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Mitochondrial actions of melatonin – an endeavor to identify their adaptive and cytoprotective mechanisms

Introduction

A relationship between melatonin, perceived by most researchers as a regulator of circadian and annual rhythms, and mitochondria, usually regarded as the site of respiration and, nowadays, also as a key organelle in the initiation of apoptosis, may not be evident to everyone at first glance. However, a deeper sight reveals remarkable connections, which may appear unexpected under the aspects of melatonin's classical roles. First, it seems important to understand that melatonin is not only the hormone of the pineal gland, but is also produced in numerous other tissues and cells. Apart from the retina as a structure of common origin with the pineal, both originating from the intermediate brain, entirely different extrapineal sites of melatonin synthesis are known, such as gastrointestinal tract, bone marrow, various leukocytes, skin, Harderian gland (in rodents), membraneous cochlea, and, perhaps, certain brain regions. In total, extrapineal melatonin exceeds the amounts found in the pineal and in the circulation by orders of magnitude, so that its quantities in the tissues must be of importance (summarized in: Hardeland and Poeggeler, 2007, 2008a; Hardeland, 2008a,b).

With regard to the unlikelihood that organs like the gut or the Harderian gland could mediate signals related to darkness, and to the low-amplitude or almost absent circadian variations of melatonin in these organs (Hardeland et al., 2003; Hardeland and Poeggeler, 2008a; Hardeland, 2008b), tissue melatonin has to be involved in other, non-classical mechanisms. Since tissue melatonin displays a dynamics, a metabolism and local activities different from those in the circulation, and since the indoleamine can even be taken up from the food or undergo enterohepatic cycling, it should not be exclusively qualified as a hormone, but has also to be seen in its additional roles as a paracoid and auto-/intracoid tissue factor (Tan et al., 2003b; Hardeland and Pandi-Perumal, 2005). With regard to the extreme pleiotropy and versatility of melatonin (Pandi-Perumal et al., 2006; Hardeland, 2008b), its functions in the tissues should be multiple ones. However, there is one action that can be observed in many places in the body, namely, antioxidative protection (Reiter et al., 2003a,b, 2007; Hardeland, 2005). But, a close look on the details of such effects shows that many reports refer to pharmacological concentrations of melatonin, that direct radical scavenging only partially explains the observed protection, already under aspects of stoichiometry and high concentrations of other antioxidants, and that signaling effects of melatonin by upregulating antioxidant and downregulating prooxidant enzymes are mostly more or less tissue-specific – what is frequently ignored – and not always sufficiently profound to provide a sound basis of interpretation (Hardeland, 2005).

Therefore, other explanations for the protective actions, which exist without any doubt, have to be sought, especially when actions in the physiological range of melatonin shall be understood. With regard to the unsatisfactory interpretations that are only based on detoxification of radicals already formed, another idea was followed, namely, that of radical avoidance (Hardeland et al., 2003; Hardeland, 2005). Several possibilities of reducing radical formation under the influence of melatonin have been summarized in those reviews, including (i) improvement of internal coordination of circadian rhythms, since their disruption or dysphasing causes increases in radical formation in organisms as different as *Drosophila* (Coto-Montes and Hardeland, 1999) and Syrian hamsters (Coto-Montes et al., 2001), (ii) damping of glutamate- or NO-stimulated neuronal activity, thereby avoiding Ca^{2+} overload, and, in particular, (iii) several mitochondrial effects (Hardeland et al., 2003; Hardeland, 2005; Hardeland and Poeggeler, 2008a). Mitochondria are a major source of free radicals, due to electron leakage from the electron transport chain to molecular oxygen, to NO formation, to generation of peroxynitrite by interaction of the superoxide anion ($O_2^{\bullet -}$) with •NO, and to peroxynitrite-derived radicals such as $\bullet NO_2$, •OH and $CO_3^{\bullet -}$ (the carbonate radical). Other secondary radicals, especially the hydroxyl radical (•OH) and its organic radical reactions products, can be formed from H_2O_2 produced by mitochondrial and cytosolic superoxide dismutases. Already from this point of view, interference of melatonin with the mitochondrial metabolism should be of potential significance for radical-avoiding effects. Metabolic effects of the indoleamine in the mitochondria are, however, not restricted to the control of antioxidant and prooxidant enzymes. In fact, melatonin was shown to modulate the activities of several components of the electron transport chain (ETC) (Fig. 1).

These considerations are supported by various observations of protective actions of melatonin at the mitochondrial level including ETC improvements (reviews: Acuña-Castroviejo et al., 2001, 2002, 2003, 2007; Reiter et al., 2002b; León et al., 2004, 2005; Hardeland, 2005), as will be discussed in detail. However, such findings describing

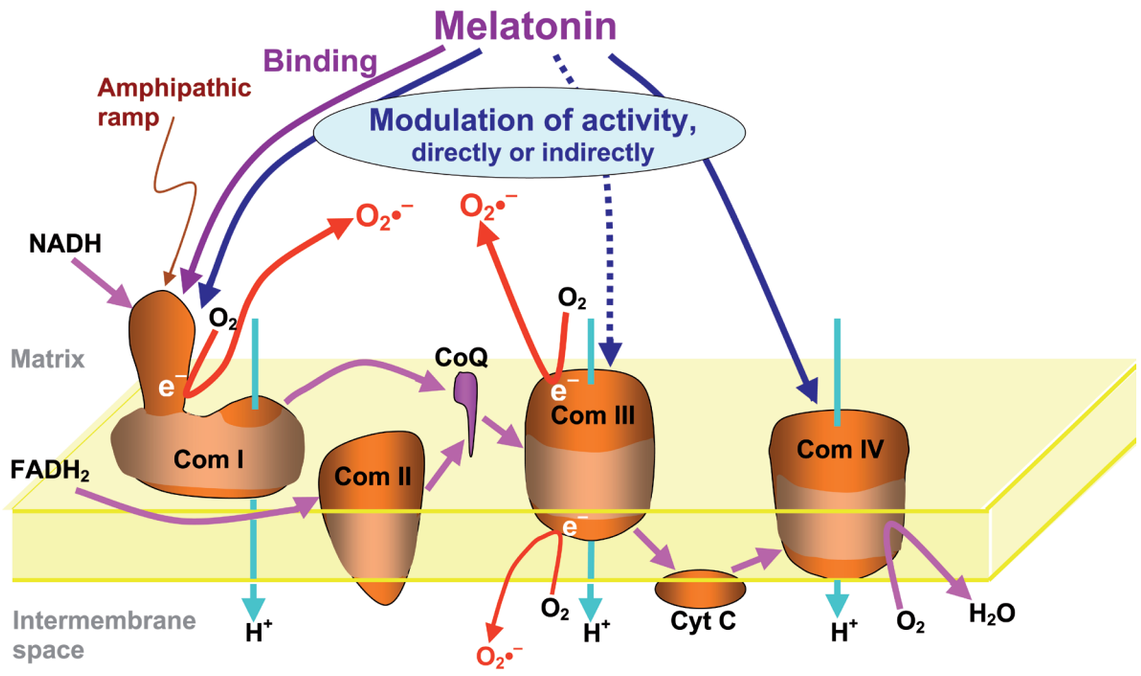

Fig. 1: Overview of melatonin's actions at the electron transport chain. Arrows, pink: electron transfer; turquoise: proton pumping; red: superoxide anion formation by electron leakage; violet; binding; blue: modulation (dotted arrow: effect not generally observed); light-colored parts of the ETC complexes: membrane domains of subunits. Abbreviations: Com = complex; CoQ = coenzyme Q; Cyt C = cytochrome C. Radical scavenging by melatonin, its metabolites or by melatonin-stimulated enzymes is not depicted, but contributes to indirect modulation.

protection under certain experimental conditions have to be carefully analyzed with regard to their very meaning. One has to discriminate between effects obtained upon administration of mitochondrial, radical-promoting toxins or under inflammatory conditions and those seen in still physiological situations, e. g., during senescence. Moreover, it makes a difference whether experiments are aiming to interfere with mitochondria-dependent apoptosis induction or only intend to modulate electron flux. And finally, changes observed in ATP formation and in the activities of mitochondrial complexes when studied in submitochondrial particles may not reflect physiological electron flux of intact mitochondria in the living cell. Even experiments in isolated mitochondria may not tell the truth, because of differences in energizing these organelles *in vitro* and *in vivo*. If, just for experimental convenience, mitochondria are mainly energized via complex II, the circumvention of the bottleneck of electron flux at complex I will lead to results differing from those obtained when the ETC is mainly fed through complex I. The role of the primary and secondary bottlenecks will be of utmost importance for understanding radical formation in mitochondria. This is intimately related to the dynamics of electron flux and leakage, which has recently been beautifully demonstrated by showing the flash-like generation of superoxide anions by intact individual mitochondria (Wang et al., 2008), shedding light on the non-steady state behavior of the ETC and the all-or-none characteristics of electron leakage reflecting distinct waves of overflow rather than a homeostatic process.

Protection of mitochondria by melatonin: the conventional view

The general consideration of melatonin as a potent antioxidant acting at different levels of free-radical detoxification should lead to the assumption that this indoleamine is also protective in mitochondria. Such actions of melatonin are attractive under several aspects, (i) the above-mentioned role of mitochondria as a major source of free radicals including their implication in aging processes, (ii) the importance of mitochondrial diseases (Lane, 2006), and (iii) the involvement of mitochondria in apoptosis. In fact, protection in a more conventional sense has been repeatedly described. In addition to similar actions in other compartments, melatonin was reported to attenuate also in mitochondria lipid peroxidation, in particular, to protect cardiolipin, to reduce oxidative protein and DNA modifications, to support the preservation of ultrastructure, to favor the resistance against oxidotoxins and enhanced radical generation in the course of ischemia-reperfusion

or UV-B radiation (Karbownik et al., 2000a,b; Milczarek et al., 2000; Acuña-Castroviejo et al., 2001; Okatani et al., 2001, 2003a; Wakatsuki et al., 2001; Reiter et al., 2003b; Watanabe et al., 2004; León et al., 2005; Duan et al., 2006; Petrosillo et al., 2006; Luchetti et al., 2007).

Oxidative stress can, of course, also lead directly to the impairment of electron flux through the ETC. In fact, corresponding observations, including protection and restoration of ETC function by melatonin, have been made with excitotoxins, such as kainic acid, and other neurotoxins, such as 6-hydroxydopamine or MTPT, which also cause oxidative stress and are indirectly or directly associated with ETC dysfunction (Dabbeni-Sala et al., 2001a,b; Mohanan and Yamamoto, 2002; Yamamoto and Mohanan, 2003; Khaldy et al., 2003; Thomas and Mohanakumar, 2004; Yalcin et al., 2004; Chen et al., 2005). A conventional view would interpret such findings as a result of protection from oxidative stress, but the more important question may be that of a more direct interference of melatonin with the ETC that exceeds prevention of damage to respiratory complexes and ubiquinone oxidation.

The electron transport chain as a direct target of melatonin?

The paucity of an interpretation only based on antioxidant actions becomes particularly evident in a potentially fundamental study by Martín et al. (2000). In isolated mitochondria challenged by 100 μM *t*-butylhydroperoxide, melatonin was shown to normalize the glutathione redox equilibrium (GSH/GSSG) and to prevent oxidative inactivation of glutathione peroxidase and glutathione reductase. Notably, these effects were not observed with other antioxidants, such as *N*-acetylcysteine, ascorbate or Trolox, the more water-soluble tocopherol analog, except for a normalization of the GSH/GSSG ratio at elevated Trolox concentrations of 1 mM, whereas melatonin was effective at 100 nM or lower (1 – 10 nM). At first glance, these findings seem to fit the conventional view of a redox effect of melatonin by interacting with oxidants, but a closer look shows that the results raise new, important questions. (i) If the other antioxidants are incapable of protecting in the same way, this may be explained in the case of a highly hydrophilic compound like ascorbate by its poor access to the mitochondrial interior, but not for Trolox, which is amphiphilic like melatonin. So why is Trolox by orders of magnitude less efficient than melatonin? (ii) Why are nanomolar concentrations of melatonin sufficient for preventing damage by 100 μM *t*-butylhydroperoxide, a compound which is also amphiphilic and easily penetrates membranes? Without any doubt, these findings cannot be interpreted by 1:1 interactions of melatonin with an oxidant molecule or radical, on a stoichiometric basis. There are only two possible explanations: Either melatonin interacts repeatedly with the oxidants, or it displays additional actions of an entirely different nature. A repetitive interaction could not be easily explained in terms of a radical scavenging cascade, since one molecule of melatonin, together with its reaction products, would only eliminate – in the extreme, but not in any case – up to 10 free radicals (Rosen et al., 2006) or other single-electron acceptors. This would not suffice for an efficacy over three orders of magnitude. Repetition might also be possible on the basis of redox cycling. Participation of melatonin in kind of a – in this case, protective – redox cycling might be deduced from non-additive, potentiating effects observed with the combination of melatonin and other, conventional antioxidants (Poeggeler et al., 1995; Tan et al., 2003a). However, the extent of such potentiations is, again, not sufficient for explaining the effects observed by Martín et al. (2000). Hence, it seems necessary to seek for other mechanisms by which melatonin could prevent mitochondrial oxidative damage beyond purely chemical redox interactions with reactive intermediates already formed. In this context, it should be noted that similarly low concentrations of melatonin in the nanomolar range were sufficient for stimulating complex I and IV activities in submitochondrial particles (Martín et al., 2002; Acuña-Castroviejo et al., 2003). Again, this could be hardly explained by elimination of oxidants on a stoichiometric basis. Hence, it seems highly attractive to assume interactions of melatonin with the ETC.

The ETC is a major source of free radicals and undergoes electron transfer reactions with suitable partners. Electron leakage from the ETC to molecular oxygen mainly occurs at complexes I and III (Choksi et al., 2004; Miwa and Brand, 2005; Panee et al., 2007). At complex III, this process is related to electron bifurcation from ubiquinol (Staniek et al., 2002) and has more recently been attributed to the Qo site, from where electrons are released to reduce oxygen especially when the intramonomer electron transfer between the two b_L hemes is interrupted (Gong et al., 2005). Electron dissipation and, thus, superoxide anion formation occurs at complex III on both sides of the inner mitochondrial membrane (Miwa and Brand, 2005). In complex I, the iron-sulfur cluster N2 has been identified as the site of electron leakage (Genova et al., 2001, 2004; Lenaz et al., 2002, 2006; Ohnishi et al., 2005). The alternative of direct electron transfer to oxygen vs. indirect transfer via mediation by semiquinones (Genova et al., 2001, 2003, 2004) now tends to be in favor of the direct process (Lenaz et al., 2006), but this issue remains controversial. One of two possible semiquinone binding sites is located close to the FeS cluster N2, at the matrix side of the inner membrane (Ohnishi, 1998). Electron leakage may take place at or near this site and,

therefore, superoxide anions are preferentially formed, in this case, at the matrix side. A so-called amphipathic ramp extends in this region into the matrix, so that interactions with hydro-, lipo- and amphiphilic compounds are possible. With regard to its consequences, this structural peculiarity may be regarded as double-edged. On the one hand, the extrusion towards the matrix makes the amphipathic ramp particularly vulnerable to secondary radicals and other oxidants/nitrosants of higher reactivity than the relatively harmless and, with suitable reaction partners, also reducing superoxide anion. On the other hand, this area may be accessible to regulatory molecules influencing electron flux. With regard to melatonin, both aspects may be of potential importance.

At this stage of our considerations, the question arises how melatonin could already protect, as observed by Martín et al. (2000), at very low, quasi catalytic concentrations, which would not require detoxification in a stoichiometric proportion to the radicals formed. If one would assume diminution of electron leakage by protection of ETC components from oxidation by free radicals, the minute amounts of melatonin required could only be effective if specifically bound on-site to the vulnerable molecules or parts of them. One may be reluctant in considering this a probable possibility. A second scenario might be based on a primary protection of enzymes like glutathione peroxidase and glutathione reductase, which would avoid hydroxyl radical formation from hydrogen peroxide and maintenance of a favorable GSH/GSSG ratio, respectively. This would indirectly protect the vulnerable ETC sites. But again, in the light of the low amounts of melatonin, it would be difficult to understand how these enzymes should be preferably protected and melatonin not be consumed in other redox reactions. A third possibility, which would also require experimental substantiation, might consist in direct electron exchange reactions of melatonin with the ETC. Based on quasi-catalytic melatonin concentrations, such a model could only work if melatonin undergoes a redox cycling of electron donation by the indoleamine and electron acceptance by a melatonyl radical. A fourth model might assume regulatory actions of melatonin at specific control sites in the ETC. In this case, binding at low concentrations would not primarily imply the participation of melatonin in redox reactions, but the indoleamine would rather influence throughput and dynamics of electron flux.

All these considerations do not require the upregulation of genes for mitochondrial proteins, nor the modulation of mitochondrial capacity by changing total mitochondrial volume – or amount of inner membrane – per cell. These processes may be influenced by melatonin as well, and could be of importance for long-term maintenance of mitochondrial functions including aspects of aging, but they have been omitted in this section, because protection by low concentrations of melatonin has already been observed in isolated mitochondria and in submitochondrial particles, as outlined above.

Melatonin and mitochondrial nitric oxide

At first glance, the notion that nitric oxide (NO) can be either beneficial or dangerously detrimental to mitochondria seems to be highly illogical. Since natural selection does not favor adverse mechanisms, the question should be that under which conditions NO is protective or the opposite. NO is known to reversibly inhibit electron flux through the ETC (Dungel et al., 2008). This is not surprising for a heme ligand, but additional, irreversible effects come into play when NO levels are strongly elevated. While basal or slighly enhanced levels of NO may only moderately reduce flux rates, high levels as occurring under conditions of septic shock can fully suppress respiration (Dungel et al., 2008). Of course, any strong suppression of electron flux should already be unfavorable under the aspect of ATP deficiency. Nevertheless, the causes of damage to the ETC have to be separately analyzed and will be discussed next. Under the viewpoint of long-term consequences including longevity, the other side of the coin seems more intriguing, namely, why a moderate limitation of electron flux by NO should be beneficial. The answer may be sought in studies on electron flux in ETC mutants of *Caenorhabditis elegans*. Several mutations which decrease electron flux markedly affected life span. However, the relationship between electron flux and longevity is not that simple. A mutation in a complex I subunit, *gas-1*, and also another one in the SDHC subunit of complex II, *mev-1*, exhibited reduced rates of oxidative phosphorylation, but the mutants were short-lived. In the *clk-1* mutation, which affects formation of the *C. elegans*-specific coenzyme Q_9 (CoQ_9) and leads to accumulation of other ubiquinone analogs (demethoxy-CoQ_9 and rhodoquinone$_9$) (Kayser et al., 2004b), reduced oxidative phosphorylation was instead associated with increased longevity (Kayser et al., 2004a). Therefore, electron flux *per se* is not an indicator of longevity. The *clk-1* mutation may only reflect some general slowing down of temporal processes, in the absence of demonstrable changes in radical formation and damage (Braeckman et al., 2002). Combinations of mutations predicted to have reduced complex I activity exhibited unexpectedly long life-spans (Kayser et al., 2004a). Whether this has to be interpreted in the same way, remains to be elucidated. On the other hand, the suppression of *gas-1* normalized the rate of oxidative phosphorylation, reduced oxidative damage to mitochondrial proteins and prolonged life (Kayser et al., 2004a). Altogether, these findings may appear to be puzzling, but they are nevertheless informative. First, one can state that decreases in

electron flux can, in principle, be beneficial, and this conclusion may be applied to moderate reductions by NO as well. Second, a most important question would be that of where the electrons are directed to when electron flux is reduced at different steps within the ETC, as occurring in the different mutant strains. In other words, are electrons leaking out at enhanced rate when electron flux is – partially or intermittently – interrupted at specific sites of the ETC? Finally, protection by moderate levels of NO has also to be seen in its relation to a secondary modest, but sustained mitochondrial depolarization associated with the decreased rate of respiration. The change in the membrane potential leads to a reduction of mitochondrial Ca^{2+} uptake, an effect reported to be protective in cardiomyocytes (Rakhit et al., 2001). This may be of similar value to neurons and other cells of high metabolic activity.

The opposition of beneficial vs. deteriorating effects, as discussed here for NO, finds its counterpart in the electron flux itself, as already indicated by the *C. elegans* mutants: both a decrease and an increase can be of advantage. However, this is, from a logical point of view, by far not contradictory. It rather depends on the starting point and on the tolerable margins whether a decrease or an increase is of advantage. If electron flux and, consequently, proton potential and oxidative phosphorylation are too much decreased, as may be occurring in mitochondrial dysfunction, enhancements should be beneficial. This holds even more for situations of inflammation, in the extreme, for a septic shock, when the ETC blockade by NO has to be reverted. On the other hand, age-related changes in activity ratios of the ETC complexes (Kwong and Sohal, 2000) can lead to unfavorable elevations of electron flux and leakage, so that reductions may be beneficial. For instance, complex IV activity was reported to be increased in cerebral mitochondria of aging mice. Interestingly, this change was reverted by melatonin to levels characteristic for 3 months-old animals (Sharman and Bondy, 2001).

A situation-dependent duality of either decreasing or increasing the rate of respiration has, in fact, been observed for the actions of melatonin. In isolated liver mitochondria, oxygen consumption was decreased by 10^{-7} M melatonin (Reyes-Toso et al., 2003). Similar findings were obtained when rats received melatonin via the drinking water (Reyes-Toso et al., 2006). In these normal, adult rats, melatonin did not affect the basal, state 4 respiration, but reduced the substrate-stimulated state 3 respiration, results which were interpreted in terms of curtailing an eventual overstimulation of the ETC (Reyes-Toso et al., 2003, 2006), and one might add: the curtailing of unnecessary and potentially harmful electron leakage. Recently, reductions in oxygen consumption and mitochondrial membrane potential by melatonin were demonstrated in isolated mitochondria, along with decreases in the production of superoxide anions and hydrogen peroxide (López et al., 2009).

More detailed information is available about effects of melatonin in situations requiring elevations of electron flux, particularly in septic shock. The involvement of NO in the inflammatory response leads to extremely high levels of the gaseous mediator, so that cells require a reversal of the resulting respiratory block to survive. In a murine model of septic shock, several studies conducted on skeletal muscle (Escames et al., 2006b; López et al., 2006a), heart (Escames et al., 2007) and diaphragm (López et al., 2006a,b) showed unanimously ETC failure including decreases in ATP synthesis, associated with oxidative damage to mitochondria, which were all widely reverted by melatonin. In all these cases, the involvement of NO was clearly demonstrated, since dysfunction and all other deleterious changes were absent in knockout mice deficient in iNOS, the inducible subform of nitric oxide synthase. Moreover, rises in the activity of mitochondrial iNOS and nitrite levels on wildtype mice were directly demonstrated, which were, correspondingly, absent in the knockouts (López et al., 2006b).

Mitochondrial dysfunction, as far it is caused by NO, is not sufficiently described by the reversible inhibition of electron flux directly caused by the interaction of NO with the ETC. The irreversible mitochondrial damage typically seen in septic shock is currently believed to be mainly caused by chemical reactions of an NO metabolite, peroxynitrite (Boveris et al., 2002; Escames et al., 2006a; Alvarez and Evelson, 2007) (Fig. 2). Superoxide anions formed by electron leakage from the ETC are only partially eliminated by the mitochondrial Mn-superoxide dismutase, since they have similar affinities to this enzyme and to •NO, i.e., to the NO radical, which is that one of the three NO congeners that is primarily synthesized by the NOS isoforms. The resulting peroxynitrite anion ($ONOO^-$) represents a highly reactive compound, which, among other reactions, can form adducts, with either a proton or CO_2 (which is highly abundant in mitochondria!). These adducts decompose to give $\bullet NO_2$ and a hydroxyl radical (•OH), in the first case, or to $\bullet NO_2$ and a carbonate radical ($CO_3\bullet^-$), in the second one. Moreover, the combination of $\bullet NO_2$ and •NO can lead to N_2O_3, a strongly nitrosating agent. Nitrosation, by NO congeners or N_2O_3, leads to the formation *S*-nitrosothiols including *S*-nitrosoglutathione and *S*-nitrosocysteine, compounds which can transnitrosate protein thiols in ETC subunits (Brown and Bal-Price, 2003; Dahm et al., 2006). Complex I seems to be particularly vulnerable to *S*-nitrosation, and this modification was found to result in increased superoxide anion formation as a consequence of enhanced electron leakage (Dahm et al., 2006). Hydroxyl and carbonate radicals deriving by decomposition of the respective peroxynitrite adducts are capable of oxidizing proteins, which is detected as increased protein carbonyl in mitochondrial proteins as a consequence of nitrosative

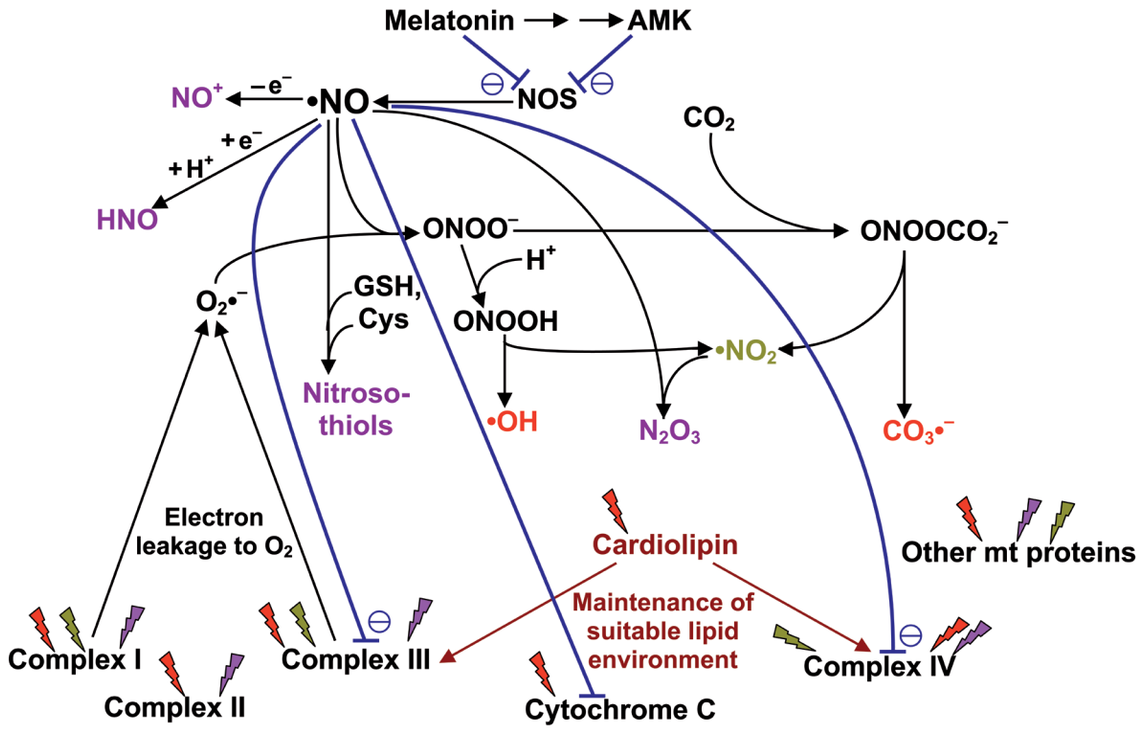

Fig. 2: Mitochondrial metabolism and effects of nitric oxide. Black arrows: metabolic reactions; blue lines with ⊖ symbols: inhibitions; brown arrows and lettering: role of cardiolipin; compounds in violet color: nitrosating agents; compounds in red color: strong oxidants; $\bullet NO_2$ in olive color indicates nitration in conjunction with hydroxyl or carbonate radicals. Correspondingly, colored flash symbols indicate sites of particular vulnerability to oxidation (red), nitrosation/transnitrosation (violet) and nitration (olive). Scavenging of reactive intermediates is not included. Melatonin and AMK (*N*[1]-acetyl-5-methoxykynuramine) are potent scavengers of hydroxyl (•OH) and carbonate ($CO_3\bullet^-$) radicals, both compounds can eliminate reactive nitrogen species by nitrosation or nitration. While nitroso- and nitromelatonin are relatively unstable and re-donate reactive nitrogens, AMK can form stable nitrosated and nitrated products. Other abbreviations and formulas: Cys = cysteine; GSH = reduced glutathione; HNO = protonated nitroxyl; mt = mitochondrial; NOS = nitric oxide synthase; $ONOO^-$ = peroxynitrite.

stress. Moreover, $\bullet NO_2$, especially in conjunction with the electron/hydrogen abstracting hydroxyl or carbonate radicals, can nitrate proteins, in particular, by forming 3-nitrotyrosine. All these protein modifications initiated by an excess of NO can lead to damage of the ETC and apoptosis (Brown and Bal-Price, 2003). In summary, NO and its reactive metabolites seem to be responsible for severe forms of mitochondrial dysfunction and cell death, not only in sepsis, but also, more specifically, in astrocyte- or microglia-dependent excitotoxicity, when high amounts of NO are released from the glia (Brown and Bal-Price, 2003).

Toxicity by NO and resulting reactive nitrogen metabolites is efficiently antagonized by melatonin, even in the severe situation of septic shock. Experiments by the group of D. Acuña-Castroviejo have clearly shown that iNOS is downregulated by melatonin, and a mitochondrially targeted, presumably modified subform of this enzyme was assumed to be responsible for protection in the sepsis model (Escames et al., 2006a,b, 2007; López et al., 2006a,b, 2009). The suppression of NO formation was associated with elevated activities of ETC complexes, improved ATP formation, maintenance of the mitochondrial membrane potential and reduced formation of superoxide and hydrogen peroxide (López et al., 2009). In this context, it should be noted that melatonin additionally downregulates expression and activity of neuronal NOS (nNOS) (León et al., 1998, 2006; Reiter et al., 2002a; Chang et al., 2002, 2005; Tjong et al., 2008). Therefore, similar protective effects as observed with the suppression of iNOS-dependent mitochondrial effects in sepsis should be assumed for the central nervous system, especially in relation to excitotoxicity involving elevated Ca^{2+} influx and nNOS activation. However, the role of melatonin in modulating nNOS seems to be more complex. One of melatonin's brain metabolites, *N*[1]-acetyl-5-methoxykynuramine (AMK), was reported to inhibit nNOS (Entrena et al., 2005) more efficiently than melatonin (León et al., 2006). Demonstrable, but not yet half-saturating effects were already detected at 10^{-11} M. In addition to these enzymological actions, AMK was shown to efficiently scavenge all three NO congeners (Hardeland et al.,

2007a), thereby forming – contrary to the easily decomposing *N*-nitrosomelatonin – a stable adduct, 3-acetamidomethyl-6-methoxycinnolinone (AMMC) (Guenther et al., 2005; Hardeland et al., 2007a,b). In fact, AMK was shown to also protect mitochondria at nanomolar concentrations (Acuña-Castroviejo et al., 2003).

As a bottomline of the melatonin-ETC relationship, we can state that the indoleamine is, on the one hand, capable of attenuating electron flux when it is unfavorably rising, as in aging animals, but, on the other hand, it can prevent the NO-mediated suppression of ETC functions, as occurring during inflammation and excitotoxic insults. In this regard, it seems to act in an adaptogenic way, by returning electron flux into a metabolic bandwidth that avoids both ATP deficiency and wasting, but certainly attenuates electron leakage and radical damage under either of these conditions.

Which insights can be obtained from submitochondrial particles and isolated mitochondria?

Working with mitochondria becomes difficult in the moment, in which more is intended than just determination of some enzyme activities. In the context discussed here, reliable information is required about metabolic flux rates, membrane potentials and ion concentrations of intact mitochondria within the living cell or, even better, in the tissue. There are only a very few parameters which can be followed in such intact systems, e.g., the membrane potential and oxygen consumption. Nevertheless, one has to be aware that indirect determinations of the membrane potential by means of principally suitable dyes are not entirely free of artifacts, related to dye distribution, biological effects of the accumulated sensor compound etc. It has been much more convenient to study mitochondrial functions in the isolated organelles or even in submitochondrial particles. We have referred to investigations of this type in the previous sections, and the conclusion has been that melatonin can exert protective or other beneficial effects in isolated mitochondria and its substructures as well.

Several additional observations can be added. Enhancements of complex I and IV activities by melatonin have been repeatedly described (Martín et al., 2002; Okatani et al., 2002a,b, 2003b; Acuña-Castroviejo et al., 2003; León et al., 2005), and also a comparable upregulation of complex III (López et al., 2009); all these are parameters that can be studied in submitochondrial particles only. Corresponding improvements of oxidative phosphorylation and effects on the membrane potential, as observed in isolated mitochondria, have been mentioned in a previous section. It should be emphasized that, under moderate experimental conditions avoiding extremes like septic shock, both oxygen consumption and membrane potential were usually decreased by melatonin (Reyes-Toso et al., 2003, 2006; López et al., 2009), i.e., parameters determined in isolated mitochondria, whereas the activities of the respiratory complexes were found to be increased, except for the study by Sharman and Bondy (2001) in aged mice. Enhancements in the activities of ETC complexes in conjunction with decreased oxygen consumption and membrane potential do not appear to be generally plausible, although explanations have been forwarded which interpreted this as an improvement of oxidative phosphorylation efficacy. It may be justified when changes in the ratio of ADP/oxygen consumption are demonstrated. However, this can be the case only if electrons "wasted" by leakage represent a large fraction of the total amount of electrons entering the ETC. One might be inclined to doubt this for normal physiological conditions, although it seems to occur under extreme conditions of oxidative or nitrosative stress and in late stages of aging.

While several of the findings on melatonin in isolated mitochondria and submitochondrial particles may be encouraging by showing that the indoleamine, in fact, exerts actions at the level of these organelles, it seems necessary to discuss the meaning of results obtained on subcellular systems. First, the mode and time of melatonin administration makes a considerable difference, even beyond the – in this context frequently neglected – chronobiological variation. If melatonin is administered to intact animals and determinations are carried out in subsequently isolated mitochondria or particles thereof, the meaning of a melatonin effect may be entirely different from a result obtained by adding melatonin to preparations of isolated, more or less purified organelles. Of course, the duration of the exposure to exogenous melatonin should play an additional role. When given to the intact animal, especially after repeated administration over consecutive days, gene expression may be of utmost importance. In such a case, the rise in the activity of an ETC complex could have resulted from *de novo* protein synthesis, which is impossible after addition of melatonin to isolated mitochondria. In fact, the expression of subunits 1 – 3 of complex IV was shown to be upregulated by melatonin (Acuña-Castroviejo et al., 2003). Similar effects on subunits 1 and 3, as well as on subunits ND1 and ND4 of complex I, were reported by Anisimov et al. (2006).

Next, one has to keep in mind that any study on mitochondrial fractions suffers, at least potentially, from isolation artifacts. During homogenization, the integrity of mitochondria can only rarely be preserved. While it may, with difficulty, be possible to isolate very short mitochondria, successful isolation is not realistic for long, fused mitochondria or for branched networks, such as those generally found in muscle syncytia and the cells of other

tissues. Whether or not mitochondria are disrupted during preparation, they undergo losses of membrane potential during this process and have to be re-energized by providing suitable substrates. Not only the transient loss of activity can be a source of artifacts, but also the mode of energizing can become a cause of misinterpretations. It makes a fundamental difference whether mitochondria are primarily energized by feeding via complex II or via complex I. Since complex I represents the first bottleneck of electron transport, investigators are frequently energizing to a high extent via complex II using high concentrations of succinate. An imbalance between these two routes may, however, lead to backward electron flow at complex I and enhanced electron leakage with its secondary consequences for radical formation. As will be discussed in a subsequent section, the primary and secondary bottlenecks of electron flux have to be considered for appropriate interpretations.

Finally, it should become clear what the rise in the activity of an ETC complex, as measured in a submitochondrial particle, really means. Such findings can never reflect *in vivo* flux rates, but rather and only flux capacities (cf. Hardeland and Poeggeler, 2008a). They may result either from enhanced stabilization of its components, by maintenance of active conformations and avoidance of inter-subunit disruption of electron transfer, or from previous upregulation of gene expression. If systems generating large amounts of reactive oxygen and/or nitrogen species are compared in the absence or presence of melatonin, higher activities obtained with the indoleamine may also reflect prevention of oxidative destruction.

So, with a sigh, we would wish we knew more about electron flux in the living cell under the influence of melatonin. Nevertheless, the fact that melatonin modulates parameters determining the rate of mitochondrial respiration and counteracts the destruction of its essential components is reason enough for future efforts.

Studies on senescence-accelerated animals and the links to aging

Following earlier assumptions on the involvement of mitochondria in the aging process (Harman, 1972), the free radical theory of aging (Harman, 2003) has developed towards a model centered on mitochondrial damage and dysfunction (Miquel et al., 1992; Poeggeler, 2005; Gruber et al., 2008), first under the premisses that these organelles are both major source and target of destructing radicals, later also under the perspective of metabolism modification and avoidance of electron leakage. Since melatonin can detoxify free radicals, prevent their formation and additionally modify mitochondrial metabolism, its connection to aging has become a matter of particular interest. Without wanting to repeat the discussions on efficacy and suitability of this indoleamine in detail, along with age- and disease-related decreases in endogenous melatonin (for reviews see: Poeggeler, 2005; Srinivasan et al., 2005, 2006), we would like to focus on some mitochondria-specific aspects we believe to be of potential importance.

Although studies on the normal aging process in wild-type animals are of highest value and, in the end, indispensable, another suitable approach is based on melatonin treatment of senescence-accelerated animals, such as SAMP8 mice, which can be compared with the normally aging SAMR1 mice sharing the genetic background. While several investigations have dealt with aspects only indirectly related to mitochondria, such as suppression of inflammation (Rodríguez et al., 2007c), improvement of neurological parameters (Morioka et al., 1999; Gutierrez-Cuesta et al., 2007; Caballero et al., 2008; Cheng et al., 2008) and antiapoptotic effects (Gutierrez-Cuesta et al., 2008), various others have directly studied mitochondrial functions (Okatani et al., 2002a,b,c, 2003b; Rodríguez et al., 2007a,b, 2008). Although these studies differ with regard to tissue (liver, cerebral cortex, heart, diaphragm), mode of melatonin administration (via drinking water or by i.p. injection), and age (oldest animals tested 10 or 12 months), and despite some variations in detail, the results collectively show the same changes: Especially at advanced age, SAMP8 animals exhibit reductions in the respiratory control index (RCI), in state 3 respiration, in dinitrophenol-uncoupled respiration (reflecting to a certain extent respiratory capacity), in the ADP/oxygen ratio, frequently also in the ATP level, and decreases in the activities of complexes I and IV. In one study (Okatani et al., 2003b), state 4 respiration was found to be increased in old SAMP8 mice, a finding that would be in line with the rises in complex IV activity, as observed by Sharman and Bondy (2001) in normal aging mice. The respiratory effects were associated with declines in the GSH/GSSG ratio and glutathione peroxidase activity, and with rises in lipid peroxidation and protein carbonyl. All these changes were, in most cases, reverted by melatonin, which additionally increased half-life and maximal life span in the SAMP8 mice (Rodríguez et al., 2008).

The results obtained in this animal model are not fully in accordance with other studies already mentioned (Reyes-Toso et al., 2003, 2006) on melatonin effects in isolated mitochondria from middle-aged rats, in which state 4 respiration remained unaffected, whereas state 3 respiration was reduced by melatonin. However, the aging process may make the difference. On the other hand, these results on SAMP8 mice are, at least partially, in line with changes observed in ETC complex activities in normal aging animals (Kwong and Sohal, 2000). Moreover, some of the results on the control SAMR1 mice showed

similar tendencies during normal aging, as observed in a more pronounced way in SAMP8.

The studies on senescence-prone mice contain one detail that is easily overlooked, but may be of importance. In one of the investigations (Okatani et al., 2003b), melatonin (10 mg/kg) was administered intraperitoneally 1 h before sacrifice. This short period of time is certainly not sufficient for substantial changes of complex I and IV activities by *de novo* synthesis of subunits. Therefore, the effect has to be explained in a different way, which may include protection from damage, substrate stabilization or other interactions of melatonin with the ETC. Of course, one has also to consider here the very high dosage applied. From our point of view, the time courses of changes induced by melatonin deserve further attention and have to be accompanied by approaches which discriminate between gene expression and direct interactions with the ETC, either on a redox basis or via a flux-controlling regulation mechanism.

Although senescence-accelerated animals are of supreme gerontological interest, they represent a very specific animal model, and the general validity of findings has to be always demonstrated for the aging processes in normal animals. Some differences, e. g., concerning age-related mitochondrial membrane potential, complex IV activity and melatonin effects on respiratory rate, may be seen in relation to the peculiarities of SAMP8 mice. Any acceleration of aging has a specific cause and changes in oxidative or nitrosative damage and mitochondrial malfunction may be secondary to the primary defect. For instance, some of the most severe human progerias, such as Hutchinson Gilford progeria syndrome, are laminopathies in which the stability of the nuclear lamina is primarily affected (Liu and Zhou, 2008).

Moreover, the changes observed in aging tissues may cell-specifically differ with regard to their causes, even if mitochondrial dysfunction is demonstrated. Among the changes observed, it is not always easy to discriminate the primary and secondary effects of aging. In a study on pancreatic acinus cells (Camello-Almaraz et al., 2008), aging was associated with a decreased response to secretagogues like acetylcholine and cholecystokinin, which was associated with reductions in the amplitude of cholecystokinin-elicited Ca^{2+} oscillations. This could be related to losses in intracellular Ca^{2+} pools, in the capacitative Ca^{2+} entry, and the mitochondrial membrane potential was found to be partially depolarized. Also in this case, melatonin was capable of restoring normal function including entry capacity, size of Ca^{2+} pool, excitability and mitochondrial membrane potential. Although Ca^{2+} distribution and mitochondrial uptake can be critical to many cells, especially to glia cells and neurons, the most important question still remains to be answered: Why and how is melatonin normalizing these parameters, by first improving mitochondrial function with consequences for Ca^{2+}, or by primarily improving Ca^{2+} distribution with consequences for the mitochondria?

Organ specificity of causes for age-related mitochondrial dysfunction may be of greater importance than usually believed. For instance, cardiolipin, which is required for a functional environment of complexes III and IV, was reported to be decreased in the aging rat brain (Petrosillo et al., 2008), but no such changes were found in aging rat cardiomyocytes (Hoppel et al., 2002; Moghaddas et al., 2002). In the brain, melatonin was shown to protect cardiolipin from oxidation, whereas this was not tested in the studies on the heart. Cardiomyocytes are interesting in this regard from an additional point of view, as a profound difference was shown to exist between subsarcolemmal and interfibrillary mitochondria. While the subsarcolemmal subpopulation did not show signs of dysfunction, the aging interfibrillary mitochondria exhibited decreased complex III and IV activities and increased electron leakage, especially from the Qo site of complex III (Fannin et al., 1999; Lesnefsky et al., 2001, 2006; Hoppel et al., 2002; Moghaddas et al., 2002, 2003; Lesnefsky and Hoppel, 2008). The impairment of electron transport was largely attributed to a defect at the Qo site (Moghaddas et al., 2003), but seems to be independent, under normal aging conditions, from cardiolipin. However, cardiolipin is highly vulnerable to peroxidation and, under conditions of otherwise induced oxidative stress, damage to this lipid can impair complex III and IV activities, so that protection from oxidation would prevent dysfunctions of the electron transport (Lesnefsky and Hoppel, 2008). In fact, melatonin exhibited this effect, which was especially described for the brain (Petrosillo et al., 2008). Support of fatty acid availability by administration of acetylcarnitine was found to be another means of protecting complex III and IV functions (Lesnefsky et al., 2006).

Studies on melatonin certainly represent only one single section of mitochondria-directed gerontology. Therefore, changes in mitochondrial activity and longevity as observed in other studies should be also considered for eventual relationships to melatonin. While some investigators have mainly focussed on complex III and IV dysfunction, the role of proton pumping and electron leakage at complex I deserves, in our opinion, particular attention. Inhibition of proton pumping by complex I enhanced superoxide formation, but ETC uncoupling reduced electron leakage (Dlasková et al., 2008a,b). These findings clearly support the idea that interruption or momentary retardation of proton and/or electron flux enhance electron leakage, whereas acceleration as caused by uncoupling leads to suction of electrons and, consequently, reduction of superoxide formation. We shall return to this point in a following section, but mention here that this relationship between proton pumping/electron flux and electron leak-

age has been discussed in terms of a vicious cycle of crucial importance for aging (Dlasková et al., 2008b).

Another actual line of research is that on aging-suppressor genes. Although the relationship to melatonin is poorly discernable at the present state of knowledge, and although a detailed discussion of the actions of their gene products would exceed the scope of this article, a brief consideration seems worthwhile and should be understood as an invitation to investigate possible connections. Here we confine our discussion to two groups of proteins, the klothos and the sirtuins. In either case, relationships to mitochondrial functions have been reported, and signaling pathways as well as endpoints of regulation partially merge with those of melatonin.

Overexpression of klotho extends the life-span of mice (Kurosu et al., 2005), upregulates the mitochondrial Mn-superoxide dismutase (Yamamoto et al., 2005), complex I (Sato et al., 2005) and IV activities, along with decreases in superoxide anion generation, lipid peroxidation and mitochondrial DNA fragmentation (Haruna et al., 2007). Klotho activates FoxO forkhead transcription factors, negatively regulates the insulin/insulin-like growth factor 1 (IGF-1) signaling pathway (Yamamoto et al., 2005; Li et al., 2008) and acts as a fibroblast growth factor 23 (FGF 23) co-receptor (Wolf et al., 2008; Wang and Sun, 2009). Together with another family member, β-klotho acting at FGF 19 and FGF 21 (Sinha et al., 2008; Kurosu and Kuro-o, 2009), numerous signaling pathways and their respective downstream factors, including PKC, Akt, p53/p21, Wnt, to mention a few, can be affected (Wang and Sun, 2009; Wolf et al., 2008; Bonafè and Olivieri, 2009). Interestingly, several longevity pathways converge on the insulin/IGF-1 signaling route (Bonafè and Olivieri, 2009). This holds also, at least, partially for melatonin signaling, but not in every case synergistically, but the endpoints, especially at the mitochondrial level, are frequently the same.

Sirtuins (SIRT) are known to promote longevity in numerous organisms, from yeast to insects and vertebrates. In mammals, seven subforms, SIRT1 to SIRT7, are known, at least three of which (SIRT3, SIRT4 and SIRT5) are mitochondrially localized (Lombard et al., 2007). Like klotho, SIRT3 can act in a FoxO-specific way, namely, by interacting with the mitochondrial FoxO3a homolog, daf-16 (Jacobs et al., 2008). SIRT3 prevents mitochondrial lysine hyperacetylation (Lombard et al., 2007) and obviously functions as an NAD^+-dependent lysine deacetylase (Hallows et al., 2008). In fibroblasts, it was shown to physically interact with the complex I subunit, the 39-kDa protein NDUFA9, to enhance complex I activity and ATP levels (Ahn et al., 2008). The functional relationship of sirtuins to mitochondria goes beyond their presence within these organelles and comprises additional regulatory effects. Apart from influences on NO formation, SIRT1, which also modulates the insulin/IGF-1 pathway, activates FoxO subforms and, thereby, antioxidant enzyme expression, and it seems to have additional effects on mitochondrial electron transport capacity, including the amount of mitochondrial volume per cell (Dilova et al., 2007; Guarente, 2008). With regard to mitochondrial effects of sirtuins and common endpoints with melatonin, we had suggested to investigate their connection to the indoleamine (Hardeland et al., 2008). A recent publication by Gutierrez-Cuesta et al. (2008) provides, in fact, first indications for an upregulation of SIRT1 in SAMP8 mice by melatonin.

Apoptosis and the mitochondrial effects of melatonin

Antiapoptotic effects of melatonin have been repeatedly described and, occasionally, also proapoptotic or other cytocidal actions, these especially in the context of melatonin's oncostatic properties (e. g., Scott et al., 2001; Trubiani et al., 2005; Wenzel et al., 2005). Prevention of apoptosis should not be surprising when melatonin is used in pharmacological concentrations to antagonize oxidative stress, because of the potent antioxidant actions of the indoleamine. In such cases, other antioxidants like ascorbate, Trolox or the Trolox-lipoic acid hybrid, LaT 3a, can be similarly effective (Vassilopoulos and Papazafiri, 2005), although the actions may be – partially – of indirect nature by involving stabilization of hypoxia-inducible factor 1α (Hif-1α). Melatonin's antioxidant properties may be likewise decisive in models using ischemia/reperfusion (Okatani et al. 2003a; Watanabe et al., 2004; Duan et al., 2006) or radiation-induced radical formation (Luchetti et al., 2006), but additional effects may contribute to protection, including signaling via the MAP kinase pathway (Han et al., 2006; Luchetti et al., 2009). Rescue of lymphoid cells from glucocorticoid-induced apoptosis may be judged differently and be based on interference of respective signaling pathways, although downstream processes such as counteraction of Bax, Bcl-2 upregulation and their consecutive steps may be the same (cf. Hoijman et al., 2004; Presman et al., 2006). Especially in leukocytes, antiapoptotic actions can be mediated by signaling mechanisms initiated by melatonin membrane receptors (Radogna et al., 2006, 2007, 2008). This may be attributed to the specific immunomodulatory role of melatonin, an aspect beyond the scope of this article. Mitochondria, as key organelles in apoptosis induced by intracellular signals, are involved in numerous cases studied, but we do not want to focus here primarily on the usual parameters like expression or activities of members of the Bcl-2 family, Bax translocation and the usual downstream events of cytochrome C release, apoptosome formation, caspase activation etc.,

but rather discuss the aspects which are directly related to electron flux, proton pumping and changes of the mitochondrial membrane potential ($\Delta\Psi_m$).

Although melatonin may also gradually decrease $\Delta\Psi_m$, as outlined in a previous section, thereby curtailing state 3 respiration and, presumably, also excessive electron leakage, the maintenance of $\Delta\Psi_m$ can be, in other situations, a highly desired effect, in particular, when processes such as ETC blockade by NO or calcium overload, either due to overexcitation, to protein misfolding or to damage by free radicals, can lead to a collapse of the mitochondrial membrane potential in the absence of antagonizing effects. In this regard, melatonin was repeatedly shown to prevent a fatal decline in $\Delta\Psi_m$, independently of cell type and highly efficient against various types of noxes (Hardeland and Pandi-Perumal, 2005). In cardiomyocytes, astrocytes and striatal neurons, it prevented calcium overload (Andrabi et al., 2004; Jou et al., 2004), counteracted the collapse of the mitochondrial membrane potential induced by H_2O_2 (Jou et al., 2004, 2007), doxorubicin (Xu and Ashraf, 2002) or oxygen/glucose deprivation (Andrabi et al. 2004). Although doxorubicin exerts numerous other prooxidant effects, which are also counteracted by melatonin (Oz et al., 2006; Oz and Ilhan, 2006), its primary mitochondrial actions affecting electron flux, leakage and membrane potential should be seen in the focus. Under this aspect, the protective effects by melatonin against doxorubicin are in line with those against other mitochondrial toxins, such as rotenone (Saravanan et al., 2007; Lin et al., 2008), also in combination with Ca^+ elevations (Sousa and Castilho, 2005), and 1-methyl-4-phenylpyridinium (MPP^+) (Chen et al., 2005; Chetsawang et al., 2007; Lin et al., 2008). Numerous additional effects were described for the protection against MPP^+ toxicity, which cannot be summarized here in total. In these cases, a strict discrimination with regard to cause and consequence would be required, from our point of view. However, the interference of melatonin with MPP^+ at complex I was also directly addressed (Absi et al., 2000).

In most reports on antiapoptotic actions of melatonin at the mitochondrial level, the prevention of mitochondrial permeability transition appeared to be a secondary effect observed at the end of a cascade. However, direct inhibition of the mitochondrial permeability transition pore (mtPTP) was also demonstrated (Andrabi et al., 2004), an effect that should suffice for rescuing cells from apoptosis. Melatonin diminished mtPTP currents, with an IC_{50} of 0.8 µM, a concentration which would require mitochondrial accumulation of melatonin, something which is principally possible and has already been discussed with regard to the amphiphilicity of melatonin (Hardeland and Pandi-Perumal, 2005). Uptake of melatonin by mitochondria has been demonstrated (Messner et al., 1998; López et al., 2009), and diurnal elevation of melatonin to nocturnal blood levels led to a preferential accumulation in these organelles, when compared to other cell compartments using protein as a reference value (Messner et al., 1998). The direct mtPTP inhibition, as shown by Andrabi et al. (2004), should be interpreted on the basis of a low affinity binding site. The major problem emerging from these results is, however, that of identifying physiological conditions, under which such an effect could be relevant. What may be welcome experimentally and, perhaps, from a therapeutic point of view when applying pharmacological concentrations of the indoleamine, might be highly undesired under normal conditions, since melatonin should not generally suppress apoptosis everywhere and at any time when its level is sufficiently high. Apoptosis is of high biological value and indispensably required – beyond development, elimination of damaged, virus-infected or cancer cells – already for avoiding hyperplasias.

Under these premisses, the counteraction of mtPTP opening by melatonin may be a mitochondria-related effect of possible therapeutic value, but it does not seem to be the central phenomenon by which melatonin could exert beneficial physiological effects, e. g., during normal aging, actions which should rather be of adaptogenic nature and take place under moderate conditions and with low quantities of melatonin. Therefore, we should re-focus on the possible interactions of melatonin with the ETC under non-toxicological conditions.

How does melatonin interact with the electron transport chain?

The existence of melatonin effects as observed at low concentrations in isolated mitochondria, already discussed in a previous section, leads to the necessity of finding explanations beyond receptor-mediated signaling, upregulation of gene expression or stoichiometric radical detoxification. Immediate influences on electron flux, proton pumping and adjustment of $\Delta\Psi_m$, along with reductions of electron leakage are imaginable in two ways. Either melatonin, or one of its metabolites, (i) interacts with the ETC by electron exchange, thereby modulating net electron flux, re-cycling electrons and thereby bridging, in terms of an electron shuttle, between components of the ETC, or (ii) the indoleamine acts via a regulatory high-affinity binding site.

The first possibility was previously discussed in a model (Hardeland et al., 2003; Hardeland, 2005; Hardeland and Poeggeler, 2008b) that was oriented at electron exchange reactions known from several substituted nitrones and from the electron-exchange reactions of oxygen species. In analogy to electron uptake by molecular oxygen at complex I or III and the known re-donation of electrons by superoxide anions or hydrogen peroxide to

cytochrome C, a parallel, competing electron cycle was assumed, in which electron donation by melatonin would generate a melatonyl radical capable of competing with oxygen for electrons, e. g., at N2 of complex I. Alternately, the melatonin metabolite AMK might react in a similar way. Both melatonin and AMK are potent electron donors and their resonance-stabilized radicals should possess a sufficiently long lifetime for taking up electrons at suitable sites of the ETC. Interactions of melatonin with cytochrome C as a putative site of electron donation have, in fact, been described (Semak et al., 2005). However, the study, which was conducted in the presence of hydrogen peroxide, showed oxidation of melatonin to hydroxylated compounds and, in a pseudo-catalase reaction, the formation of N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), the AMK precursor. This finding may be not surprising because no electron acceptor of the ETC was present in the system. With regard to its known redox properties, AMK may be also capable of reducing cytochrome C. As already stated, this model was proposed in analogy to findings with nitrones, which can be highly efficient in protecting mitochondria and have been shown to considerably extend lifetime in an invertebrate animal model, especially when made sufficiently amphiphilic by suitable substituents that facilitate the entrance to the organelles (Poeggeler et al., 2005; Durand et al., 2007). Unfortunately, they are unsuitable in vertebrates because of metabolic decomposition. For the moment, the validity of this model for melatonin or AMK is entirely open and would require further experimental support.

Meanwhile, indications for a regulatory effect of melatonin at the ETC have emerged. Melatonin was found to bind with high affinity at complex I (data by Pappolla MA, Poeggeler B and Pucci B, cited in Hardeland and Poeggeler, 2007; Hardeland et al., 2008; Hardeland, 2008a). In rat brain mitochondria, a dissociation constant of 150 pM and a total number of specific binding sites of 30 fmol/mg protein have been determined. Displacement studies with the N2-specific ligands capsaicin and dopamine indicated a localization at the amphipathic ramp of complex I.

The consequences of melatonin binding to complex I remain to be experimentally identified in detail, but, for the moment, interpretations may be given which are based on the observations of melatonin effects on electron flux and reductions of electron leakage. For this purpose, it seems worthwhile to consider the bottlenecks of electron flux as well as the possibilities of backward electron overflow in situations of retarded forward transport (cf. Fig. 3). The major bottlenecks of electron flux are located at complexes I and IV of the ETC (Lenaz et al., 2006). Compared to the other ETC components, complex I represents the entrance bottleneck, which may be circumvented by feeding electrons via complex II. Regulation at the entrance to a pathway is a common means for controlling metabolic

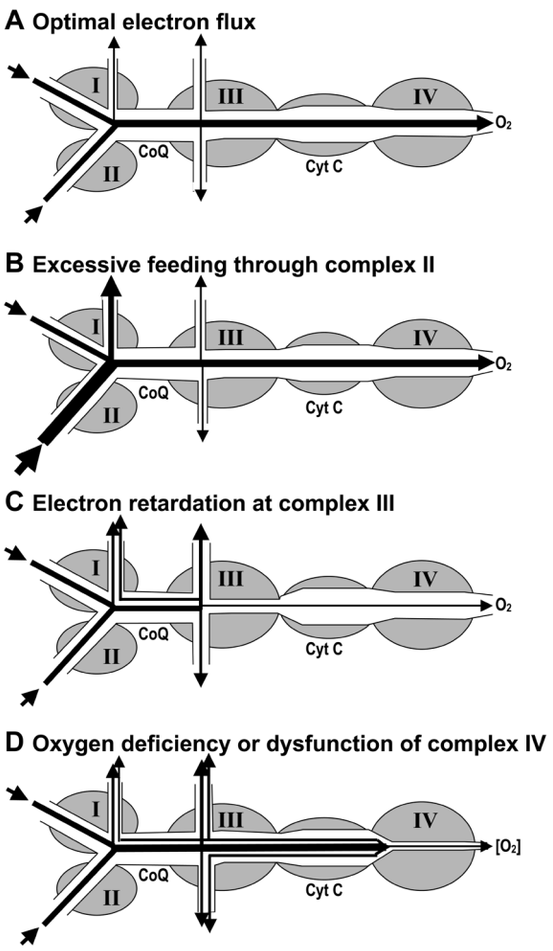

Fig. 3: Variations of electron flux and the role of bottlenecks in different physiological and pathophysiological situations. Abbreviations: I, II, III and IV = ETC complexes I, II, III, IV; CoQ = coenzyme Q; Cyt C = cytochrome C. Upward directed arrows indicate electron leakage to the matrix, downward directed arrows electron leakage to the intermembrane space. Forward and backward fluxes are indicated by separate arrows.

throughput. In this regard, the observation that melatonin curtails state 3 respiration (Reyes-Toso et al., 2003, 2006) could be interpreted in terms of a melatonin-dependent reduction of electron uptake at complex I, which would reduce electron leakage and, thus, radical formation. However, melatonin may not just act as a usual negative ligand, but might exerts its effects in a conformation-dependent way allowing rises in electron flux when the proton-moving force is reduced and $\Delta\Psi_m$ is declining too much for sufficiently maintaining ATP production. Such self-regulatory, conformation-based mechanisms should be built-in in the ETC, already independently from melatonin, but may be controlled by the indoleamine.

A second bottleneck in the ETC is unavoidable at complex IV, because of frequently occurring limitations in

oxygen supply. Reduced oxygen availability, including impaired vascular function associated with aging and degenerative disorders, causes retardation of electron transport, eventually backward electron flow and increases of electron leakage, at complexes I and III. Electron input should not exceed capacity, which becomes limited by oxygen deficiency. Leakage at complex III may be particularly important in complex IV dysfunction (Fig. 3), whether caused by lack of oxygen or by damage to complex IV subunits or to cardiolipin also required for normal function. Interruption of electron transfer between the two b_L hemes in complex III can occur because of damage to complex III subunits or, again, to cardiolipin, or as a consequence of reduced electron flow downstream of complex III. In either case, electron dissipation at the Qo site should result.

Upon additional feed-in via complex II, backward electron flow is possible at complex I, so that its by-passing can be only favorable if complex IV is not rate-limiting. In this case, the iron-sulfur cluster N2 is the major site of electron leakage. While cysteines in the respiratory chain are anyway prone to oxidative damage (Moosmann and Behl, 2008), the amphipathic ramp of N2 is particularly vulnerable to reactive oxygen species, because this peptide arm containing both hydrophilic and lipophilic domains extrudes to the matrix and is, therefore, particularly exposed to water, oxygen and reactive intermediates. Damage at this site not only reduces electron throughput, membrane potential and ATP production, but can also become, via electron dissipation, the starting point of a vicious cycle, which might ultimately limit life span (Dlasková et al., 2008b).

A prediction of such a model considering the bottlenecks will also be that experimental results can be entirely divergent when isolated mitochondria are differently energized, by using different substrate ratios that change the relative electron feeding via complex I or complex II, and that a surplus of electron input is not necessarily favorable because of possibly enhanced electron leakage.

Moreover, it seems important to dismiss the conception of mitochondrial "homeostasis" and to replace this by a more dynamical view. The demonstration of discontinuous superoxide flashes (Wang et al., 2008) clearly shows that electrons are not steadily flowing at a constant rate, but that they are fed in, intermittently until overflow, eventually in forward or backward direction, and that electrons can experience kind of a traffic jam in the ETC. This situation should become more dramatic, or at least, more dynamic, when a cell is stimulated, experiences enhanced Ca^{2+} influx and mitochondrial uptake, is exposed to rising NO levels and switches from state 4 to state 3 respiration. The demonstrated effects of melatonin on electron flux and membrane potential, as outlined above, should now be re-analyzed with regard to their influences on the dynamical behavior of electron flux.

Conclusion

Although numerous publications have reported beneficial effects of melatonin at the mitochondrial level, findings do not unanimously point into the same direction. While melatonin was usually found to reduce damage by free radicals, to favor glutathione availability and reduction, and to improve respiration efficacy, its effects on the ETC were divergent. Although the activities of ETC complexes were reported to be enhanced in some models, especially those related to inflammation, it either decreased or increased electron flux and $\Delta\Psi_m$ in other studies. To resolve these seemingly contradictory results is a challenge. This can be only done by considering the different experimental situations under which the data were obtained. Of course, it makes a difference whether melatonin is given in situations of elevated or unfavorably/dangerously decreased electron flux. The first of these situations can be found at state 3 respiration, but this may exceed this classic, physiological situation. In an aged animal or human, partially dysfunctional mitochondria may be forced to work at a higher rate of electron flux to attain the ATP levels required. Improvements of electron conductance through the ETC by reducing intermittant retardation of the flux, as caused by damage to ETC components or to cardiolipin, and/or by attenuating electron dissipation, may allow the ETC to become more efficient again and to use less of electrons for maintenance of normal respiratory functions. In the second situation, namely, marked decreases in ETC function, as seen in the extreme under septic shock and bearing the danger of a $\Delta\Psi_m$ collapse, elevations of electron flux and proton pumping should be beneficial and avoid apoptosis. To what extent such stimulations, as in fact observed, can be attributed to counteractions against NO and peroxynitrite-derived free radicals remains to be analyzed in detail and may be a matter of the specific experimental approach. Moreover, improvements of GSH availability may contribute in the same situation to the avoidance of mitochondrial malfunctions.

However, it should become evident that all these effects – apart from their dependence on the respective experimental model – cannot be simultaneously identified in reductional approaches. While submitochondrial particles cannot reflect electron flux through the intact ETC, this should be possible in isolated mitochondria, but this system does not yet reflect electron flux *in vivo*, since it depends on the more or less artificial methods of energizing. More studies are needed on mitochondria in the living cell, although many important parameters cannot be followed or only with difficulty.

Mitochondrial effects of melatonin should substantially exceed those found in short-term experiments with subcellular preparations. With regard to the physiological relevance, attention should be directed to actions already

observed at low concentrations. Apart from direct interactions, as assumed for the N2 site, other effects should be based on signaling cascades initiated by melatonin's membrane receptors or on direct genomic effects by nuclear receptors. Up- and downregulations of anti- or prooxidant enzymes, especially their mitochondrially targeted subforms, mostly require gene expression and will be seen only after sufficiently long exposure to melatonin. The same holds for the expression of aging-suppressor genes, such as those for klotho and for sirtuins, which await further elucidation with regard to their connections to melatonin. And even a sirtuin that is not mitochondrially localized, such as SIRT1, may favor mitochondrial function substantially by stimulating mitochondrial growth (Guarente, 2008). Such processes can be modulated by small molecules, as assumed in this last case for resveratrol. Similar influences of melatonin should be investigated (Hardeland et al., 2008).

The principal messages of our considerations can be summarized under three aspects. (i) Melatonin's mitochondrial actions are multiple ones and complex. The complexity, comprising overlapping antioxidant, radical avoiding and electron-flux modulating effects, deserves further experimental dissection. (ii) Melatonin can directly interact with the ETC, by binding to complex I, but, perhaps, also by undergoing electron exchange reactions. (iii) Melatonin can either increase or decrease electron flux and membrane potential, depending on the experimental situation including gerontological aspects. This duality of possible actions should not be seen as a contradiction in itself, but rather as a remarkable property of an adaptogenic molecule, which has been classified, according to Poeggeler, as a mitochondrial metabolism modifier ("MMM") (cf. Hardeland et al., 2003). Melatonin's capability of maintaining mitochondrial activity within a certain physiologically favorable bandwidth, also described as safeguarding of electron flux and mitochondrial membrane potential, in conjunction with reductions in electron dissipation at both edges of the metabolic spectrum, may be regarded as a valuable contribution to enduring functionality over long periods within the total life span.

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