfate, to yield the main urinary metabolite 6-hydroxymelatonin sulfate.

Recently, using state-of-the-art methods, Ma et al. [88] showed that 6-hydroxymelatonin sulfate only comprises about 37.1% of melatonin metabolized in mice. The proportions of urinary melatonin metabolites in mice are 6-hydroxymelatonin sulfate, 37.1%, > NAS, 3.1%, > 2-hydroxymelatonin, 2.2%, > C3-OHM, 0.5%, > AFMK, 0.01%, =AMK, 0.01%. This indicates that either a major portion of melatonin up to 57% is metabolized to currently unknown metabolites or the above-mentioned metabolites

are distributed in the body rather than excreted in urine. Based on data available, we speculate that a major portion of melatonin is likely transformed to AFMK and its derivatives. The urinary excretion of AFMK does not represent its real production as this molecule is not as highly hydrophilic as 6-hydroxymelatonin sulfate and, thus, its urinary excretion is limited. In addition, AFMK can further degrade to AMK and several other molecules. Currently, methods used to quantity these AFMK derivatives are not available.

In some tissues, especially in CNS, melatonin may be exclusively metabolized to AFMK and its derivatives. Hirata et al. [93] observed that after the injection of ^{14}C-melatonin into the cisterna magna, the only detectable metabolite (20–40 min later) in neural tissue was AMK and no 6-hydroxymelatonin was detected. In urine, 35% of the total radioactivity was associated with AMK. This observation was under normal conditions and the pyrrole-ring cleavage to form AFMK and AMK is catabolized largely by the indoleamine 2,3-dioxygenanse. Under conditions of high oxidative stress, especially when high levels of $O_2^{\bullet -}$, H_2O_2 are available, myeloperoxidase activation and direct interaction of melatonin with ROS/RNS would result in larger quantities of melatonin oxidatively cleaved to AFMK. This is the case in patients with meningitis. The AFMK concentrations of the CSF in these patients are in excess of 13,200 pg/mL (50 nm/L) [107], which are three orders of magnitude higher than that of daytime serum melatonin levels in humans. In normal subjects in this study, AFMK levels were under the detection limit [the lowest detection limit of AFMK in CSF was 2640 pg/mL (10 nm/L); this value is also two orders of magnitude higher than that of daytime serum melatonin levels in humans].

Leukocytes are another important site for AFMK formation. Activated leukocytes dramatically promote AFMK formation up to fivefold over basal levels [102]. In animal studies, AFMK has been detected in the retina of rats and it appears that these levels parallel the levels of melatonin, i.e. the highest values of both are during the scotophase [105]. Using radioimmunoassay, AFMK is detectable in the serum of rats 30 min after intraperitoneal injection of melatonin [106]. These findings are consistent with AFMK being an important melatonin metabolite in vivo. In addition to brain and leukocytes, the lungs, skin and red blood cells would be expected to generate AFMK. The lungs contain large numbers of alveolar macrophages and have high oxygen levels. Activated macrophages combined with the high availability of O_2 rapidly converts melatonin to AFMK. Skin has the capacity to synthesize melatonin and is continuously exposed to environmental stresses including the UV irradiation and chemical exposures. Fischer et al. [38] reported that AFMK is a major melatonin metabolite of keratinocytes under UV-B exposure. Red blood cells contain abundant hemoglobin and oxygen. Hypervalent hemoglobin can effectively oxidize melatonin to form AFMK. Recently, Budu et al. [114] observed that AFMK modulated the cell cycle of malarial parasites. AFMK levels in red blood cells of mice infected with malaria increased roughly 300-fold from the ring stage (0.1 ± 0.1 nmol/L) to the schizont stage (29 ± 5 nmol/L) when incubated with melatonin. The elevated AFMK levels reflect the dramatically increased oxidative stress in infected red blood cells.

In cellular organelles, mitochondria are the favorite site for AFMK formation. Mitochondria contain high levels of melatonin and cytochrome C. Cytochrome C can use melatonin as its substrate to biotransform it to AFMK [87]. To prove whether a major portion of melatonin is converted to AFMK in high-rank animals such as in mammals, further investigations are warranted.

Variations of melatonin bioavailability in humans

Melatonin as a food supplement is widely used in the USA and in several other countries. Its sales once exceeded vitamin C in the USA [115]. However, the pharmacokinetics of melatonin, especially its bioavailability in humans, has not drawn sufficient attention. In animal studies, particularly in laboratory rodents, the pharmacokinetic parameters of melatonin including its half-life ($t_{1/2}$), clearance rate (*Cl*) and bioavailability are uniform and exhibit small individual variations. Compared with rodents, the bioavailability of melatonin in humans is poor. A clear study using deuterium melatonin as a reference standard revealed that the bioavailability of orally consumed melatonin was as low as 1% in some subjects with an obvious sexual difference. The bioavailability of melatonin in females is double that of males, i.e. 16.8 ± 12.7% vs. 8.6 ± 3.9%, respectively [116]. The low bioavailability of melatonin is attributed to its first pass effect through the liver. However, that a considerable amount of melatonin may be degraded by gastrointestinal CYP450 1B1, may participate in the hepato-enteric cycling or be consumed by the nonenzymatic mechanisms including an interaction with ROS/RNS could not be ruled out. High levels of melatonin in bile of rats [39] and in the biliary tract of humans [117] have been reported; this suggests that a portion of orally taken melatonin may enter the hepato-enteric circulation.

In addition to its low bioavailability, substantial individual variations in melatonin bioavailability have been observed. This variation can be as high as 37-fold. The average individual variations calculated from available data in terms of melatonin's bioavailability in humans differ by about 18-fold (Table 1). The largest individual variations are probably due to the heterogenic properties of cytochrome C P450 subtype gene expression in humans.

Table 1. Bioavailability and individual variations of melatonin in humans

Studies	Range of bioavailability	Folds of difference	Mean value of bioavailability
Waldhauser et al. [118]	3–76%[a]	25.3	22.0%[b]
Di et al. [119]	10–56%	5.6	33.0%
DeMuro et al. [120]	7.3–20.3[b]	3.0	14.3%
Fourtillan et al. (117)	1–37%	37	8.7% (male) 16.8% (female)
	Average	17.7	18.9%

[a]Calculated by Lane and Moss [121], [b]calculated by the authors.

The low bioavailability (average 18.9%) and large individual variation (average 17.7-fold) may well explain the different responses in subjects who take melatonin orally. Currently, the most popular melatonin formula commercially available is a 3 mg tablet. For some subjects, this dose when taken to benefit sleep may induce drowsiness the day after; for others, whose bioavailability is low, this dose may not be sufficient to treat insomnia or related disorders. To obtain the optimal effects of melatonin treatment, individualization of dose is suggested based on the serum or salivary melatonin levels after melatonin administration or adjusting the dose depending on the responses of the subjects.

Drug interactions also influence the bioavailability of melatonin. For example, co-administration of melatonin with CYP1A2 inhibitor, fluvoxamine (also a serotonin reuptake inhibitor), in healthy subjects, results in a 17-fold increase in melatonin blood levels [122]. These data also indirectly indicate a very low melatonin bioavailability in humans, probably $<6\%$ in this study. When melatonin is taken with 200 mg caffeine, equivalent to a large cup of coffee, its bioavailability increases 140% presumably because both are substrates of CYP1A2 [123]. We also observed that concomitantly when melatonin was taken with vitamin E and vitamin C in human subjects, the bioavailability of melatonin increased (Tan & Reiter, unpublished observations). Clarifying the pharmacokinetics of melatonin and its interaction with other substances will help to understand dose differences in a variety of situations and between individuals.

Functions of melatonin metabolites

Melatonin is a highly conserved molecule. Its presence can be traced back to photosynthetic prokaryotes, *Rhodospirillum rubrum* [124], unicellular organisms, marine algae (dinoflagellate *Gonyaulax polyedra*) [125] and yeast [126] (*Saccharomyces cerevisae*). In these organisms, a primitive and primary function of melatonin is to protect against oxidative stress. Later in evolution, melatonin produced by the pineal gland evolved to be a chemical signal of dark/light, to mediate seasonal physiological functions, immunostimulation and other receptor-mediated functions in multicellular organisms. Since the report of melatonin as a potent free radical scavenger and an antioxidant in 1993 [6], in excess of 1500 scientific publications have directly or indirectly confirmed this observation. These data have been thoroughly summarized in reviews [127–130]. Here, we briefly review the potential activities of known melatonin metabolites.

While 6-hydroxymelatonin is a major urinary melatonin metabolite, little was known of its function until melatonin was discovered to be an antioxidant. Due to the structural similarity, scientists have examined whether 6-hydroxymelatonin also exhibits significant antioxidant capacity. Indeed, 6-hydroxymelatonin was found to reduce lipid peroxidation in the liver, muscle and brain of rats induced by oxidative stress [131]. Recently, reports regarding an antioxidant properties of 6-hydroxymelatonin are on the rise. 6-Hydroxymelatonin is reported to protect against DNA damage, i.e. reducing oxidative 8-hydroxy-2-deoxygaunosine formation induced by an environmental pollutant, chromium [132] and $^{\bullet}OH$ generated by Fenton reagents [133]. Additionally, it protects against cisplatin-induced nephrotoxicity in rats [134] and prevents neuronal and hepatic toxicities caused by cyanide, quinolinic acid, iron and alpha-naphthylisothiocyanate [135–138]. The protective mechanism of 6-hydroxymelatonin on tissue damage is attributed to its direct free radical scavenging and antioxidant capacities [139–141]. Recently, 6-hydroxymelatonin was reported to strongly inhibit cytochrome C release from the mitochondria and to suppress the activity of caspase 3 as well as to stabilized the mitochondrial membrane potential thereby protecting against neuronal death induced by oxygen–glucose–serum deprivation in cultured cells [142].

One report claimed that, via redox cycling, 6-hydroxymelatonin caused oxidative DNA damage with double-strand breaks [143]. This is not totally unexpected, as 6-hydroxymelatonin has an unshielded hydroxyl group, under certain conditions, this molecule may participate in pro-oxidative reactions which would result in oxidative damage. Similar reactions have been postulated for the hydroxylated indoles by Tan et al. [7].

Cyclic 3-hydroxymelatonin was first identified to be an oxidative melatonin metabolite in the urine of rats and humans [78]. Interactions of melatonin with a variety of ROS/RNS produce C3-OHM. Its functions and its distribution in organisms are not clear. The structure of C3-OHM is similar to that of an inhibitor of cholinesterase, physostigmine. It has been speculated that C3-OHM may be useful in disorders related to cholinergic transmission impairment, e.g. Alzheimer's disease [92]. C3-OHM has been found capable of reducing two ABTS cation radicals per molecule [91] and preventing oxidative DNA damage caused by Fenton regents [133]. C3-OHM is two- to threefold more potent than its precursor, melatonin, in terms of reducing hypervalent hemoglobin to its basic form (Tan & Reiter, unpublished observations). Interestingly, the formation of C3-OHM is always accompanied by AFMK. The equilibrium of C3-OHM and AFMK depends on the ratio of oxidants to melatonin. The higher the ratio, the more AFMK than C3-OHM is produced. The co-existence of C3-OHM and AFMK implies that the C3-OHM is readily converted to AFMK, probably by interacting with and scavenging ROS/RNS. Thus, the ratio of C3-OHM to AFMK may be an indicator of the level of the oxidative stress in a system. Organisms could use this ratio as a signal to modulate their antioxidative defense system by up- or downregulation of antioxidant enzymes.

AFMK and AMK are major melatonin metabolites. AFMK and AMK have similar structures with AMK lacking one formyl group. Cyclic voltammetry indicated that AFMK has the capacity to donate two electrons as a reductant [144]. A structural and reaction products analysis showed that one AFMK molecule could donate 4 or more electrons when it interacts with reactive radicals at low concentrations [108]. The antioxidative capacity of AFMK has been examined in different conditions. It was found that AFMK scavenges $O_2^{\bullet-}$ with a similar potency to melatonin [145]. AFMK reduces lipid peroxidation, oxidative DNA

damage induced by a variety oxidative stressors and prevents neuronal cell injury caused by H_2O_2 and amyloid β-peptide [141, 144, 146, 147].

In terms of scavenging ROS and preventing protein oxidation, AMK has greater efficiency than does its precursor, AFMK [148]. In addition to its direct antioxidant capacities, AMK effectively inhibits neuronal nitric oxide synthase activity and reduces intracellular NO levels [149, 150]. Its ability to reduce NO formation has been referred to as free radical avoidance activity [151]. AMK, like its precursor melatonin, promotes mitochondrial complex I activity to elevate ATP production by lowering electron leakage and inhibiting opening of the mitochondrial permeability transition pore [151, 152].

The anti-inflammatory and immunoregulatory activities of AFMK and AMK have also attracted significant attention. AMK was observed to inhibit the biosynthesis of prostaglandins [153]. AFMK (0.001–1 mM) inhibits tumor necrosis factor-alpha and interleukin-8 formation caused by LPS in neutrophils and peripheral blood mononuclear cells. The formation of AFMK during melatonin oxidation was speculated to be an important event in the cross-talking between neutrophils and monocytes [154]. A mechanistic study has revealed that AFMK and AMK selectively inhibit gene expression of a proinflammatory enzyme, cyclo-oxygenase 2 (COX-2) [155]. AFMK also regulates the cell cycle of malaria parasites as does its precursor melatonin [114].

Numerous physiological actions of AFMK and AMK summarized here indicate that some functions of melatonin may be mediated or amplified by its metabolites. Due to the generation of many structurally unrelated derivatives during melatonin metabolism, multiple physiological functions of melatonin are predictable. It is expected that research regarding the physiological and therapeutic roles of AFMK and AMK will increase rapidly.

Concluding remarks

Melatonin is a conserved molecule and it is present in virtually all creatures from prokaryotes to humans. The original function of melatonin was to serve as an antioxidant to protect organisms from ubiquitous oxidative stresses. Other receptor-related functions of melatonin were acquired during evolution. Melatonin synthesis is likely inducible by low-intensity stressors, e.g. DR in rats and monkeys and exercise in humans. In accordance with its protective effects, melatonin levels in organs or tissues which are frequently exposed to the hostile environmental insults, such as the gut and skin, and organs which have high oxygen consumption, such as the brain, are reported to be higher than originally thought. A major urinary metabolite of melatonin in vertebrates is 6-hydroxymelatonin sulfate which is catabolized either in the liver or in other organs and tissues. However, the primitive and primary melatonin metabolites might be AFMK. AFMK is the only detectable melatonin metabolite in unicellular organisms and in several metazoans, and 6-hydroxymelatonin sulfate has not been found in these evolutionarily low-ranked species. In addition to these low-rank species, AFMK has also been detected in mice, rats and humans.

AFMK is generated from melatonin via several pathways including enzymatic, pseudo-enzymatic and interaction with a variety of ROS/RNS. Considering the cascade of reactions that includes AFMK, a melatonin molecule can scavenge up to 10 ROS/RNS. Unquestionably, the structurally different metabolites of melatonin exhibit free radical scavenging activity. This phenomenon of melatonin and its metabolites sequentially interacting with ROS/RNS is referred as scavenging cascade reaction of melatonin. This cascade makes melatonin highly effective as a novel free radical scavenger and antioxidant. In addition to the direct effects of melatonin and its metabolites with ROS, AFMK and AMK also have the ability to downregulate pro-oxidative and proinflammatory enzymes including inducible nitric oxide synthase and COX-2 and to carry out free radical avoidance functions. The variety of biologically activate metabolites explains why melatonin exhibits a variety of physiological functions. As the oxidative status of organisms can modify melatonin metabolism, the ratio of different melatonin metabolites could serve as an indicator of oxidative status of organisms and can also serve as signals to trigger in vivo responses of organisms.

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