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Antioxidative Protection by Melatonin

Multiplicity of Mechanisms from Radical Detoxification to Radical Avoidance

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Melatonin has been shown to protect against oxidative stress in various, highly divergent experimental systems. There are many reasons for its remarkable protective potential. Signaling effects comprise the upregulation of antioxidant enzymes, such as superoxide dismutases, peroxidases, and enzymes of glutathione supply, downregulation of prooxidant enzymes, such as nitric oxide synthases and lipoxygenases, and presumably also the control of quinone reductase 2. Other mechanisms are based on direct interactions with several reactive oxygen and nitrogen species. Among these reactions, the capacity of easily undergoing single-electron transfer reactions is of particular importance. Electron donation by melatonin is not only an aspect of direct radical scavenging, but additionally represents the basis for formation of the protective metabolites AFMK (*N*^{1}-acetyl-*N*^{2}-formyl-5-methoxykynuramine) and AMK (*N*^{1}-acetyl-5-methoxykynuramine). Recent investigations on mitochondrial metabolism indicate that melatonin as well as AMK are capable of supporting the electron flux through the respiratory chain, of preventing the breakdown of the mitochondrial membrane potential, and of decreasing electron leakage, thereby reducing the formation of superoxide anions. Radical avoidance is a new line of investigation, which exceeds mitochondrial actions and also comprises antiexcitatory effects and contributions to the maintenance of internal circadian phase relationships.

Key Words: Antioxidants; free radicals; kynuramines; melatonin; mitochondria.

Introduction

Numerous publications have demonstrated protective actions by melatonin, and most of them are related to the attenuation of oxidative damage. Although these findings have been reviewed repeatedly *(1–22)*, recent developments in this area merit another close look at the mechanisms. Several new aspects of antioxidative protection have emerged, which seem to be of high relevance and may fill the gap between the initially discovered radical-scavenging properties, plus some additional regulatory actions on redox metabolism, and the beneficial effects observed at the levels of cells, tissues, and organisms.

Without going into detail of previously published and reviewed data, one should distinguish between various types of experiments on the antioxidant properties of melatonin and the meaning as well as the limits of their results. In the beginning, the chemical property of melatonin of easily and efficiently scavenging several oxygen free radicals was the focus of interest *(23–33)*. In addition, the early investigations already showed that both pharmacological and physiological concentrations of melatonin were capable of protecting DNA from damage by hydroxyl radicals *(34,35)*. However, in subsequent protection studies, melatonin was rarely administered to vertebrates in physiological concentrations; alternatively, the role of naturally occurring levels was preferably studied by comparing untreated/sham-operated with pinealectomized animals *(36–38)*. Protection experiments in dinoflagellates using high, but physiologically possible, levels of melatonin *(39–41)* have to be interpreted differently, because these unicellular organisms produce this indoleamine in considerably higher concentrations. In vertebrates, however, the gap between the physiological levels of melatonin and the concentrations required in the majority of protection experiments was perceived as an increasing problem. This was considered important as other antioxidants are present in cells and body fluids in much higher concentrations. So which might be the role of melatonin as an antioxidant if it is by far exceeded by other, at first glance similarly acting, compounds?

In fact, many of the protection experiments were designed in a way that superphysiological levels of the hormone were already required from a conceptual point of view (cf. refs. *4* and *6*). When oxidative stress was generated experimentally, by administration of oxidotoxins or by irradation (UV or X rays), the treatment had to override the physiological protection mechanisms, so that no effect by naturally occurring (or any antioxidant) melatonin levels could be expected. Nevertheless, the concentrations used varied over many orders of magnitude, and one has to distinguish carefully between results obtained at different dosages. The use of

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melatonin in the millimolar range may only make sense in chemical experiments designed to identify products from interactions with oxidants and to clarify oxidative reaction mechanisms. In biological experiments, such concentrations must be considered suprapharmacological and would never be applied to an organism.

Nonetheless, a substantial body of evidence remains for a physiological role in antioxidative protection, based also on results obtained at nanomolar concentrations. However, as will be outlined below, this is not simply and only a matter of some regulatory receptor–mediated actions leading to upregulation of antioxidant and downregulation of prooxidant enzymes. Moreover, protective effects that are demonstrable at nanomolar levels do not immediately exclude the significance of the intially discovered radical scavenging properties, for the only reason that physiological melatonin concentrations might appear insufficient for such effects. This view would, first, neglect the fact that radical reactions generate new metabolites, some of which also have been shown to exert protective effects. Second, electron donation by melatonin—which becomes obvious as radical scavenging—can be readily followed by electron donation from another donor to the melatonyl radical thereby formed. This is assumed to be the basis for quasi-catalytic processes requiring only very low amounts of melatonin, a concept that is actually discussed in terms of diminution of radical formation by interaction with components of the mitochondrial respiratory chain *(38)*. Both of these aspects may have a considerable potential for the understanding of protection by melatonin at physiological concentrations. They deserve future attention.

Single-Electron Transfer Reactions

The most conspicuous finding in the oxidation chemistry of melatonin was that of hydroxyl radical scavenging *(1,27–29,31,33)*. In this regard, melatonin turned out to be considerably more efficient than the majority of its naturally occurring structural analogs *(27,31,33,42)*, indicating that the substituents of the indole moiety strongly influenced reactivity and selectivity. Rate constants determined were in the range between 1.2×10^{10} and 7.5×10^{10} M^{-1} s^{-1}, depending on the methods applied *(42–47)*. Regardless of differences in the precision of determination, melatonin has been shown, independently by different groups, to be a remarkably good scavenger of this radical species. This property may be crucial for protection in pharmacological and other experimental in vitro situations. To what extent this may contribute under physiological conditions, depends on local concentrations and may be a matter of debate.

A hydroxyl radical can interact with an aromate in different ways, (i) by abstracting an electron or, as a variant, a hydrogen atom, or (ii) by adduct formation. Both are possible with melatonin, but our considerations of damage by free radicals should not be restricted to the hydroxyl radical as the most reactive oxygen species formed in biological material.

From a broader perspective, single-electron donation may be regarded as the basic and most important type of redox reaction melatonin can undergo. This statement does not exclude additional possibilities of reaction with oxidants, but it places emphasis on a chemical property of highest significance not only for broad-spectrum radical scavenging *(48)*, but also non-enzymatic product formation and the potential of interacting with the respiratory chain. The preference for single-electron transfer has been unambiguously demonstrated by cyclic voltammetry *(48)*. Moreover, it represents the common key step of interaction with free radicals of both high and low reactivity, as far as they are capable of directly oxidizing by electron abstraction. This holds for hydroxyl radicals *(27–29)*, for carbonate radicals *(49,50)*, and also for several organic radicals formed from biologically occurring photocatalysts, such as protoporphyrin IX *(30,32,51,52)*, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid *(53,54)*. However, the alternative—direct radical addition—has been observed or theoretically predicted only with hydroxyl radicals *(48,55–57)* and nitric oxide *(48,58–60)*. Electron abstraction was also concluded to be a primary step of melatonin oxidation in a pseudoenzymatic reaction catalyzed by oxoferrylhemoglobin *(61)*. Single-electron donation by melatonin is most obvious in the interaction with the ABTS [2,2'-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid)] cation radical *(42,62,63)*, a free radical having a remarkably low reactivity and, therefore, a half-life of many days. Despite its low reactivity, the ABTS cation radical efficiently and rapidly oxidizes melatonin as well as many other compounds and represents a convenient test substance for initial reactivity studies. The main metabolites formed by electron exchange with ABTS cation radicals are the same as those found in many other oxidation systems *(63)*, namely, AFMK (N^1-acetyl-N^2-formyl-5-methoxykynuramine) and, temporarily, cyclic 3-hydroxymelatonin (Fig. 1). This is reminiscent of the observations with other partners of single-electron exchange, such as protoporphyrinyl cation radicals *(30,32,51,52)* and substituted anthranilyl radicals *(53,54)*. Different results are, however, obtained with systems in which hydroxyl radicals are generated at high rates, e.g., by pulse radiolysis, especially at relatively low concentrations of superoxide anions. In the latter type of oxidation experiments, the main products are hydroxylated indoles and their derivatives, such as 3-, 6-, or 7-hydroxymelatonin *(48,56,64)*, cyclic 3-hydroxymelatonin *(48,55)*, or an indolinone formed from 2-hydroxymelatonin *(57)*, a substance also detected in other systems, but only in low amounts *(65)*.

These results from different oxidation systems shed light on several important facts, which are not obvious at first glance and which may have not been seen by any investiga-

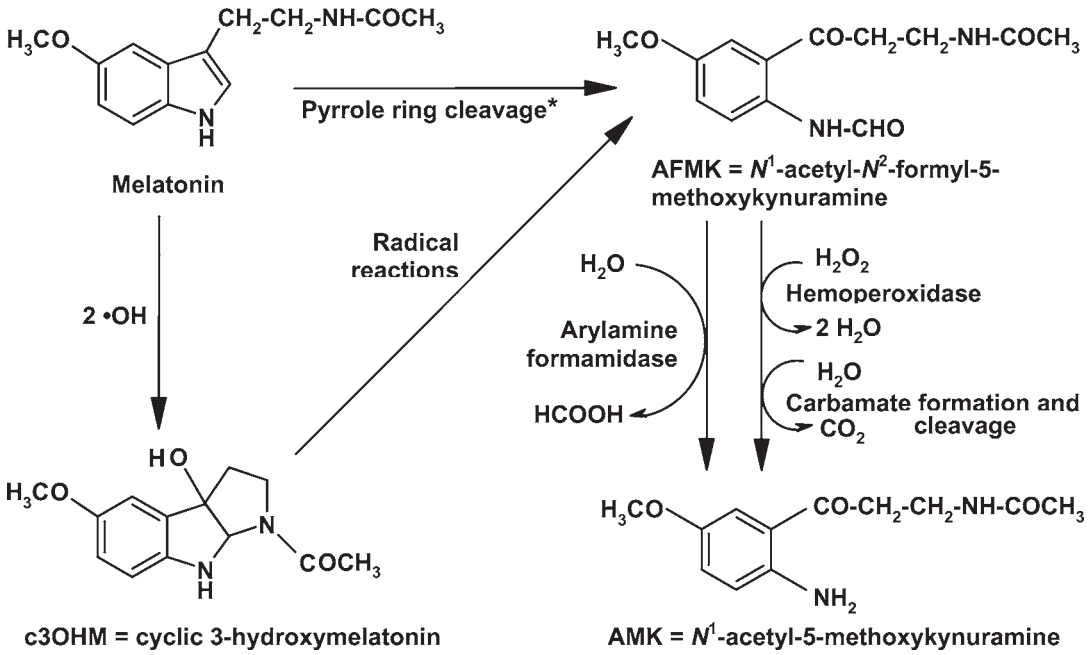

Fig. 1. The kynuric pathway of melatonin oxidation. *Numerous oxidation reactions involving scavenging of various free radicals, singlet oxygen, or ozone, several photocatalytic, pseudoenzymatic and enzymatic reactions lead to AFMK (for details see text). For conversion of c3OHM to AFMK see ref. *63*. Two enzymatic reactions of AMK formation are known to date; an additional, radical-mediated reaction may also exist.

tor. (i) Reactivity of a free radical is not necessarily a measure for its capability of undergoing reactions with a particular substance, neither in an artificial chemical system nor in biological material, and also not for its capacity of destroying biomolecules. The hydroxyl radical, frequently the focus of interest because of its extremely high reactivity, is, for the same reason, very short-lived and short-reaching. A long-lived low-reactivity radical may in the end be more harmful, because it has a longer lifetime available for finding a reaction partner in a diffusion-controlled system. However, the hydroxyl radical is capable of generating various secondary radicals, especially in biological material, e.g., organic radicals and carbonate radicals, which may represent transducers of damage to distant sites. With different compounds, we have repeatedly seen much higher conversion rates when we incorporated hydrogen carbonate into an oxidation system, which formed carbonate radicals at the expense of hydroxyl radicals; this was, in fact, also observed with melatonin *(49,50,54)*. (ii) The spectrum of products formed depends to a high degree on the composition of the system, particularly the relative concentrations of different radical species. Under natural conditions, hydroxyl radicals are not the quantitatively dominating reactive oxygen species, whereas superoxide anions are present in considerably higher concentrations. For this reason, a preferential formation of hydroxylated indoles and their derivatives, as described for certain oxidation systems, can be regarded as the result of an artificial situation in which hydroxyl radicals dominate, owing to the chemists's attempt of studying exclusively the interaction with a single radical species. However, in systems also containing relatively higher amounts of superoxide anions and reflecting, in this regard, near-physiological conditions *(50)*, the principal metabolite has always been the substituted kynuramine AFMK *(50–54,66)*. (iii) The biological or pharmacological value of a radical scavenger is not only determined by its own reactivity and radical specificity, but also by the mode of termination of the radical reaction chain and the products formed thereby *(50,67)*. One should be aware that, by interacting with a free radical, an organic radical scavenger becomes a free radical itself. It can be decisive whether this new radical propagates a radical chain or soon undergoes a termination reaction by combining with another radical *(28,32,42,50,67)*. Moreover, a hydroxylated indole deriving from adduct formation may behave as a prooxidant when interacting with another free radical, because of the formation of an *O*-centered indolyl radical that propagates the chain reaction, whereas AFMK does not show this prooxidant behavior and represents a relatively stable compound *(66,68)*, owing to its preference for two-electron transfer reactions, as demonstrated by cyclic voltammetry *(69)*. Among the various free radicals tested thus far, AFMK was only attacked at substantial rates by hydroxyl radicals (cf. data of refs. *66,68,* and *69*).

The primary intermediate deriving from melatonin by a single-electron transfer step is the melatonyl cation radical.

Its formation, orginally predicted from mechanistic considerations *(28,32)*, was directly demonstrated independently by two groups *(67,70,71)*. A corresponding cation radical was found during oxidation of indole-3-propionic acid, a structural analog of melatonin *(46)*. It is, of course, possible that a neutral melatonyl radical is alternatively formed by hydrogen abstraction, or that the neutral radical is formed by proton release from the cation radical *(63)*. However, for several reasons, the existence of a cation radical intermediate appears to be of particular interest: (i) In the presence of sufficient amounts of superoxide anions, this oxygen species can easily combine with the cation radical, thereby allowing electron pairing and neutralization of charges. The product derived is AFMK (Fig. 1). (ii) The cation radical is clearly demonstrable in reaction mixtures containing peroxynitrite, in both the absence and presence of hydrogen carbonate *(70,71)*. Thus, this intermediate is also involved in scavenging of radicals formed from reactive nitrogen species, including the carbonate radical resulting from cleavage of the peroxynitrite-CO_2 adduct, a conclusion which was also drawn on the basis of product analyses and chemiluminescence data in another oxidation system *(50)*. (iii) The melatonyl cation radical may be crucial for understanding mitochondrial effects already observed at nanomolar concentrations of the indoleamine *(38)*.

Multiple Levels of Protection

As already pointed out, direct detoxification of free radicals may only play a quantitatively significant role if melatonin concentrations are sufficiently high. This can be the case in toxicological experiments in the presence of exogenous melatonin. There has been a debate as to whether physiological levels of melatonin could be locally high enough for this type of protection. This may occur in melatonin producing tissues, such as pineal gland, retina, skin, and—especially in rodents—the Harderian gland. In particular, this latter extrapineal site of melatonin synthesis is a good candidate for such an assumption, because it is generating free radicals at high rates, owing to the presence of 5-aminolevulinic acid and protoporphyrins in amounts much higher than in any other tissue. For this reason, rodent Harderian glands exist at the borderline of survival, and cell groups are regularly subject to physiological cell death, so that it can be used as a physiological model of oxidative stress *(38, 72,73)*. Moreover, melatonin concentrations correlate with protoporphyrin levels when compared on a species- and gender-dependent basis, and melatonin administration can protect from damage by additional, exogenous 5-aminolevulinic acid *(74)*.

Another area of discussion concerns the possibility of subcellular melatonin accumulation, so that the indoleamine might attain local concentrations for allowing on-site protection of, e.g., the DNA. Some experiments aiming to carefully control the problem of melatonin displacement during experimentation indicated this *(75,76)*, but nuclear accumulation was not seen in other studies *(77,78)*. Similar assumptions of mitochondrial accumulation are also not supported by those investigations *(77,78)*. The issue may be regarded as an open question.

In several non-vertebrate organisms, especially those which are capable of synthesizing the precursor, tryptophan, via the shikimic acid pathway, much higher concentrations of melatonin were measured, *(32,79–82)*. These may well suffice for protection by direct radical scavenging. This is presumably the case in a dinoflagellate *(39–41)* and may be assumed for some high-melatonin plants and fungi.

In this context, the aspect of bioavailability should also be considered in terms of the physical properties of melatonin. Contrary to the majority of other antioxidants, which are either strongly hydrophilic or lipophilic, its amphiphilicity allows the molecule to cross any membrane and to act in any cell, body fluid, cell compartment and in aqueous and lipid phases as well *(11,38,83,84)*.

Nevertheless, the problem of stoichiometry between oxidants formed and available melatonin concentrations remains, at least for the majority of vertebrate tissues, even when radical scavenger cascades are considered, in which several scavengers are sequentially generated from melatonin *(63,68)*. However, indirect antioxidant effects via receptor-mediated processes are not affected by this difficulty. In fact, melatonin was shown to upregulate several antioxidant enzymes (Fig. 2), frequently glutathione peroxidase *(1,9,12,13,85–99)*, sometimes—and presumably indirectly via oxidized glutathione (GSSG)—glutathione reductase *(9,12,13,100)*, in some tissues Cu,Zn- and/or Mn-superoxide dismutases *(12,13,88–90,96,97,99–105)* and, rarely, catalase *(13,99,103,105,106)*. Stimulation was most consistently observed in glutathione peroxidase, which was enhanced wherever tested in both mammalian and avian brain; upregulation was seen in other tissues as well, but varied according to sources and conditions. Surprisingly, rises in glutathione peroxidase activity were also reported for rat erythrocytes *(93,107)*, i.e., in cells lacking induction mechanisms. The possibility that glutathione peroxidase is not only upregulated at the transcriptional level, but may be additionally activated, deserves further attention. In accordance with the stimulatory actions, a sequence of circadian maxima was observed in the chicken brain, in which the melatonin peak preceded that of glutathione peroxidase, which was followed by the maximum of glutathione reductase *(108,109)*. Enzyme maxima were clearly melatonin-dependent, because they were abolished by constant light, an effect that should not be expected in the case of direct control by a circadian oscillator. A catalase rhythm, however, was not suppressed by light *(109)*. The actions of melatonin on glutathione metabolism seem to exceed the effects mentioned. Stimulations reported for glucose-6-phosphate dehydrogenase *(9)* and γ-glutamylcysteine synthase *(13,19)* may indirectly support the action of glutathione peroxidase

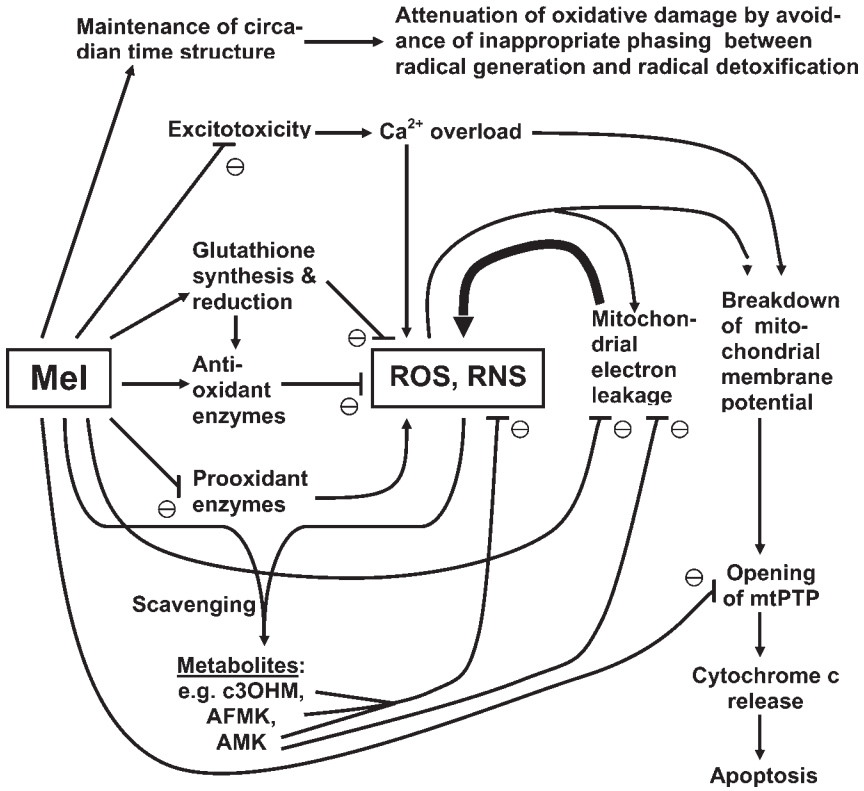

Fig. 2. Overview of the potentially most important actions of melatonin at different levels of antioxidative protection. Abbreviations: Mel, melatonin; c3OHM, cyclic 3-hydroxymelatonin; AFMK, N^1-acetyl-N^2-formyl-5-methoxykynuramine; AMK, N^1-acetyl-5-methoxykynuramine; ROS, reactive oxygen species; RNS, reactive nitrogen species; mtPTP, mitochondrial permeability transition pore; ⊖ inhibitory effect.

by providing reducing equivalents (NADPH) for the action of glutathione reductase and by increasing the rate glutathione synthesis, respectively.

Contrary to the enzymes of glutathione metabolism, no general rule can be detected in the effects of melatonin on superoxide dismutase subforms and catalase. Although stimulation has been repeatedly described, some of them were only of limited extent, others were only demonstrated at the mRNA level, but, more important, very often no effects *(91, 92,95,98,110,111)* or even decreases *(94)* in enzyme activities were observed. This may be partially a matter of tissue differences, but one should also clearly see the complexity in the regulation of the respective enzymes. Frequently, they exhibit compensatory rises in response to oxidative stress. When melatonin counteracts experimentally induced stress, the result may be a normalization of enzyme activity, i.e., lower values compared to animals treated with oxidotoxins, rather than inductions. Such normalizations were, in fact, repeatedly described *(94,112,113)*. However, in cases of stronger oxidative stress, active centers of enzymes may be destroyed by the free radicals generated and normalization of enzyme activities by melatonin administration becomes interpreted as rises *(90,106,112)*.

An additional aspect of melatonin's actions on antioxidant enzymes deserves further attention. In two neuronal cell lines, physiological concentrations of melatonin were not only shown to induce glutathione peroxidase and superoxide dismutases at the mRNA level, but concomitantly altered the life-time of these mRNAs *(96)*.

Melatonin was also shown to downregulate prooxidant enzymes (Fig. 2), in particular, 5- and 12-lipoxygenases *(13, 114–116)* and NO synthases *(4,6,9,13,14,38,117–122)*. The almost generally observed attenuation of NO formation is particularly important in terms of limitations to rises of the strongly prooxidant metabolite peroxynitrite and of the free radicals deriving from this compound, namely, $\bullet NO_2$, carbonate ($CO_3\bullet^-$), and hydroxyl radicals. Suppressions of both lipoxygenase and NO synthase may additionally set limits to inflammatory responses, although the immunomodulatory actions of melatonin are certainly more complex.

Another, widely unexplored area of signaling by melatonin in the context of antioxidative protection concerns the role of quinone reductase 2 *(38,123–125)*. This enzyme, which is implicated in the detoxification of potentially prooxidant quinones, binds melatonin at upper physiological concentrations, so that it had originally been regarded as a melatonin receptor. Its precise function under the influence of melatonin is not yet understood.

The multiple levels of antioxidative protection by melatonin are not comprehensively described by direct radical

detoxification and regulation of pro- or antioxidant enzymes. Another poorly understood, but possibly important field is that of interactions with other antioxidants. In both chemical and cell-free systems, melatonin was repeatedly shown to potentiate the effects of ascorbate, Trolox (a tocopherol analog), reduced glutathione, or NADH *(31,48,63,126)*. These findings, which can be clearly distinguished from additive effects, are surprising as they indicate multiple interactions, via redox-based regeneration of antioxidants transiently consumed. Although the details require further elucidation, such results may be taken as a strong hint for quasi-catalytic electron-transfer reactions between the different antioxidants. One might be inclined to regard such potentiations as nothing else but in vitro effects, without any relevance for living cells. Nevertheless, melatonin was also shown in vivo, under conditions of long-lasting experimental oxidative stress, to prevent decreases in hepatic ascorbate and α-tocopherol levels *(98)*.

Two other facets of indirect antioxidative effects by melatonin are frequently omitted from the discussions of protective mechanisms, although they may be highly important in practice. Especially in the central nervous system, strong excitatory responses associated with high calcium influx and NO release or, in the extreme, excitotoxicity are usually associated with enhanced radical generation. Melatonin was shown to possess strong anticonvulsant properties and to efficiently counteract the actions of various excitotoxins *(127)*. When analyzed in detail, neuroprotection by melatonin against excitotoxins turned out to be a superposition of antiexcitatory and direct antioxidant effects (Fig. 2), as shown for glutamate *(128)* and its agonists, ibotenate *(129)*, kainic acid *(36,130)*, domoic acid *(131)*, and, in particular, also for quinolinic acid *(132–134)*. Nevertheless, it is important to note that antiexcitotoxic effects already attenuate radical formation and, thereby, represent indirect antioxidant actions.

Indirect antioxidative protection in terms of radical avoidance may also be assumed for the chronobiological role of melatonin as an endogenous coordinator of rhythmic time structures. The importance of appropriate timing for maintaining low levels of oxidative damage has been overlooked for quite some time. However, it turned out that temporal perturbations as occurring in short-period or arrhythmic circadian clock mutants lead to enhanced oxidative damage, effects observed in organisms as different as *Drosophila (38,135)* and the Syrian hamster *(38,136)*.

A Chronobiological Problem, the Significance of Tissue Melatonin, and Formation of Protective Metabolites

In the average animal cell, with the main exception of phagocytic leukocytes, the major sources of oxygen free radicals are mitochondria; reactive nitrogen species and other peroxynitrite-derived radicals depend on NO synthesis. As an important consequence, free radicals are predominantly formed when cells or tissues are in an enhanced state of activity. This was directly demonstrated in living, exercising muscles and also became evident in neuronal NO formation. Therefore, circadian maxima of free radical formation should be associated with maxima of locomotor and neuronal activities *(38)*. However, melatonin as released from the pineal gland always peaks nocturnally, regardless of whether animals are day- or night-active. Thus, the problem arises why melatonin should exert its protective effects mainly in circadian phases of low radical generation in day-active animals, whereas it would be in phase with maximal radical formation in dark-active species. This problem extends to receptor-mediated processes, and, in fact, upregulation of glutathione peroxidase by melatonin is nocturnal both in the night-active rat and the day-active chicken *(1,9, 12,13,85–99)*. This relationship is less evident in NO formation. In various mouse or rat organs including brain, kidney, testes, and lungs, rhythms of NO formation mostly peaked at night *(137,138)*, and, thus, did not correlate inversely with melatonin. Still, the possibility remains that nocturnal melatonin limits the rises in NO.

The temporal relationships, appearing at first glance as a major problem for ascribing to melatonin a physiological role in antioxidative protection, turns out to be much less severe after a look at tissue melatonin. Although melatonin may somehow also contribute to the antioxidant status in the blood plasma *(139)*, protection mainly takes place in tissues and cells. Tissue concentrations of melatonin are only partially known. Where determined, the amounts are much higher than in the circulation *(4)*. This holds especially for the gastrointestinal system, which contains, compared to the pineal gland, several hundred times more of melatonin, to which several sources contribute, such as synthesis by enterochromaffin cells, uptake from the blood, and uptake from the lumen including enterohepatic cycling *(82,77,78,140, 141)*. Another decisive difference to levels in the circulation concerns the amplitude of the gastrointestinal melatonin rhythm, which is much smaller (minimum/maximum ratio rarely higher than 1:2) and sometimes even at the borderline of demonstrability *(140,141)*. In the Harderian gland, the melatonin rhythm is also weakly expressed *(4)*. Therefore, one should not judge melatonin's role in tissues by extrapolating from levels and rhythmicity in the blood plasma. Low-amplitude rhythmicity in the tissue, however, strongly weakens the chronobiological argument of melatonin being in antiphase with preferential radical formation in day-active animals. This would especially hold if melatonin's oxidative metabolites formed by interactions with free radicals would contribute to protection. If melatonin concentrations are rhythmically varying in tissues by not more than a factor of 2 or even less, the amounts of oxidants produced become rate limiting for the levels of oxidation products from mela-

tonin, such as AFMK and its deformylated metabolite AMK (N^1-acetyl-5-methoxykynuramine). Such products might oscillate in phase with radical formation, an assumption that remains, however, to be demonstrated.

This idea may gain relevance because kynuramines including AFMK and AMK have been shown to be biologically active *(142)*. Protection by AFMK has been described, in terms of inhibition of 8-hydroxy-2-deoxyguanosine formation, reduction of lipid peroxidation, and rescuing of hippocampal neurons from oxidotoxic cell death; however, pharmacological concentrations of the kynuramine were applied in these studies *(69,143)*. Its deformylated product AMK seems to be even more interesting, for several reasons: first, it is a radical scavenger of considerably higher reactivity than AFMK *(66,68,144)* and, second, it acts as a cyclooxygenase inhibitor much more potent than acetylsalicylic acid *(145)*, according to unpublished data (B. Poeggeler, personal commun.) with specificity for cyclooxygenase 2. It might, therefore, contribute to the attenuation of oxidative stress both directly and, by interference with inflammatory responses, indirectly. A third, mitochondrial effect will be discussed below. A major obstacle for judging the role of AMK is the lack of information on its physiological levels.

Nevertheless, the formation of kynuramines from melatonin seems to be highly important especially in non-hepatic tissues. A statement frequently read in earlier literature that almost all melatonin is metabolized in the liver to 6-hydroxymelatonin, which is subsequently conjugated and excreted, can, if at all, only be valid for the circulating indoleamine, but not for tissue melatonin. Extrahepatic P_{450} monooxygenase activities are too low for a quantitative turnover via this route, and the high amounts of gastrointestinal melatonin enter the circulation only to a limited extent, e.g., as a post-prandial response; most of it is either released to the lumen *(77,78,140,141)* or has to be metabolized in another pathway. According to recent estimations, about 30% of overall melatonin degradation is attributed to pyrrole-ring cleavage *(146)*, although the rate may be considerably higher in certain tissues. AMK was identified as a main metabolite of melatonin in the central nervous system *(147)*. When melatonin was injected into the cisterna magna, about 35% was recovered as AMK. Under the conditions used, AFMK and AMK were the only products formed from melatonin in the brain and no 6-hydroxymelatonin was detected. These findings have re-gained high actuality, after melatonin was shown to be released directly to the third ventricle, via the pineal recess, in much higher quantities than in the blood *(148)*. The high turnover in the kynuric pathway of melatonin catabolism is the more remarkable as it cannot be explained on the basis of the enzymes capable of catalyzing the formation of AFMK: (i) indoleamine 2,3-dioxygenase, anyway using tryptophan as the main substrate, exhibits sufficiently high activities only after inflammatory stimulation of microglia *(149–151)*; (ii) myeloperoxidase, which can also catalyze pyrrole-ring cleavage of melatonin *(146)*, is also associated with activated phagocytes. To assume free radical reactions as the main cause of kynuric melatonin degradation in the brain is, therefore, highly suggestive.

It is a remarkable fact that AFMK is formed by many different mechanisms (summarized in refs. *48,52,* and *66*), including enzymatic reactions (see above), pseudoenzymatic catalysis by oxyferrylhemoglobin or by hemin, by interactions with free radicals, e.g., combinations of hydroxyl radical and $O_2^{\bullet-}$, or $CO_3^{\bullet-}$ and $O_2^{\bullet-}$, or organic cation radicals and $O_2^{\bullet-}$, by singlet oxygen, by ozone, by O_2 under photoexcitation of melatonin, and by radical reactions of cyclic 3-hydroxymelatonin. This preference for the pyrrole-ring cleavage pathway is exceptional among indolic compounds. Although kynuric products are also formed from other indoles, especially the radical-based mechanisms usually lead to much higher amounts of non-kynuric products from other indoleamines, including the most closely related structural analogs, such as 5-methoxytryptamine, *N*-acetylserotonin, *N*-acetyltryptamine, 2-iodomelatonin, 6-hydroxymelatonin, and even 6-methoxymelatonin (*42,152*; unpublished data by J. Rosen and R. Hardeland). These findings strongly underline the significance of the combination of 5-methoxy and *N*-acetyl residues and of the absence of other substitutents.

The primary cleavage product AFMK is relatively inert toward radicals of lower or intermediate reactivity *(66,68)*, which is easily explained by its preference for two-electron transfer reactions as demonstrated by cyclic voltammetry *(69)*, but, not surprisingly, it interacts with the hydroxyl radical *(69,143)*. Therefore, protective effects by high concentrations of AFMK observed in living cells *(69)* have be explained—if not by unknown signaling mechanisms—either by the high dosage or by conversion to the more potent AMK. In biological material, deformylation to AMK is highly likely, as already shown by the higher amounts of AMK compared to AFMK detected in the urine after melatonin injections *(147)*. To date, two enzymatic deformylating mechanisms are known, (i) by arylamine formamidase *(28,66)* and (ii) by the ubiquitously present hemoperoxidases (including catalase) *(66,153)*. In the latter case, the enzyme catalyzes the formation of an imino intermediate, which is hydrated to give an unstable carbamate releasing CO_2 (Fig. 1). AMK is not only considerably more reactive *(66,68, 154)*, but undergoes single-electron transfer *(66,68)* and addition reactions including NO scavenging, which give rise to various new products (R. Hardeland et al., unpublished data). The pharmacology of AMK and its metabolites, as well as the exploration of their protective potency, are still at their beginning. Actually, our knowledge is largely limited to the inhibition of cyclooxygenase and to in vitro protection of proteins from destruction by peroxyl radical-initiated processes *(68)*, but a mitochondrial effect of AMK

(see below) seems to indicate the most promising route for future research.

Mitochondrial Effects

Protective actions of melatonin are actually attracting particular interest, especially with regard to three aspects: (i) mitochondria as a major source of free radicals including their assumed role in aging processes, (ii) the increasing perception of the importance of mitochondrial dieseases, and (iii) the key role of mitochondria in apoptosis. Protection in a more general sense such as attenuation of mitochondrial lipid peroxidation, prevention of oxidative protein and DNA modifications, preservation of ultrastructure, resistance against toxins, etc., were repeatedly described (e.g. refs. *19,91,* and *155–158*). Such studies also demonstrated influences of melatonin on redox-active compounds in mitochondria, such as decreases in NO *(159,160)* and restorations of normal levels of reduced glutathione *(91,156)* and coenzyme Q10 *(161)*. These effects may have been somehow expected with regard to previously known antioxidative actions, but additionally, activities in the respiratory chain were observed, which are far from being self-evident. In fact, results obtained were not at all uniform and varied according to experimental conditions, especially with regard to the absence or presence of oxidotoxins. In the latter case, melatonin was usually found to increase respiratory activity and ATP synthesis, mostly in relation to rises in the measurable activities of complexes I and IV *(13,37,155, 158–160,162–164)*. However, there are some divergencies regarding the enhancement of complex I and IV activities by melatonin, because just the opposite was observed in other situations. Complex IV activity in cerebral mitochondria, which is reportedly increased in aging mice, was diminished by melatonin to levels characteristic of 3-mo-old animals *(165)*. Decreases in oxygen consumption by liver mitochondria in vitro were described for 10^{-7} *M* melatonin *(166)*. Contrary to this, complex I and IV activities were found to be stimulated by melatonin in hepatic mitochondria of senescence-accelerated mice *(167–169)*. Moreover, a recent study indicated a stimulatory action of melatonin on gene expression of complex IV components *(160)*.

What is the really important message from these investigations? Presumably it is not the more or less of ATP produced or O_2 consumed. First, one can state that some of the effects were observed when melatonin was administered in the drinking water *(165,167,168)*, i.e., at concentrations lower than those applied to counteract oxidotoxins in the majority of in vitro experiments. But even in vitro, mitochondrial effects of 1 n*M* melatonin were demonstrable *(37)*, findings underlining the physiological importance of mitochondrial protection, compared to many other protective effects observed at the highest melatonin levels only and explained by direct radical scavenging. Second, it seems necessary to focus on those situations that are really dangerous for a cell in pathophysiology and aging, namely, breakdown of the mitochondrial membrane potential as a consequence of overexcitation, calcium overload—eventually due to protein misfolding—leading to initiation of apoptosis via opening of the mitochondrial permeability transition pore (mtPTP), in other words, by processess that can be induced by excitatory actions of NO and damage by oxygen free radicals as well. It is therefore important to know that melatonin, in cells as different as cardiomyocytes, astrocytes, and striatal neurons, was able to prevent calcium overload *(170, 171)*, to counteract the collapse of the mitochondrial membrane potential induced by H_2O_2 *(170)*, doxorubicin *(172)*, or oxygen/glucose deprivation *(171)*, and to inhibit mtPTP opening *(170,171)*. It is not yet clear to what extent physiological levels would suffice for these effects. In addition to the antioxidant actions, melatonin seemed to directly inhibit mtPTP currents (Fig. 2), with an IC_{50} of 0.8 μ*M (171)*, a concentration which would require mitochondrial accumulation of melatonin.

This is, however, not entirely impossible, owing to melatonin's amphiphilicity (see above), which might allow a certain enrichment in/at the mitochondrial inner membrane. Amphiphilicity as a necessity for entering the site is another prerequisite for melatonin's actions at the respiratory chain. Again, the redox properties of melatonin seem to be crucial for understanding these effects, especially with regard to single-electron transfer reactions. This should not be seen primarily in terms of direct scavenging of radicals already formed, but rather of decreasing the rates of radical formation by interfering with the respiratory chain. In fact, melatonin has been shown to diminish mitochondrial H_2O_2 *(173)*, which is formed from superoxide anions generated by single-electron donation to oxygen, mainly by the iron–sulfur cluster N2 in complex I *(174)*. This profoundly important link between radical formation and energy metabolism would explain many effects, already at lower, physiological concentrations of melatonin if this compound and/or one of its metabolites can be shown to undergo single-electron exchange reactions with the respiratory chain. Because melatonin as well as AMK can reduce complexed iron, we assume that cytochrome c, normally mediating between complexes III and IV and acting as an electron acceptor from outside the respiratory chain, e.g., from $O_2^{\bullet -}$ formed in complex I *(175)*, may be also a site of electron donation by melatonin and/or AMK. In a recently published model orginating from an idea by B. Poeggeler based on comparable results with amphiphilic nitrones, we suggest a mechanism by which $O_2^{\bullet -}$ formation at complex I is decreased by competing, electron-accepting melatonyl or AMK cation radicals; these are, in turn, formed by electron donation to the respiratory chain *(38)*. Such a mechanism (Fig. 3) would only require very low, quasi-catalytic amounts of melatonin or AMK, in accordance with the effects demonstrated with nanomolar con-

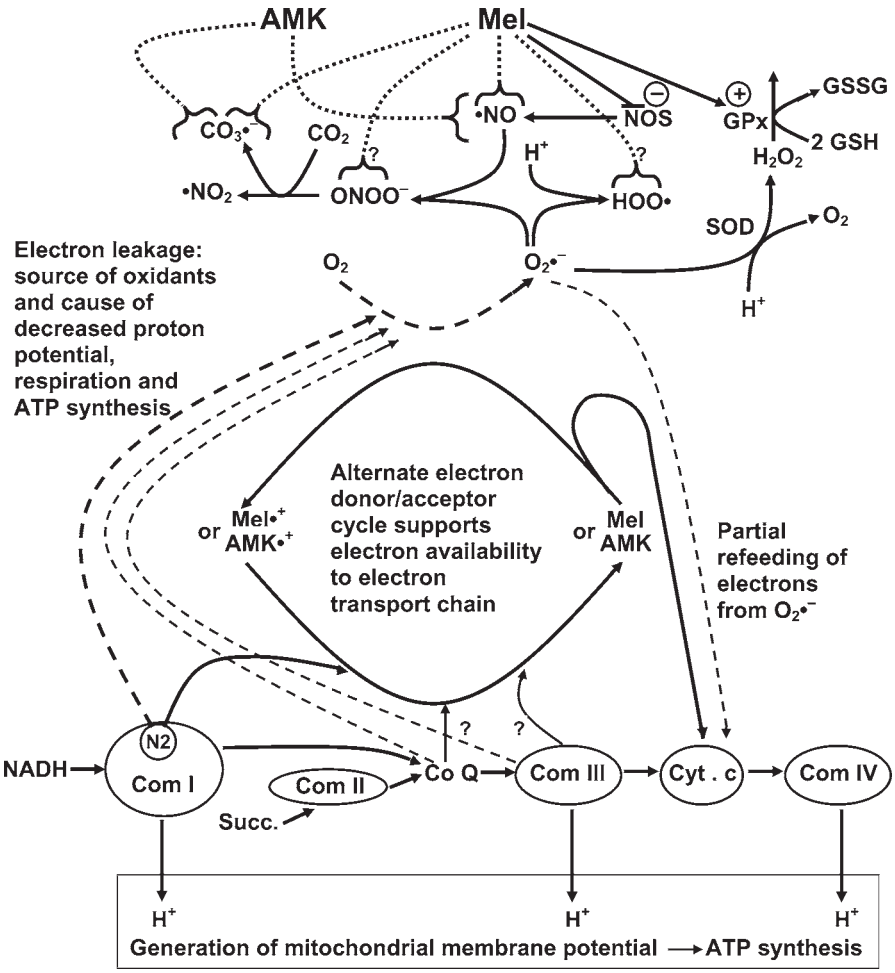

Fig. 3. Model of the main interactions of melatonin and its metabolite AMK with mitochondrial metabolism. Dashed lines: electron leakage and partial refeeding; dotted lines: scavenging of oxidants. Abbreviations: AMK, N^1-acetyl-5-methoxykynuramine; Com, complex; Co Q, coenzyme Q; Cyt.c, cytochrome c; GPx, glutathione peroxidase; Mel, melatonin; NOS, NO synthase; SOD, superoxide dismutase; Succ., succinate.

centrations *(37)*. Because the recycled electrons do not get lost for the respiratory chain, this would also explain improvements in complex IV activity, oxygen consumption, and ATP production. The prediction by our model of a protection by AMK to mitochondria was recently confirmed *(160)*. AMK was shown to exert effects on electron flux through the respiratory chain and ATP synthesis very similar to those observed with melatonin.

Concluding Remarks

In the future, research on antioxidant effects of melatonin should follow several lines. First of all, protection as demonstrated in numerous publications at pharmacological but sometimes also physiological levels has to be explained on the basis of concentrations present in the respective tissues. Contours of the mechanisms are actually becoming visible, but explanations have to go beyond upregulation of antioxidant and downregulation of prooxidant enzymes. It seems promising to strongly focus on mitochondrial effects, which appear to be crucial for the understanding of antioxidative protection by melatonin and its metabolites, in particular AMK. Avoidance of radical formation may turn out to be more important than scavenging of radicals already produced. It will also be necessary to investigate the antioxidant and other pharmacological properties of melatonin metabolites in detail, not only those of AFMK and AMK, but also of the products formed from AMK, some of which have already been identified (R. Hardeland et al., unpublished data). Antioxidative protection by the pleiotropically acting indoleamine melatonin is now presenting itself as a highly complex phenomenon (Figs. 2 and 3), but the ensemble of mechanisms, whether already known or still to be elucidated, is worth of becoming fully understood in all details, especially with regard to the remarkable safety of the compound, which has been administered without problems for years, even to patients, at dosages of up to 30 mg per day, as stated in an actually running study on amyotrophic lateral sclerosis *(176)*. It seems that the application of melatonin will soon gain a broader basis of understanding.

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