Review

Mitochondria: the birth place, battle ground and the site of melatonin metabolism in cells

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ABSTRACT

It was a surprising discovery when mitochondria, as the power houses of cells, were also found to synthesize the potent mitochondrial targeted antioxidant, melatonin. The melatonin synthetic enzyme serotonin N-acetyltransferase (SNAT) was found in matrix and also in the intermembrane space of mitochondria. We hypothesize that the melatonin synthesis occurs in the matrix due to substrate (N-acetyl co-enzyme A) availability while the intermembrane space may serve as the recycling pool of SNAT to regulate the melatonin circadian rhythm. Another surprise was that the melatonin membrane receptors, including MT1 and MT2, were also present in mitochondria. The protective effects of melatonin against neuronal injury induced by brain ischemia/reperfusion were proven to be mainly mediated by mitochondrial melatonin receptors rather than the cell surface membrane receptors which is contrary to the classical principle. In addition, melatonin metabolic enzyme has also been identified in the mitochondria. This enzyme can convert melatonin to N-acetylserotonin to strengthen the antitumor effects of melatonin. Thus, mitochondria are the generator, battle ground and metabolic sites of melatonin. The biological significance of the strong association between mitochondria and melatonin should be intensively investigated.

Key words: mitochondria, melatonin, oxidative stress, serotonin N-acetyltransferase (SNAT), plant, bacteria, archaea.

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1. INTRODUCTION

Melatonin is a phylogenic ancient molecule. Its presence can be traced to bacteria, algae and fungi (1). It is widespread in the domains of bacteria and eukaryotes. Its presence in domain of archaea has not yet been proven. From the evolutionary point of view, melatonin should also be present in archaea. A homolog of melatonin synthetic gene N-acetyltransferase (*NAT)* has been reported in archaea (2) and a structure of NAT in another archaea (*Thermoplasma volcanium*) has been identified belonging to N-acetyltransferase family member; it has the capacity to bind acetyl coenzyme A (3). Acetyl coenzyme A is the substrate of mammalian SNAT that is used for melatonin synthesis. Archaeal *NAT* gene is clustered closer to bacteria rather than to the eukaryotes

(4); this indicates its potential origin and that it may have been horizontally transferred from bacteria. It has been speculated that the melatonin synthetic gene, particularly *SNAT* in eukaryotes, was also transferred from bacteria. This transference may be *via* the process of symbiosis of the protoeukaryotes with bacteria being hypothesized as the precursors of mitochondria (5). As a result, the *SNAT* of bacteria is then integrated into the nuclei of eukaryotes. In most vertebrates, only one homolog of *SNAT* gene is detected and it is probably transferred from the proteobacteria (6).

In contrast, in plants many *SNAT* homologs have been identified in different species (7-9). This indicates potentially multi-origins of *SNAT* genes in plants. For example, some plant *SNAT* genes may have originated from cyanobacteria (10–12). The DNA sequences and protein structures of *SNAT* from bacteria to vertebrates have evolved significantly, especially in the regulatory parts of this enzyme (4); however, the structure of their product, melatonin, has not been modified. This is likely attributed to melatonin's important function as an optimal antioxidant. The structural modification might jeopardize its free radical scavenging activity and antioxidant capacity of this molecule. A primary function of melatonin in all organisms serves as the first-line antioxidant to protect against environmental and internal oxidative stress. Other functions of melatonin are acquired during evolution (1).

In vertebrates, melatonin also serves as the signalling molecule to transduce environmental photoperiodic information to their neuroendocrine system (13-15). Thus, the animals can synchronize their daily or seasonal physiological activities coupled to their environments. These physiological activities include immunity, locomotive, sleep, reproduction, etc. (16-21). These actions of melatonin have developed during different stages of evolution since bacteria do not require melatonin to transduce photoperiodic information. Unicellular organisms can directly detect these changes.

In eukaryotes, mitochondria are the major source of reactive oxygen species (ROS) and they require specific onsite protection (22–24). Melatonin is a suitable selection for this purpose due to its antioxidative potency and its availability (25). It is calculated that a melatonin molecule may scavenge up to 10 ROS (26, 27). In addition, it also stimulates the activity of the mitochondrial antioxidative enzymes, Mn-SOD in animals and also in plants (28-31). Mitochondria can synthesize melatonin *de novo* and they possess an uptake mechanism to maintain high levels of melatonin. To better understand the importance of melatonin in mitochondria, in this review, we briefly discuss the recent developments regarding mitochondrial melatonin synthesis, metabolism and some activities of melatonin on mitochondrial functions as well as their dynamics.

2. MITOCHONDRIA ARE THE MAJOR SITES OF MELATONIN SYNTHESIS IN ALL CELLS

Melatonin synthesis in vertebrates is different from that in plants or some unicellular organisms such as yeast. Yeasts use glucose as a starting material to synthesize melatonin (32). Plants use CO_2 as the initial molecule for melatonin biosynthesis (33). However, the sole starting material of melatonin synthesis in vertebrates is tryptophan (34), an essential amino acid. Therefore, the melatonin synthetic machinery in vertebrates is much simpler than that in fungi and plants. The melatonin synthetic pathway of vertebrates is comprised of only 4 enzymes. They are tryptophan 5-hydroxylase, aromatic amino acid decarboxylase (AADC), serotonin N-acetyltrasferase (SNAT)

and N-acetylserotonin methyltrasferase (ASMT). Under the enzymatic actions of these 4 enzymes in sequence, melatonin is synthesized from tryptophan (Fig. 1).

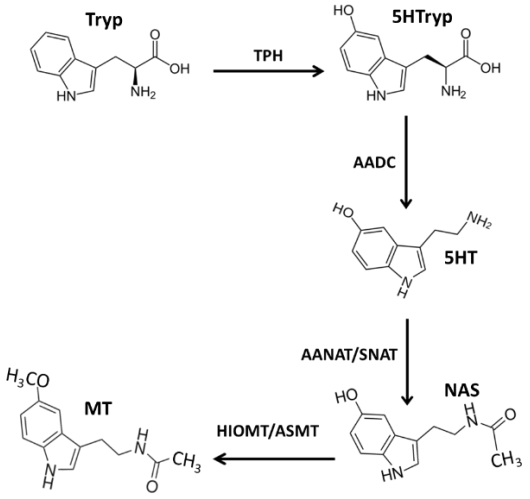

Fig. 1. Melatonin synthetic pathway in vertebrates.

Tryp: tryptophan, TPH: tryptophan 5-hydroxylase, 5HTryp: 5-hydroxyltryptophan, AADC: aromatic amino acid decarboxylase, 5HT: 5-hydroxyltryptomine (serotonin), SNAT (formal AANAT): serotonin N-acetyltrasferase, NAS: N-acetylserotonin, ASMT (formally HIOMT): N-acetylserotonin methyltrasferase, MT: melatonin.

The most likely precursors (proteobacteria) of mitochondria have the capacity to produce melatonin (35, 36). Based on evolutionary evidence and other factors, we have hypothesized that mitochondria are the major sites of melatonin production in organisms (5). This is completely different from the previous idea which hypothesized that melatonin is synthesized in the cytosol of cells. Since then, melatonin synthesis has been identified in mitochondria of different cells including oocytes, pinealocytes, neurons, endothelial cells and plant cells (37-40), Not only do isolated mitochondria generate melatonin but they also possess SNAT, the rate limiting enzyme for melatonin synthesis; it is found exclusively to co-localize with mitochondria (Fig. 2).

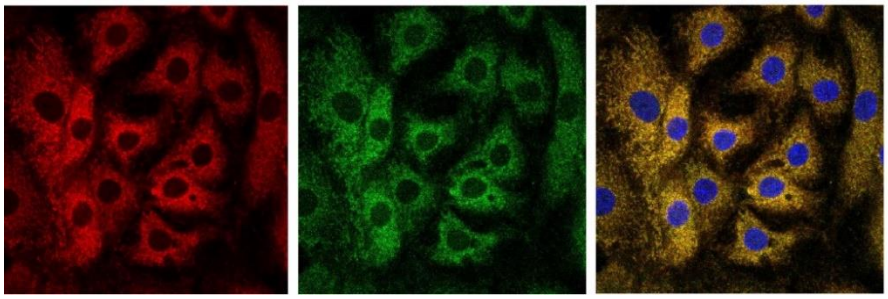

Fig. 2. Localization of SNAT in rat choroid plexus endothelial cells.

Left panel: fluorescence images of SNAT (red), middle panel: staining of mitochondria (MitoTracker Green FM), right panel: the merging of SNAT staining and mitochondrial staining, the nuclei are blue stained with fluorescent dye Hoechst 33342. The results indicate that the SNAT is exclusively co-localized with mitochondria in rat choroid plexus endothelial cells. Modified from (40).

The overwhelming evidence confirms that melatonin is generated by mitochondria. This observation does not naturally preclude the possibility of the cytosolic melatonin production since the red blood cells, which are devoid of mitochondria, have the capacity to produce melatonin (41). However, N-acetyl-coenzyme A is an irreplaceable substrate of SNAT. The N-acetyl-coenzyme A is mainly synthesized in mitochondria. Judging from the substrate availability, particularly the N-acetyl-coenzyme A, the melatonin synthetic efficiency in cytosol would be much lower than that in mitochondria. Bearing this in mind, we believe that the amounts of cytosolic synthesized melatonin is minimal compared to the amounts of mitochondrial melatonin under most circumstances.

One major question is at which sites in mitochondria, i.e., matrix or intermembrane space is melatonin formed? With the use of a protein degradation procedure, Suofu *et al*. (39) deduced that the SNAT was located in the matrix of mitochondria; they claimed that melatonin was synthesized exclusively in the matrix of mitochondria. In contrast, Yang *et al* observed that the SNAT was present in the mitochondrial intermembrane space close to the outer membrane (42). This seems to support the observations of Kerenyi *et al* (43, 44) that SNAT product was observed in the mitochondrial intermembrane space of pinealocytes. Based on the current data, it is difficult to localize melatonin synthesis exclusively in the matrix or in the intermembrane space of mitochondria. In our opinion, the SNAT localization cannot be used as the sole index of melatonin synthetic site.

The *SNAT* gene is not encoded by mitochondrial DNA but by nuclear DNA. Thus, the SNAT protein generally is produced in the cytosol and then it is imported into mitochondria. Most of the mitochondrial precursor proteins have a signal sequence at their N terminus (transient peptide). The mitochondrial-targeted transient peptides are both necessary and sufficient for import of the proteins into mitochondria that contain them (45, 46). This transient peptide is not detected in the vertebrate SNAT.

Although the mechanisms are currently unknown, we do know that the SNAT can be translocated from the cytosol into mitochondria. When SNAT is imported into the matrix, it must across the intermembrane space. Thus, the SNAT detected in the intermembrane space is a necessary process and this does not mean that the enzyme also functions in that location. In addition, the reverse process that the SNAT translocation from the matrix into the intermembrane space may also exist by using the same or different translocators. This reversed transport, if it exists, can serve as the regulatory mechanisms for melatonin synthesis.

Since N-acetyl-coenzyme A is synthesized and enriched in matrix, from a point of view of enzymatic kinetics, the matrix is a more suitable location for melatonin synthesis than that is in the intermembrane space. We speculate that the intermembrane space can be used as the SNAT recycling pool to regulate melatonin production required by cells. This is especially likely in pinealocytes. Circadian rhythm of melatonin synthesis in the mitochondria of pinealocytes with a high activity in the dark and lower level during the day was observed (39). Melatonin circadian rhythm is dependent on the activity of SNAT. The regulation of SNAT activity occurs at multiple levels. In ungulates and primates, the SNAT activity is generally regulated by the enzyme's phosphorylation and dephosphorylation. Phosphorylation increases the stability of SNAT and enhances its activity. In rodents, its regulation is mediated by up or downregulation of the *SNAT* gene expression (47). Even rough mitochondria from rat brain did not exhibit a rhythm in the presence of AANAT, according to the Suofu et al (39). However, the same authors reported the presence of the 14-3-3zeta scaffolding protein in mitochondria, a protein that stabilizes

phosphorylated AANAT. Therefore, it might be possible that mitochondrial AANAT may cycle on the basis of phosphorylation and stabilization, as known from the primate and ungulate pineal. However, a cycle of intramitochondrial phosphorylation would have to be demonstrated. Here, we suggest an alternative mechanism to explain the melatonin rhythm, that is, to alter the SNAT activity by translocation from the matrix to intermembrane space or *vice verse*. For example, if the pinealocytes are required to produce less melatonin such as during the day, the SNAT is shunted from the substrate enriched matrix to the intermembrane space and *vice verse*. This mechanism might be more economical regarding energy consumption than phosphorylation/dephosphorylation and gene regulation.

N-acetylserotonin methyltransferase (ASMT) is also localized in the mitochondria. It can be deduced that the last step of melatonin formation may be carried out by SNAT under some conditions rather than by ASMT. This alternative pathway for melatonin synthesis has been confirmed in plants (48). In that study, the results indicated that during normal condition, a majority of melatonin was the product of ASMT as the last enzyme; however, under stressful conditions, the major part of melatonin was generated by catalytic activity of SNAT as the final enzyme (49).

The discovery of melatonin synthesized by mitochondria provides a plausible explanation for the observation that virtually all cells have the capacity to produce melatonin because they have mitochondria. The obvious question is whether the cells with more mitochondria, such as muscles and hepatocytes generate more melatonin than other types. The answer is currently unknown. However, the bile of vertebrates contains extremely high levels of melatonin. An example is that the level of melatonin in shark bile is several orders of magnitude higher than that in the serum of animals (50). Whether this high level of melatonin is from hepatocytes which contain large quantities of mitochondria is a question to be answered.

3. MELATONIN METABOLISM IN MITOCHONDRIA

In animals the circulatory melatonin is primarily metabolized in hepatocytes by the $CP_{450}A_2B$ to form 6-hydroxymelatonin which is then sulfated and excreted in the urine. A small portion of melatonin is degraded by other tissues including skin and brain by either $CP_{450}A_2B$ or 2,3-indolamine dioxygenase to form 6-hydroxymelatonin or N^1 -acetyl-N^2 -formyl-5-methoxykynurenine (AFMK) (51–53). AFMK has been identified in urine of rats and humans (53, 54) and its blood level might associate with breast cancer of human (50); however, its urinary amounts were far below its tissue or CSF levels (55, 56). The urinary excretion probably is not the major metabolic route of AFMK judging from its water solubility.

All enzymatic actions of melatonin metabolism occur outside mitochondria since these enzymes are believed to be only present in cytosol. In addition to the enzymatic metabolism, melatonin can also interact with a variety of ROS to form a spectrum of products (27, 57-59). These actions can occur both in cytosol and mitochondria since mitochondria are the primary source of ROS formation. Whether melatonin can be enzymatically metabolized in mitochondria relies on whether its metabolic enzymes are also present in these organelles. Semak *et al* (60, 61) initially observed that melatonin was metabolized to form AFMK in mitochondria. However, this AFMK was not converted by 2,3-indolamine dioxygenase which is the standard enzyme for AFMK formation. The AFMK in mitochondria is the product of melatonin interaction with cytochrome C. Cytochrome C is not a classic enzyme but an electron carrier of the electron

transport chain (ETC) in mitochondria; thus, this reaction is classified as the pseudo-enzymatic reaction of melatonin (62).

It is not surprising that cytochrome C metabolizes melatonin. Cytochrome C is a phylogenic ancient molecule which is already present in bacteria (62). Bacteria may have selected this molecule as the protoenzyme to metabolize melatonin. As mentioned above, bacteria are the precursors of mitochondria. Mitochondria probably also preserved this primitive method to metabolize melatonin. The potential mechanism as to how cytochrome C interacts with melatonin was discussed in detail by two groups (60, 63).

Unexpectedly, several Cytochrome P_{450} (CP_{450}) enzyme isoforms are found to localize in the mitochondria. One of them, $CP_{450}1B_1$, responsible for metabolizing melatonin to form N-acetylserotonin (64). This was the first report to identify melatonin metabolizing enzyme in mitochondria. This enzyme probably is the key element responsible for the antitumor effects of melatonin in some cancer cells. Mitochondrial $CP_{450}1B_1$ deficiency led to a low level of mitochondrial N-acetylserotonin and reduced the anticancer effect of melatonin. The over expression of this enzyme increased the efficacy for melatonin to kill tumor cells. This observation raises an interesting issue, that is, a potential equilibrium mechanism occurs between N-acetylserotonin and melatonin in mitochondria. In mitochondria, due to the presence of $CP_{450}1B_1$ and ASMT (39), N-acetyl serotonin is either the precursor or the product of melatonin (Fig.3).

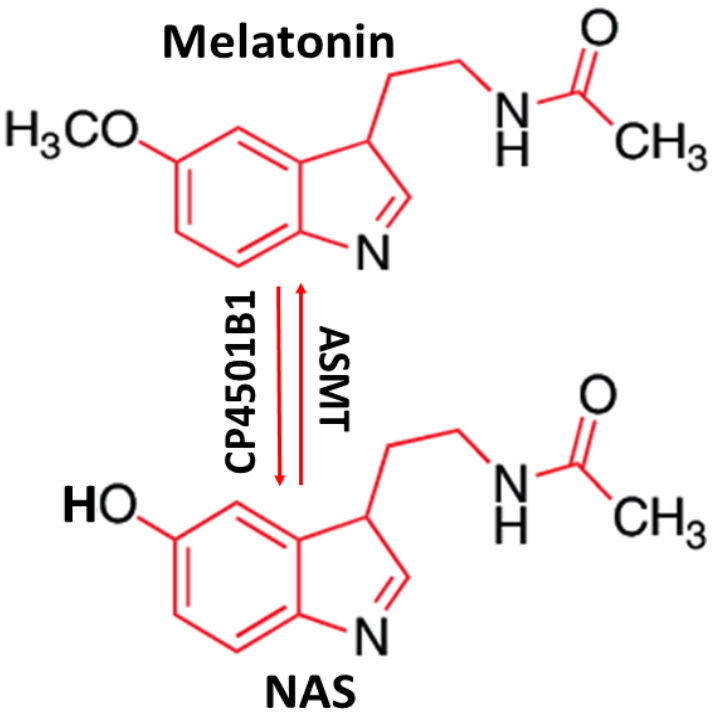

Fig. 3. The proposed equilibrium of melatonin and N-acetylserotonin in mitochondria.

The N-actylserotonin is the precursor as well as the product of melatonin. NAS: N-acetylserotonin; ASMT: N-acetylserotonin methyltransferase; $CP_{450}1B1$: Cytochrome $P_{450}1B1$.

Whether the equilibrium favors melatonin or N-acetylserotonin productions depends on the activities of both $CP_{450}1B_1$ and ASMT. For example, in tumor cells, we expect that the equilibrium is towards to N-acetylserotonin which can induce tumor cell apoptosis. In the normal cells, the reversed equilibrium is expected since melatonin protects normal cell from apoptosis and other cellular injuries (65–69). Many studies have observed that melatonin treatment has beneficial effects on normal cells but induces apoptosis in tumor cells (70–74). Under melatonin treatment, large quantity of cytochrome C is released from the mitochondria into cytosol to induce apoptosis in tumor cells (75–77) while and the cytochrome C release is inhibited in the normal cells by melatonin (75, 78–80).

Regarding the cytochrome C release which only localizes in mitochondria, this differential activity of melatonin must be initiated in mitochondria *per se* by different melatonin metabolic mechanisms between normal and tumor cells. The different melatonin metabolism in normal and tumor cells can be used as a target for cancer therapy. This character of melatonin in mitochondria is especially beneficial to the chemotherapy which causes severe side effects including injury to normal cells. Several animal studies indicated that melatonin application boosted the efficiency of chemotherapy and at the same time reduced the normal cell injuries (65, 81–85).

Interestingly, the extra-mitochondrial melatonin can be actively transported into mitochondria via the human transporters, oligopeptide transporter 1/2 (PEPT1/2), to increase its antitumor effects in tumor cells (86). The PEPT1/2 melatonin transporter system provides a mitochondrial-cytosolic melatonin recycling mechanism. The high melatonin inside the mitochondria defuses into cytosol and the PEPT1/2 move it back against the melatonin gradient. It preserves the high level of melatonin in mitochondria where it produced its optimal effects as the mitochondrial protector in normal cells and killer of tumor cells.

4. MELATONIN'S ACTIVITIES IN MITOCHONDRIA

As a bioenergy generating organelle, why do mitochondria bother to synthesize and metabolize melatonin? Clearly melatonin and its metabolites provide on-site protection to mitochondria from oxidative stress, thus, preserving optimal functions of mitochondria (87, 88). Mitochondria are vulnerable to oxidative stress due to their large quantity of ROS production. During evolution, melatonin is selected to be the first-line antioxidant in unicellular organisms such as in bacteria and because of this trait, melatonin is preserved in mitochondria. Thus, melatonin is referred as the mitochondrial targeted antioxidant (5, 89-91).

Many studies have reported the protective effects of melatonin on mitochondrial damage induced by a variety of insults in organisms. These insults include ischemia/reperfusion in different organs and tissues, neurodegenerative diseases, sepsis, hypoxia, toxins, and aging (92–98). Melatonin treatment not only maintains normal mitochondrial morphology but also preserves or even improves their function to generate ATP. These may be achieved via impacting the mitochondrial dynamics by inhibiting their fission and promoting their fusion.

In addition to producing ATP, mitochondria are also the regulator of cell apoptosis. Under stressful condition, mitochondria release the cytochrome C as a signal for initiation of apoptosis. Melatonin treatment significantly reduces cytochrome C leak from the mitochondria and protects against apoptosis in normal cells. The protective effects of melatonin on mitochondria usually are superior to other well-known antioxidants or synthetic mitochondrial targeted antioxidants (99–101). The detailed mechanisms were reviewed by several recently published articles (95, 97, 102,

103). Here, we only address a few of these aspects related to potential mechanisms which may stimulate the interest of the readers.

4.1. Effects of melatonin on mtPTP of mitochondria.

Mitochondrial permeability transition pore (mtPTP) is a structure formed by different proteins which are located in both the outer and inner membranes of the mitochondria. Many factors (usually adverse factors, for example, ROS) can lead to its opening. It seems that there is lack of the positive regulatory mechanisms to control its opening and closure. If its opening is prolonged, mitochondrial transmembrane potential ($\Delta\psi$) will collapse resulting in mitochondrial swelling, cell apoptosis or necrosis depending on the nature of the insult. Numerous studies have documented the protective effects of melatonin on mtPTP opening which, if not controlled, causes cellular injury or death. For example, melatonin treatment inhibits the mtPTP opening induced by ischemia/reperfusion, calcium overloading, amyloid β-peptide (Aβ), aluminum phosphide, 5-hydroxydecanoate, sulfur mustard, N-methyl-D-aspartate (NMDA) and aging (104–111).

It appears that melatonin inhibition of mtPTP opening is a universal phenomenon without distinction as to the causative factor. The mechanisms are not completely clarified and several of them deserve to be discussed. Andrabi *et al* (112) observed that the inhibitory pattern of melatonin on the mtPTP was similar to that of cyclosporine A (CSA), a mtPTP direct blocker. Zhou *et al* (105) observed that the dissociation of hexokinase 2 with voltage-dependent anion channel (VDAC1) in the mitochondrial outer membrane caused mtPTP opening. This dissociation is induced by mitochondrial fission under pathological conditions. For example, ischemia/reperfusion injury leads to the Drp1 activation which promotes mitochondrial fission. Melatonin treatment activates AMPKα which suppresses Drp1 activation, Drp1-dependent mitochondrial fission, dissociation of hexokinase 2 with VDAC1 and mtPTP.

The ADP/ATP carrier (AAC) is reported to serve as the mtPTP. AAC tightly binds to cardiolipin in the closed status (163). Cardiolipin is abundant in the inner mitochondrial membrane, where it constitutes about 20% of the total lipid composition and is vulnerable to ROS attack. Pro-oxidation of the bond between cardiolipin and AAC causes configurational modification of AAC to its open form, i.e., mtPTP opening. Melatonin protects cardiolipin from pro-oxidation and therefore maintains the closed configuration of AAC to inhibit mtPTP opening (113–115).

It is known that 2',3'-cAMP, which is formed during RNA degradation, is another culprit of mtPTP opening. This was proven in brain and liver mitochondria as well as in the aging rat mitochondria. Its formation is increased by adverse insults including cell injury, oxidative stress, mitochondrial dysfunction signals and aging. 2',3'-cAMP increases the Ca^{2+} sensitivity of mtPTP and promotes the mtPTP opening. In the mitochondria, 2',3'-cAMP is metabolized to 2'-AMP by 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). 2'-AMP is harmless and is the building material of RNA and DNA. Melatonin preserves the mitochondrial content of CNPase and, thus, reduces 2',3'-cAMP level causing inhibition of the mPTP opening.

4.2.Effects of melatonin on UCPs of mitochondria.

UCPs are also protein tunnels located in the inner membrane of the mitochondria [116]. Different from the mtPTP, UCPs can be positively regulated to protect the mitochondria (117). UCP_1 is exclusively present in the mitochondria of brown adipose tissue (118); other family members (homologs) of UCPs including UCPs $_{2\text{-}5}$ are distributed in the mitochondria of different

species and a variety of cell types (119, 120). Opening of the UCPs channels shunt the protons from the intermembrane space into matrix of the mitochondria. Thus, this process dissipates the energy of the proton flux to heat rather than to be used as the oxidative phosphorylation process to generate ATP. In brown adipose tissue, UCP_1 activation is mainly related to non-shivering thermogenesis especially in hibernating animals for arousing. It is also important in small mammals and human infants; however, in adult humans, its activation is closely linked with energy metabolism. Its deficiency is strongly associated with metabolic disorders and obese (121, 122). Melatonin not only promotes the recruitment of the brown adipose tissue but also upregulates the gene expression of UCP_1 and increases its activity (123).

Some studies have shown that long term melatonin administration to rats or mice makes the white adipose tissue differentiate to brite (brown-in-white)/beige adipocytes which characteristically express UCP_1 in their mitochondria (124–126). The classic brown and brite adipocytes have different origin (127). The origins of brite adipocytes have been proposed to be from *de novo* differentiation of precursor cells or differentiated from mature white fat cells or even muscle cells (128–130).

The main function of brite adipocytes is to "burn" fat and balance the excess storage of fat and reduce the body weight. Due to the effects of melatonin in promoting both brown and brite adipose tissues, melatonin has been hypothesized as an alternative remedy for obesity and other metabolic disorders (123). Many animal studies and several small scale of clinical trials support the hypothesis and have been shown to reduce body weight and improve the lipid metabolic profiles (131–135).

Activation of other UCPs rather than UCP_1 is probably not related to thermogenesis or energy metabolic modification in organisms since their activation does not significantly generate heat or consume energy. Their activation is frequently associated with readjust/tune-up of the $m\Delta\psi$. Thus, their upregulation and activation reduce the over-loaded $m\Delta\psi$, lowering ROS formation and preventing the cellular oxidative stress (the potential mechanisms is discussed in following section). Several reports have attributed melatonin's protective effects on oxidative stress in different tissues to its ability to upregulate gene expression as well as the activation of UCP_2 (136, 137).

4.3.Melatonin on ATP production in mitochondria.

The primary function of mitochondria is to produce ATP to power the bioactivities of cells. Dysfunction of the mitochondria results in an ATP deficiency and retards cellular activities and even leads to cell death. Effects of melatonin on the ATP production are complicated and depend on the condition cells experienced. The major function of melatonin is to prevent mitochondrial damage caused by oxidative stress; this action has been extensively reviewed (87, 91). Increase of ATP production under normal conditions probably is not the essential function of melatonin. As mentioned above, melatonin is the promotor of UCPs. The activation of UCPs shunts the protons stored in intermembrane space into the matrix to generate heat. These protons can be used to produce ATP and it seems that this precocious energy is wasted by the activity of melatonin. As a result, melatonin should reduce the ATP production in cells.

Couple of studies has shown that melatonin application indeed reduces the ATP production in tumor cells (138) and in transformed human skin cells (139). ATP production has a negative feedback mechanism. In the normal cells, if there is an over-supply of ATP, or a low ratio of ADP/ATP, it inhibits complex V to further generate ATP. Once the protons of intermembrane

space lose their major outlet to form ATP, the high level of protons in the intermembrane space increases the mitochondrial inner membrane potential (mΔψ) above the normal range. The high mΔψ decreases the electron flow in the ETC and mitochondria enter the stagnation status, which results in electron leakage from the ETC and forms ROS.

It was estimated that the majority of ROS are formed during the mitochondrial stagnation stage. Thus, it is necessary to bring the elevated mΔψ to its normal range by opening the UCPs as the flood gate to dissipate the over-loaded protons in the intermembrane space into the matrix. The uncoupling has been proposed as an important mechanism to reduce ROS levels (140). Melatonin upregulates gene expression and increases activities of UCPs. In this way, melatonin accelerates the electron flow in the ETC and reduces the electron leakage thereby avoiding ROS formation (141). This was referred as free radical avoidance effect of melatonin as mentioned by Hardeland (142). Sacrificing a portion of ATP productive potential to avoid the severe oxidative injuries in mitochondria might be the consequence of natural selection during evolution. The activation of UCPs is a unique effect carried by the mitochondrial-targeted antioxidant, melatonin.

Under the situations in which the cells require ATP for their biological activities including in embryo development (143–145), or under the situation of ATP production deficiency caused by mitochondrial dysfunctions or other insults, melatonin always promotes ATP production. For example, melatonin improves aging-related ATP deficiency in cybrids (96), restores the normal ATP production in a parkinsonian phenotype zebra fish which express high oxidative stress (88) and increases ATP levels in neural stem cells (NSCs) which require high energy to differentiate into oligodendrocytes and neurons (146). These are only of the few examples from many studies which documented melatonin's effect to improve the ATP production in cells and animal models under pathological conditions.

4.4.Melatonin actions on mitochondrial dynamics.

Decades ago, it was noted that the morphology of mitochondria in pinealocytes are dynamically changed during a 24-hour period (147). The main function of the pinealocytes is to synthesize and release melatonin and, thus, these changes were proposed to associate with their melatonin synthetic function. As data accumulated, it seems that the morphological changes of the mitochondria in pinealocytes are the result of the altered melatonin levels since melatonin has the capacity to modify mitochondrial dynamics. This aspect has been reviewed by Tan *et al* (91). Here we simply discuss several recent developments related to this topic.

Melatonin seems to increase mitochondrial biogenesis. In 3T3-L1 mouse embryo fibroblasts, melatonin increased the citrate synthase activity, and upregulated expression of PPAR-γ coactivator 1 α, nuclear respiratory factor-1, and transcription factor A (148); in liver and neurons, melatonin also enhanced mDNA level, mDNA copy numbers and oxidative phosphorylation proteins (149, 150). In neural stem cells, melatonin stimulated mitochondrial mass, mtDNA, complexes, mitochondrial respiration, and membrane potential as well as ATP synthesis (146). Second, melatonin targets mitochondrial fission and fusion processes. In miotic cells, for example, during the embryo development, melatonin seems not to inhibit mitochondrial fission capacity since melatonin supplement always promotes the normal distribution of mitochondria in these cells (144, 145). For post-miotic cells including neurons, myocardial cells and muscle most of the time melatonin inhibits mitochondrial fission and promotes fusion (151-153).

Generally, mitochondrial fusion increases the crestal surface area and the integrity of the mitochondria and thus, enhances their function. Functional mitochondria produce more ATP and

less ROS than fragmented mitochondria. Many studies have reported the inhibitory effects of melatonin on mitochondrial fission elements and enhancement of mitochondrial fusion process (154-156). The effects of melatonin on mitochondrial fission and fusion are attributed to the beneficial effects of melatonin on mitochondria biology and cell survival.

Mitophagy is an intracellular process used by cells to clean up the damaged or dysfunctional mitochondria by itself. Adequate mitophagy protects the cells; however, excessive mitophagy accelerates cell damage. The effects of melatonin on the mitophagy are highly complicated. Some reports documented the inhibitory action of melatonin on mitophagy (157) and others report that melatonin promotes mitophagy depending on the study conditions and experimental models (84, 158, 159). Currently, progressively more studies indicate that mitophagy-enhanced activity is the dominant effect of melatonin; this relates to the protective effects of melatonin on brain and heart ischemia/reperfusion damage, inflammation and tissue repair. Further information can be found in a recently excellent review on this topic by Boga *et al* (160).

4.5. **Melatonin membrane receptors in mitochondria.**

It is a little surprising that the melatonin plasma membrane receptor, particularly MT1, is present also in the mitochondria since this G protein-coupled receptor (GPCR) is classically characterized as cell-surface receptor which transmits extracellular signals into cells (161, 162). Suofu *at al* (39) used several different methodologies to convincingly prove that MT1 is present in mitochondria. Interestingly, the signal transducing pathway of MT1 in mitochondria is similar to cell surface membrane MT1. Both activated Gαi to block adenylate cyclase activity and reduce the cAMP levels. Based on the results from cell culture and on animal studies, they claimed that the protective effects of melatonin on the brain injury induced by ischemia/reperfusion is mediated by the mitochondrial MT1 rather than the plasma membrane MT1. This conclusion is in conflict with several observations by others (163–165) and requires further confirmation. To date, not only MT1 but also the MT2 are reportedly present in the mitochondria of the gastric endothelial cells (166). The signal transduction pathway of the mitochondrial MT2 as well as its biological consequence after its activation or inhibition are currently unknown. MT2 seems to have a strong impact on energy metabolism. An MT2 mutation has been associated with obese and diabetes (167–170).

5. CONCLUSION

The discovery that melatonin is mainly synthesized in the mitochondria had been hypothesized (5) and it seems that this is a universal phenomenon which occurs in different species and different cell types. Considering that mitochondria are the main source of ROS and melatonin is a mitochondrial-targeted antioxidant, this discovery is consistent with what might be expected. The on-site production of melatonin could provide local protection of the mitochondria. Melatonin is not only produced in mitochondria but is also metabolized in mitochondria via several pathways These include melatonin's direct interaction with ROS to form a spectrum of metabolites, and opening the indole ring by cytochrome C to form AFMK and demethylation by $CP_{450}1B_1$ to form NAS. $CP_{450}1B_1$ for the first time has been identified in mitochondria. The antitumor effect of melatonin may be attributed to the presence of $CP_{450}1B_1$. High levels of mitochondrial NAS promote tumor cell apoptosis. The discovery of mitochondrial $CP_{450}1B_1$ provides an additional explanation regarding the differential actions of melatonin in normal and tumor cells, that is,

melatonin reduces normal cell apoptosis but promotes tumor cell apoptosis. Other activities of melatonin on mitochondria include its inhibition on mtPTP, activation on UCPs, modification of mitochondrial dynamics and balancing ATP production. These also contribute to the beneficial effects of melatonin on mitochondria. Mitochondria are the birth place, battle ground and metabolic site of melatonin. The biological significance of the strong association between mitochondria and melatonin should not be underestimated.

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AUTHORSHIP

D.X. Tan initiated this topic and drafted the manuscript and R.J. Reiter contributed to discussion and editing of the manuscript.

CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest to this work

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