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Mini Review

Melatonin, mitochondria, and cellular bioenergetics

Abstract: Aerobic cells use oxygen for the production of 90–95% of the total amount of ATP that they use. This amounts to about 40 kg ATP/day in an adult human. The synthesis of ATP via the mitochondrial respiratory chain is the result of electron transport across the electron transport chain coupled to oxidative phosphorylation. Although ideally all the oxygen should be reduced to water by a four-electron reduction reaction driven by the cytochrome oxidase, under normal conditions a small percentage of oxygen may be reduced by one, two, or three electrons only, yielding superoxide anion, hydrogen peroxide, and the hydroxyl radical, respectively. The main radical produced by mitochondria is superoxide anion and the intramitochondrial antioxidant systems should scavenge this radical to avoid oxidative damage, which leads to impaired ATP production. During aging and some neurodegenerative diseases, oxidatively damaged mitochondria are unable to maintain the energy demands of the cell leading to an increased production of free radicals. Both processes, i.e., defective ATP production and increased oxygen radicals, may induce mitochondrial-dependent apoptotic cell death. Melatonin has been reported to exert neuroprotective effects in several experimental and clinical situations involving neurotoxicity and/or excitotoxicity. Additionally, in a series of pathologies in which high production of free radicals is the primary cause of the disease, melatonin is also protective. A common feature in these diseases is the existence of mitochondrial damage due to oxidative stress. The discoveries of new actions of melatonin in mitochondria support a novel mechanism, which explains some of the protective effects of the indoleamine on cell survival.

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Introduction

A major success of phylogenetic adaptation is aerobic metabolism. Remarkably, more than 90% of the body's oxygen (O_2) consumption is utilized by a single enzyme, cytochrome oxidase (C-IV) [Nathan and Singer, 1999]. Aerobic organisms use ATP generated by an oxidative phosphorylation (OXPHOS) pathway involving a multienzymatic process, which includes C-IV as an electron acceptor. Normally, there is no shortage of ATP production under aerobic conditions, since the amount of O_2 delivered to the tissues (100 mL/min) is in excess to the body's requirements. This situation is reversed when sufficient oxygen is available to the mitochondria, but they are unable to utilize it due to an inhibition at any of several points in the electron transport chain (ETC). Such disturbances are common to aging and several pathologies, including neurodegenerative diseases, ischemia/reperfusion, and sepsis, where an increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\bullet$), nitric oxide (NO) and peroxynitrite ($ONOO^-$), inhibit ETC complexes [Murphy, 1989; Beal, 1998; Lenaz, 1998; Bockowski et al., 1999].

Mitochondria are surrounded by a double system of membranes described as outer and inner membranes, which define two soluble compartments, the intermembrane space and the matrix. Their matrices contain ribosomes for protein synthesis, their own genome, the enzymes of the β-oxidation pathway, and the majority of the enzymes needed for the Krebs cycle. An important exception is succinate dehydrogenase, which is linked to the ETC in the inner membrane. Mitochondria specialize in the rapid oxidation of NADH and FADH through the ETC to produce ATP. The ETC is constituted by four complexes and the electron transfer from one complex to another is

driven by the reduced forms of the ubiquinone (UQ) and cytochrome c (cyt c) [de Grey, 1999]. During this electron transfer, O_2 may be partially reduced, yielding ROS. This review mainly centers on how melatonin counteracts ROS production by mitochondria, thereby improving OXPHOS.

Mitochondrial production of ATP

In cells under aerobic conditions, OXPHOS is responsible for production of 90–95% of the total amount of ATP, the remainder being synthesized by glycolytic phosphorylation. The adult human forms and decomposes about 40 kg ATP/day [Skulachev, 1999]. $NADH + H^+$ and $FADH_2$ produced by glycolysis, Krebs cycle, and β-oxidation of fatty acids are oxidized by the ETC transferring electrons from these precursors to O_2. The ETC comprises a series of reduction/oxidation reactions involving complex I (NADH dehydrogenase), II (succinate dehydrogenase), III (cyt c reductase), and IV (cytochrome oxidase). The synthesis of ATP via the respiratory chain is the result of two coupled processes: electron transport and OXPHOS. Electrons from $NADH + H^+$ enter C-I of the ETC at a redox potential of -0.30 V and emerge to reduce a membrane-associated ubiquinone/ubiquinol (UQ/UQH_2) pool to $+0.10$ V. Electrons from $FADH_2$ have an insufficient negative redox potential to enter C-I and instead reduce the UQ/UQH_2 pool via C-II. UQH_2 transfers electrons to C-III, which in turn reduces cyt c at a redox potential of $+0.254$ V. Cyt c then reduces the terminal acceptor C-IV, which then transfers four electrons to molecular O_2 forming H_2O. C-I, C-III, and C-IV function as proton pumps, acting in series with respect to the electron flux and in parallel with respect to the proton circuit [Nicholls and Budd, 2000]. The fall in the redox potential of electrons passing through these complexes is used to generate a proton electrochemical potential gradient, $\Delta\mu_H+$, usually expressed in electrical potential units as the proton-motive force (Δp). At 37°C, $\Delta p = \Delta\Psi_m - \Delta pH$, where $\Delta\Psi_m$ is mitochondrial membrane potential and ΔpH is pH gradient across the inner membrane. Under most conditions, $\Delta\Psi_m$ is the dominant component of Δp, accounting for 150–180 mV of the total Δp of 200–220 mV. A fourth component of the inner mitochondrial membrane, the energy-linked transhydrogenase, utilizes Δp to maintain a high level of reduction of the matrix NADPH pool by driving the following reaction to the right: $NADH^+ + NADP = NAD^+ + NADPH$. In a typical mitochondrion, the NAD pool is about 10% reduced while the NADP pool is more than 90% reduced [Nicholls and Budd, 2000].

ATP synthase is the dominant pathway for the reentry of protons into the mitochondrial matrix. The electron flux and thus respiration are controlled by ATP demand, which is referred to as the 'respiratory control'. A collapse in Δp leads not only to a cessation of mitochondrial ATP synthesis, but to a rapid hydrolysis of cytoplasmic ATP as the ATP synthase reverses in an attempt to restore Δp. This can lead to a profound ATP depletion. The Δp is the central parameter controlling fundamental cellular processes: ATP synthesis, mitochondrial Ca^{2+} uptake, and ROS generation.

Mitochondria possess a constitutive proton leak across their inner membrane [Murphy, 1989]. This leak is highly potential-dependent, but above a $\Delta p > 180$ mV, it becomes independent of Δp. This nonohmic leak plays a major role in controlling basal metabolic rate and, in addition, may limit the production of potentially dangerous ROS.

Mitochondrial production of ROS and RNS

Most of the O_2 taken up by human cells is reduced to water via the action of mitochondrial C-IV. This requires the addition of four electrons to each O_2 molecule. The intermediate steps of O_2 reduction result in the formation of $O_2^{-\bullet}$, H_2O_2, and $HO^\bullet$, corresponding to reduction by one, two, and three electrons, respectively. The $HO^\bullet$ is tremendously reactive, since its redox potential is more positive than any substance in the living cell ($+1.35$ V). It seems that ubisemiquinone ($UQ^\bullet$) could be responsible for ROS generation both at C-I and C-III level [Barja, 1999]. As well as being able to generate $O_2^{-\bullet}$ and H_2O_2, mitochondria are themselves susceptible to damage by these compounds. NAD(P)H is responsible for maintaining the matrix glutathione pool in the reduced form via glutathione reductase (GRd). Since the NAD(P)H pool is maintained in a highly reduced state by means of the Δp-driven energy-linked transhydrogenases, the magnitude of Δp may be critical for mitochondrial survival. If it is too high, C-III will generate ROS, and if it is too low, ATP levels are reduced and the NAD(P)H pool becomes oxidized [Nicholls and Budd, 2000].

Mitochondria possess a special mechanism called mild uncoupling that prevents a marked increase in Δp and, hence, $O_2^{-\bullet}$ formation. Mild uncoupling seems to be a first line of mitochondrial antioxidant defense since it reduces $O_2^{-\bullet}$ generation [Skulachev, 1999]. If, nevertheless, some $O_2^{-\bullet}$ is still formed, the next line of defense is

activated. This role is carried out by cyt c dissolved in the solution occupying the intermembrane space of mitochondria. Cyt c oxidizes $O_2^{-\bullet}$ back to O_2 (cyt $c^{3+} + O_2^{-\bullet} = $ cyt $c^{2+} + O_2$), whereas reduced cyt c can then be oxidized by O_2 via C-IV (4 cyt $c^{2+} + O_2 + 4H^+ = 4$ cyt $c^{3+} + 2H_2O$). This mechanism represents the most effective way to scavenge $O_2^{-\bullet}$, since it is merely converted back to O_2 [Skulachev, 1999]. Antioxidants, such as ascorbate, UQ, and α-tocopherol, may assist the mitochondrial antioxidative defense system, but none of them can convert $O_2^{-\bullet}$ to O_2. An important role is also played by superoxide dismutase (SOD), which converts $O_2^{-\bullet}$ to H_2O_2, which in turn is converted to water via the enzyme glutathione peroxidase (GPx) which, in the process metabolizes reduced glutathione (GSH) to its disulfide (GSSG). All O_2 reduction ceases when the hydrogen supply to the ETC is abolished. This type of anti-ROS defense may be employed when ROS increase markedly [Skulachev, 1999].

GSH participates in several redox reactions and maintains the permeability transition pore (PTP) closed by the reduction of SH groups in its inner face. The redox cycling of GSH in mitochondria is normally very active to avoid significant lost of GSH. Mitochondrial NO mainly inhibits C-IV causing a reversible inhibition competing with O_2 for its binding site. Normal tissue levels of NO and O_2 are 100–500 nM and 30 M, respectively. This ratio of NO/O_2 causes approximately half-maximal inhibition of mitochondrial respiration rate, suggesting that NO serves to tonically inhibit respiration [Brown, 1999]. But NO also reacts with $O_2^{-\bullet}$ to form $ONOO^-$, which causes irreversible inhibition of mitochondrial respiration thereby damaging all components of the ETC. Also, $ONOO^-$ induces mitochondrial swelling, depolarization, Ca^{2+} release, PTP activation, and apoptosis [Brown, 1999].

Mitochondria are also of central importance for physiological Ca^{2+} handling. They act as a reservoir for Ca^{2+}, provide much of that used by Ca^{2+}-ATPases, and Ca^{2+} regulates the activity of intramitochondrial dehydrogenases. The inner mitochondrial membrane possesses a Δp-linked uniporter to transport Ca^{2+} into the matrix. Thus, Ca^{2+} overload produces collapse of Δp, mitochondrial swelling, loss of respiratory control, and release of matrix Ca^{2+} caused by PTP opening [Nicholls and Budd, 2000]. Besides Ca^{2+}, ROS are the major PTP regulators. When the production of ROS increases, they may induce PTP opening and molecules up to 1.5 kDa are released from mitochondria into cytoplasm. Among them, cyt c becomes part of the so-called apoptosome that interacts with procaspase 9 producing the autoactivation of procaspase 9. In turn, caspase 9 proteolytically converts procaspase 3 to caspase 3, which attacks some other key proteins resulting in controlled cell death [Crompton, 1999].

Mitochondrial pathologies

Abnormalities of mitochondrial metabolism, which cause human disease, have been recognized for more than 30 years. They encompass defects of fatty acid oxidation, Krebs cycle enzymes, and the ETC and OXPHOS system. The study of mitochondrial metabolism has recently increased in neurodegenerative diseases [Beal, 1998; Schapira, 1999]. Some of these pathologies included Parkinson's disease (deficiency of C-I), Huntington's disease (deficiencies in the C-II, C-III, and C-IV) [Schapira, 1999], Alzheimer's disease (mitochondrial dysfunction and down-regulation of the mitochondrial ETC and β-amyloid-induced oxidative damage), and Friedreich's ataxia (impaired OXPHOS in skeletal muscle) [Beal, 1998]. The partial-dependence of oxidative damage in these diseases suggests the use of antioxidants in their treatment [Schapira, 1999].

During ischemia/reperfusion, damage induced by anoxia seems to be related to subsequent reoxygenation, which induces $HO^{\bullet}$ responsible for the initiation of an apoptotic program opening the PTP [Crompton, 1999]. NO generated during reperfusion due to NOS activation, and the $ONOO-$ produced when NO couples with $O_2^{-\bullet}$ seems to be the primary cause of damage to C-I and C-II during reperfusion.

Glutamate is the main excitatory amino acid in the mammalian brain. Glutamate exposure (excitotoxicity) results in a massive intracellular accumulation of Ca^{2+} that may be taken up by mitochondria with the subsequent decrease of Δp and ATP synthesis, and an increase in $O_2^{-\bullet}$ and H_2O_2 generated by mitochondria and a rise in $ONOO-$ produced from NO generated by cytosolic and mitochondrial NOS [Nicholls and Budd, 2000]. A significant reduction in mitochondrial GSH pool occurs during excitotoxicity.

Sepsis is a severe toxic state due to an abnormal immune response against bacterial infection in an organ, leading to iNOS induction and massive tissue damage. The mechanisms of NO-induced toxicity depend in part on the reversible inhibition of C-IV [Brown, 1999] and in increases of $O_2^{-\bullet}$ and H_2O_2 production by mitochondria of the diaphragm. A parallel increase in $ONOO-$ also impairs mitochondrial function and muscle contractility [Bockowski et al., 1999]. To prevent the

progression of sepsis, NOS antagonists and antioxidants have been used.

Aging is a complex process characterized as degenerative in nature, which causes progressive loss of function and an increased risk of death. The free radical theory of aging proposed by Harman [1956] suggests that aging is the result of the failure of various protective mechanisms to counteract the ROS-induced damage. The mitochondrial theory of aging states that the accumulation of impaired mitochondria is the driving force of the aging process [Miquel et al., 1980]. Aging is accompanied by structural changes in mitochondria and by a decrease in C-IV and C-V activities, which results in a bioenergetic decline that may impair energy-dependent neurotransmission, contributing to a senescent decline in memory and other brain functions [de Grey, 1999]. Oxidative stress represents an early intrinsic component of any PTP-induced apoptotic cascade [Loeffler and Kroemer, 2000]. Unifying programmed and stochastic theories of aging claim that cells are first programmed to differentiate, and then they suffer progressive disorganization because of injury as the result of chronic oxidative stress. The free radical theory of aging also provides a rationale for intervention by means of antioxidant administration [Sastre et al., 2000].

Melatonin and ROS and RNS

From a phylogenetic point of view, melatonin is a molecule present in organisms from unicells to mammals. Since tryptophan and serotonin (melatonin precursors) are present at the early stages of cell phylogeny, the presence of melatonin is also suggested. Tryptophan metabolites, including melatonin are antioxidants, and from a hypothetical point of view, the first function of melatonin may have been as an antioxidant. Melatonin, identified initially in the pineal gland [Lerner et al., 1958] was considered for several decades as a regulator of reproduction, mainly in seasonal-breeding animals [Reiter, 1980]. The discovery of different targets in the cell suggests a variety of mechanisms of action for this compound. At the present, melatonin's mechanisms of action seem to fall into three categories: receptor-mediated, protein-mediated, and non-receptor-mediated effects. Receptor-mediated melatonin events involve both membrane and nuclear receptors. Although membrane melatonin receptors have been identified and are well-characterized in humans [Conway et al., 2000], some of the receptor-related antioxidant effects of melatonin seem also to be related to its nuclear receptors [Acuña-Castroviejo et al., 1994; Becker-André et al., 1994; García-Mauriño et al., 2000]. The expression of some enzymes, mainly related to the endogenous antioxidant system of the cell, such as GPx, GRd, SOD, and iNOS [Antolín et al., 1996; Crespo et al., 1999], are under genomic regulation by melatonin. The interaction between membrane and nuclear melatonin signaling has been proposed [Carlberg and Wiesenberg, 1995].

Experimental evidence has clearly demonstrated the interaction of melatonin with Ca^{2+}-calmodulin (CaCaM), a ubiquitous protein in the cell. High-affinity binding of melatonin to CaM has been characterized [Romero et al., 1998]. The significance of melatonin–CaCaM interaction was emphasized in a series of experiments showing changes in the cytoskeletal rearrangements due to this interaction [Benítez-King, 2000]. Also, the binding of melatonin to CaCaM inhibits intracellular CaCaM-dependent enzymes, such as NOS [León et al., 2000]. Hence, melatonin inhibits NO production.

A series of experiments have provided strong evidence for the anti-excitotoxic properties of melatonin both in vivo and in vitro [Acuña-Castroviejo et al., 1995]. Both experimental and clinical anticonvulsant activity of melatonin was reported [Molina-Carballo et al., 1997; Lapin et al., 1998]. Reduced melatonin levels are related to increased brain damage after stroke or excitotoxic seizures in rats [Manev et al., 1996], whereas melatonin protected the brain against kainic acid or quinolinic acid oxidative damage [Melchiorri et al., 1995; Tan et al., 1998; Cabrera et al., 2000]. Electrophysiological experiments further demonstrated the antagonism of melatonin against the NMDA-induced excitotoxicity, an effect involving the inhibition of the nNOS [Escames et al., 1996; León et al., 1998, 2000]. Melatonin also counteracts oxidative damage in MPTP-induced Parkinson's disease [Acuña-Castroviejo et al., 1997], and in vitro melatonin was more potent than vitamins E and C, and L-deprenyl in blocking dopamine (DA) autoxidation [Khaldy et al., 2000]. Paraquat, an MPTP-related molecule, also produces strong oxidative damage when administered to animals. To the same extent as in the MPTP model, melatonin counteracted paraquat-induced damage [Melchiorri et al., 1998]. The neuroprotective effects of melatonin were also tested against neurodegenerative manifestations of Alzheimer's amyloid β-protein and showed antiapoptotic and ROS scavenging properties [Pappolla et al., 2000].

In a model of ischemia/reperfusion in gerbil due to bilateral carotid artery occlusion, NOS/NO system was inhibited after melatonin administration,

resulting in a reduction of neuronal damage associated with reperfusion [Guerrero et al., 1997]. Protection by melatonin in other models of neurotoxicity, including hyperbaric hyperoxia-exposed rats, cyanide-induced seizures in rats, and d-amino-levulinic acid-induced neuronal damage in rat brain homogenates, was also provided [Reiter et al., 1997a, 1998; Reiter, 1998]. In experimental models of sepsis induced by administration of lipopolysaccharide (LPS) to rats, melatonin administration reportedly counteracted most of the markers of cell injury, including the inhibition of iNOS expression and subsequent NO production [Sewerynek et al., 1995; Crespo et al., 1999]. Melatonin also protects against oxidative damage induced by a variety of free radical generating agents, including the carcinogen safrole, Fenton reagents, glutathione depletion, carbon tetrachloride, and ionizing radiation [Karbownik and Reiter, 2000; Reiter, 2000]. Melatonin is also effective in protecting nuclear DNA, membrane lipids, and cytosolic proteins from oxidative damage and from increased membrane rigidity [Reiter et al., 1997b, 1998; García et al., 1999].

The protective properties of melatonin have been tested in several models of aging, which involve extensive cell damage. In different models of aging, including a relative melatonin deficiency, and in age-related diseases, such as cancer and cataracts, melatonin administration has been shown to be protective [Reiter et al., 1999; Reiter, 1999]. The fact that melatonin decreases with age has been suggest as contributing to aging in mammals [Reiter, 1998].

The experimental and clinical situations summarized above have three main features: 1) high ROS production, 2) mitochondrial pathology as a consequence of oxidative damage, and 3) beneficial effects of melatonin as evidenced by a reduction in lipid peroxidation, DNA damage, and NO levels, and increased GSH and thus a rise in the GSH/GSSG ratio [Reiter, 1998; Reiter et al., 1998, 2000]. Collectively, the data demonstrate that melatonin efficiently counteracts oxygen radical pathology. The antioxidant properties of melatonin involve its scavenging of $HO^{\bullet}$, NO, $ONOO^{-}$, and possibly $O_2^{-\bullet}$ [Reiter et al., 2000; Tan et al., 2000a]. Also of importance, melatonin was recently shown to scavenge H_2O_2, the precursor of the $HO^{\bullet}$, which is formed during normal metabolism of mitochondria [Tan et al., 2000a,b]. Melatonin also reportedly regulates the expression and activity of the antioxidative enzymes GPx, GRd, SOD, glucose-6-phosphate dehydrogenase (G-6-PDH), and iNOS [Antolín et al., 1996; Crespo et al., 1999; Reiter et al., 2000]. In contrast to vitamins E and C, melatonin does not deplete the main reductive force of the cell, GSH, rather it prevents or even increases the content of GSH. When melatonin scavenges H_2O_2, the resulting metabolites, i.e., N^1-acetyl-N^2-formyl-5-methoxy kynuramine (AFMK) and N-acetyl-5-methoxy kynuramine (AMK), also are effective free radical scavengers. The continuous ROS scavenging potential of melatonin and its metabolites has been defined as a scavenging cascade reaction [Reiter et al., 2000; Tan et al., 2000a,b].

The importance of melatonin as an antioxidant depends on several characteristics: its lipophilic and hydrophilic nature, its ability to pass all biobarriers with ease, and its availability to all tissues and cells. It distributes in all cell compartments, being especially high in the nucleus and mitochondria [Menéndez-Peláez et al., 1993; Martín et al., 2000a]. In mammals, the pineal gland is one of several organs that produce melatonin. Other tissues, including the retina, cells of the immune system, gut, bone marrow, human ovary, lens, and testes, may produce melatonin for local use [Reiter et al., 2000]. Levels of melatonin are two to three orders of magnitude higher than maximal blood melatonin concentrations in bile [Tan et al., 1999] and in cerebrospinal fluid (CSF) [Skinner and Malpaux, 1999]. Thus, some organs may produce melatonin making them independent of circulating levels of the indoleamine. Physiological levels of melatonin should not be exclusively defined in terms of the concentrations of melatonin normally found in the blood.

Melatonin and mitochondria

Two main considerations suggest a role for melatonin in mitochondrial homeostasis. First, mitochondria produce high amounts of ROS and RNS. Second, mitochondria depend on the GSH uptake from the cytoplasm, although they have GPx and GRd to maintain GSH redox cycling. Thus, the antioxidant effect of melatonin and its ability to increase GSH levels [Urata et al., 1999] may be of great importance for mitochondrial physiology. The fact that the toxicity of cyanide, which blocks C-IV of the mitochondrial ETC, is counteracted by melatonin, also supports its intramitochondrial role [Yamamoto and Tang, 1996].

Binding experiments with ^{125}Iodomelatonin showed most of the specific binding (39%) to be present in the mitochondrial fraction of the cell [Poon and Pang, 1992]. Soon thereafter, a metabolic effect of melatonin on mitochondrial metabolic activity was reported [De Atenor et al.,

1994]. Also, melatonin added to J774 macrophages in culture reduced the suppression of mitochondrial respiration induced by $ONOO^-$ [Gilad et al., 1997]. Melatonin also reduced cell death induced by cysteamine pretreatment of PC12 cells, a mechanism involving mitochondrial iron sequestration [Frankel and Schipper, 1999]. Furthermore, melatonin inhibited NADPH-dependent LPO in human placental mitochondria [Milczarek et al., 2000] and protected against $O_2^{-\bullet}$ damaging U937 cells treated with 7-ketocholesterol [Lizard et al., 2000]. A protective effect of melatonin against MPP^+-induced inhibition of C-I of the ETC has been also provided [Absi et al., 2000].

The ability of melatonin to influence mitochondrial homeostasis was initially tested in vivo. Rats were injected ip with melatonin (10 mg/kg) and sacrificed at different times after treatment. Mitochondria from brain and liver tissues were prepared and the activity of the ETC complexes were measured. Under these conditions, melatonin increased the activity of the C-I and the C-IV of the mitochondrial ETC in a time-dependent manner, whereas the C-II and C-III were unaffected. The effect of melatonin was observed at 30 min after injection and the activity of these complexes returned to control values at 120–180 min after melatonin treatment [Martín et al., 2000a]. These data correlate well with the half-life of the indoleamine, suggesting a direct effect of melatonin on mitochondria. Subsequently, rats were treated with ruthenium red, an inorganic polycationic complex which acts as a non-competitive inhibitor of the mitochondrial Ca^{2+} uniport uptake system. Since Ca^{2+} is important for the activation of several mitochondrial dehydrogenases, ruthenium red administration produced an obvious impairment of mitochondrial metabolism, including the reduction of the ETC and ATP synthesis. Also, at micromolar concentrations ruthenium red is a prooxidant acting as a Fenton-type reagent by substituting for Fe^{2+} in the process of degradation of deoxyribose by H_2O_2 and generating methyl radicals by a redox cycling process involving Ru^{III}/Ru^{IV} interconversion in the presence of ascorbate or succinate. Thus, ruthenium red may cause mitochondrial uncoupling and cellular oxidative stress. The former is demonstrated by the inhibition of the ETC activity, and the latter by a decrease in the GSH-dependent redox enzymes. After ruthenium red administration in vivo, mitochondria of rat liver and brain showed a significant inhibition of the C-I and C-IV of the ETC. These effects were totally counteracted by melatonin administration at the time of ruthenium red injection, further supporting an intramitochondrial role of the indoleamine. Melatonin also counteracted the inhibitory effect of ruthenium red on GPx. These data also indicate that melatonin counteracts not only ruthenium red-induced oxidative stress, but also the uncoupling effect that the toxin causes [Martín et al., 2000a].

The protective effects of melatonin were further analyzed in isolated mitochondria prepared from rat brain and liver tissue and compared with those of other known antioxidants, such as N-acetylcysteine (NAC) and vitamins C and E [Martín et al., 2000b]. Oxidative stress was induced by incubation of mitochondria with t-BHP, which oxidizes pyridine nucleotides depleting mitochondrial GSH and inhibiting both GPx and GRd activities [Aoshima et al., 1999]. In mitochondria incubated in absence of t-BPH, melatonin (100 nM) significantly increased the content of GSH and reduced the GSSG content. After incubation with t-BHP, virtually all GSH is oxidized to GSSG, and the activity of both GPx and GRd are reduced to practically zero. In this situation, 100 nM melatonin counteracted these effects, restored basal levels of GSH and the normal activities of both GPx and GRd. Melatonin also significantly reduced the hydroperoxide production. Neither NAC nor vitamins E and C reversed t-BHP-induced oxidative stress in mitochondria, even though they were used in much higher concentrations (1 mM) than melatonin [Martín et al., 2000b].

To characterize the effects of melatonin on mitochondrial ETC activity, another set of experiments was done using submitochondrial particles prepared from mitochondria obtained from rat liver and brain. Melatonin increased the activity of the C-I and C-IV in a dose-dependent manner, the effect being significant at 1 nM. Melatonin also counteracted cyanide-induced inhibition of the C-IV, restoring the levels of cyt $a + a_3$. These effects of melatonin were of physiological significance, since the indoleamine increased the ETC activity coupled to OXPHOS, which was reflected by an increase of ATP synthesis, both in normal mitochondria and in mitochondria depleted of ATP by cyanide [Martín et al., 2000c].

These results suggest a direct effect of melatonin on mitochondrial energy metabolism and provide a new homeostatic mechanism for regulation of mitochondrial function. The findings also identify a new mechanism of action of melatonin at the mitochondrial level. Improvement of mitochondrial respiration and ATP synthesis by melatonin increases the rate of electron transport across the ETC. In turn, due to the high redox potential of

melatonin (− 0.98 V) [Reiter et al., 2000], this molecule may donate an electron to the C-I of the ETC. This effect is also supported by the observations that melatonin is highly lipophilic, and it may stabilize mitochondrial inner membranes [García et al., 1999]. Melatonin interacts with microsomal membranes to maintain membrane stability, an effect that may improve ETC activity. Melatonin also directly scavenges H_2O_2, which is abundantly produced in mitochondria from $O_2^{-\bullet}$ [Tan et al., 2000a]. Thus, melatonin improves ETC and reduces mitochondrial oxidative damage. Both effects are the basis of increased ATP production.

Concluding remarks

Considering the reported beneficial effects of melatonin against oxidative stress-related damage in different animal models and in humans, it is concluded that the beneficial effects of melatonin are related to an improvement of mitochondrial function by counteracting mitochondrial oxidative stress. Thus, melatonin improves the bioenergetics of the cell, including more efficient nuclear and mitochondrial genomic repair mechanisms, increasing GSH levels, which may account for the anti-apoptotic effects of the indoleamine, elevating ATP production and improving ATP-dependent functions, including neurotransmission (Fig. 1). These mechanisms may be the basis for the potential anti-aging properties of melatonin and indicate that its age-dependent loss and the resulting increase in oxidative stress may impair mitochondria that in turn produce more ROS increasing cell damage, which favor aging. Also, many degenerative disorders of the aged have in common an alteration in mitochondrial physiology. Regardless of whether the primary cause of mitochondrial dysfunction is oxidative damage or due to a disturbance in the ETC, the use of melatonin should be considered to improve mitochondrial energy supply to the cell. An obvious implication of the findings summarized herein is that an important target of melatonin action is at the level of the mitochondria.

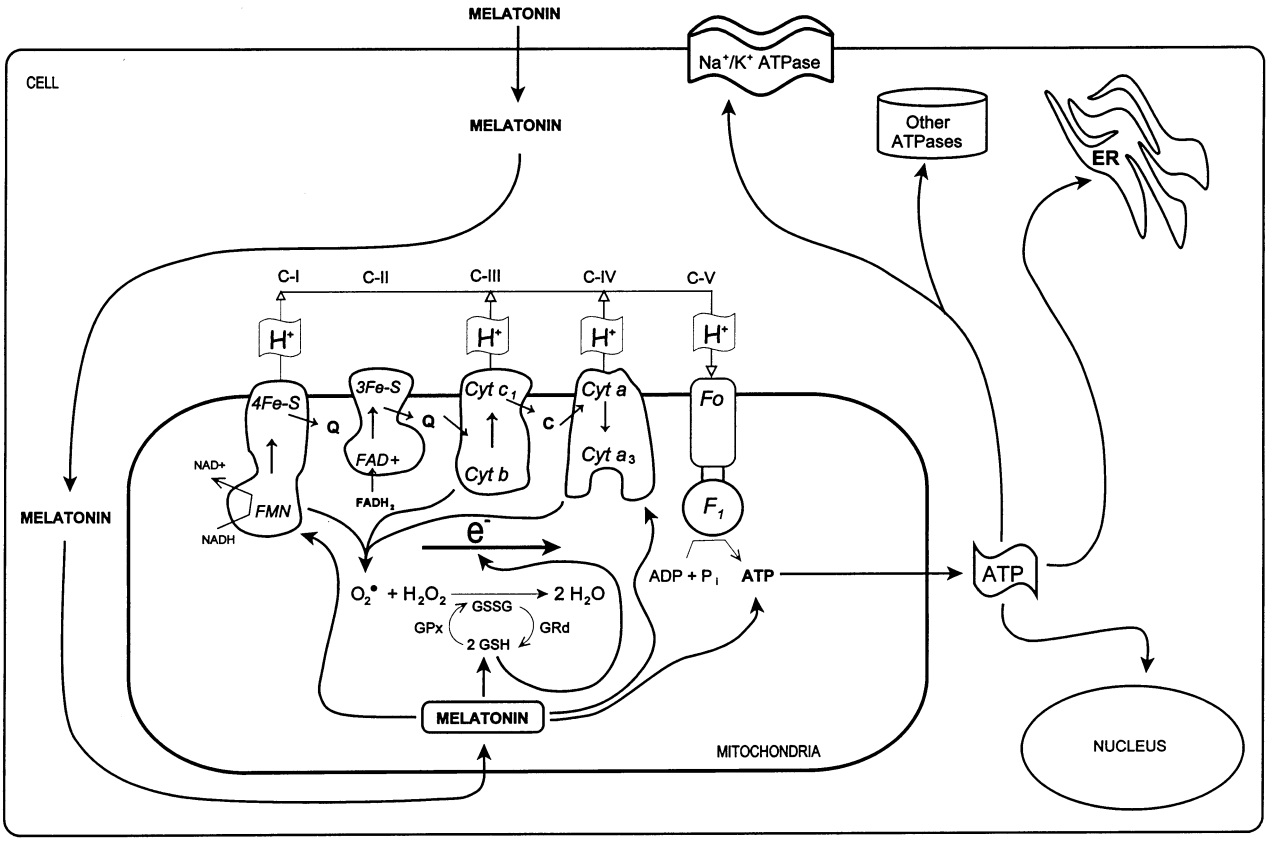

Fig. 1. Schematic diagram of the hypothesized melatonin involvement in mitochondrial ETC and in ATP synthesis. Melatonin easily crosses biological membranes (cellular and mitochondrial) to reach the mitochondrial matrix. Hence, the indoleamine increases the activity of the complexes I and IV of the ETC. Melatonin also influences glutathione redox cycling and increases the intramitochondrial content of GSH, which in turn improves the efficiency of the ETC. As a consequence of the melatonin's action at the level of the mitochondria, electron transfer along the respiratory chain is enhanced promoting ATP synthesis.

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