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ORIGINAL ARTICLE

Interactions of melatonin with mammalian mitochondria. Reducer of energy capacity and amplifier of permeability transition

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Abstract Melatonin, a metabolic product of the amino acid tryptophan, induces a dose-dependent energy drop correlated with a decrease in the oxidative phosphorylation process in isolated rat liver mitochondria. This effect involves a gradual decrease in the respiratory control index and significant alterations in the state 4/state 3 transition of membrane potential ($\Delta\Psi$). Melatonin, alone, does not affect the insulating properties of the inner membrane but, in the presence of supraphysiological Ca^{2+}, induces a $\Delta\Psi$ drop and colloid-osmotic mitochondrial swelling. These events are sensitive to cyclosporin A and the inhibitors of Ca^{2+} transport, indicative of the induction or amplification of the mitochondrial permeability transition. This phenomenon is triggered by oxidative stress induced by melatonin and Ca^{2+}, with the generation of hydrogen peroxide and the consequent oxidation of sulfydryl groups, glutathione and pyridine nucleotides. In addition, melatonin, again in the presence of Ca^{2+}, can also induce substantial release of cytochrome C and AIF (apoptosis-inducing factor), thus revealing its potential as a pro-apoptotic agent.

Keywords Melatonin · Mitochondria · Permeability transition · Ca^{2+} · Oxidative stress · Pro-apoptotic factors

Abbreviations

AdNT	Adenine nucleotide translocator
AIF	Apoptosis-inducing factor
BKA	Bongkrekic acid
CsA	Cyclosporin A
DTE	Dithioerythritol
ΔE	Electrical transmembrane potential
$\Delta\mu_{H+}$	Transmembrane electrochemical gradient
$\Delta\Psi$	Electric membrane potential
EGTA	Ethylene glycol tetraacetic acid
MLT	Melatonin
MPT	Mitochondrial permeability transition
NEM	*N*-Ethylmaleimide
RCI	Respiratory control index
RHM	Rat heart mitochondria
RLM	Rat liver mitochondria
ROS	Reactive oxygen species
RR	Ruthenium red
tBOOH	Tert-butylhydroperoxide
TPP^+	Tetraphenylphosphonium

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) (Fig. 1) is an indolamine isolated for the first time by Aaron Lerner in the 1950s from the bovine pineal gland (Lerner et al. 1958).

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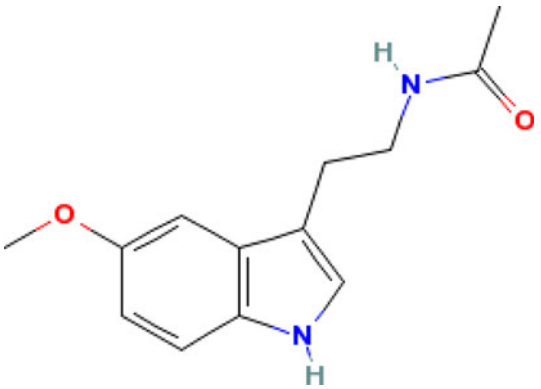

Fig. 1 Molecular structure of melatonin

Melatonin is widely found in nature, where it occurs in vertebrates, invertebrates, plants, unicellular eukaryotes, algae, and even bacteria. It is distributed ubiquitously throughout an organism, due to its amphiphilic nature which makes it permeable to all cellular compartments (Slominski et al. 2008).

Melatonin is a hormone produced especially by the pineal gland or epiphysis, where the enzyme tryptophan hydroxylase converts the amino acid tryptophan to 5-hydroxytryptophan. By the intervention of 5-hydroxytryptophan decarboxylase, this pathway leads to the production of serotonin, which is a substrate for *N*-acetyltransferase. *N*-acetylserotonin is methylated to form melatonin, the production of which is controlled by the suprachiasmatic nucleus of the hypothalamus. In addition, its biosynthesis and release are inhibited by light, stimulated by darkness and also regulated by noradrenergic activity (Gutiérrez et al. 2006). In mammals, the concentrations of circulating melatonin vary over a 24-h period, its levels in blood generally being high during the night and low during the day (Pandi-Perumal et al. 2008, and references therein).

Melatonin receptors are amply distributed throughout the body, and their expression differs among various target organs. Its membrane-bound receptors include melatonin receptors types 1 and 2 (MT1 and MT2) (Slominski et al. 2008).

Previous studies on rats indicated that melatonin might be a wonderful integrator which could make people younger and healthier, and perhaps even improve their sexual performance (Arendt 1996; Bergstrom and Hakanson 1998).

Melatonin also seems to regulate the internal body clock in case of great changes in time zones (e.g., jet lag) (Herxheimer 2006). Indeed, different studies show that the circadian rhythm of many species of reptiles, birds and mammals, including humans, may be synchronized by doses of exogenous melatonin (Cassone 1990; Buscemi et al. 2006).

Melatonin has also been claimed to inhibit the growth of cancer cells in vitro, although it can promote melanoma growth in hamsters. On the strength of these preliminary observations with opposite meanings, the possible clinical uses of melatonin as adjuvant treatment in chemotherapy or immunotherapy are too premature for results to be evaluated properly (Arendt 1996, and references therein).

The effects of melatonin on mitochondria are difficult to summarize, for several reasons: they were obtained under differing experimental conditions, often with de-energized organelles, and show ample variations according to the organ, tissue and cell type examined (Srinivasan et al. 2008; Büyükavcı et al. 2006).

It has been reported very recently that melatonin inhibits cardiolipin peroxidation in rat heart mitochondria (RHM) and prevents the mitochondrial permeability transition (MPT) and cytochrome C release (Petrosillo et al. 2009). It has also been found that the indolamine induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells (Bejarano et al. 2009) but reduces this process in human leukocytes (Espino et al. 2010). The differential actions exhibited by melatonin on mitochondria and apoptosis in normal and cancer cells were also previously reported in a review article (Sainz et al. 2003).

In addition, as regards the physiological and pathological concentrations of melatonin, its levels in various body fluids and cells are not necessarily in equilibrium with those in the blood (Reiter and Tan 2002) and this hormone, as also observed in other compounds like flavonoids and polyphenols (De Marchi et al. 2009; Salvi et al. 2002; Battaglia et al. 2008), can show alternative actions as a reducing or oxidative agent, depending on the structural features of the target molecules and their environment, the incubation time and the concentrations used (Osseni et al. 2000).

Some studies on sub-mitochondrial particles deriving from brain and liver tissues show that doses of melatonin significantly increase the activity of respiratory chain complexes I and IV at the nanomolar level. Although these concentrations are similar to the physiological ones found in plasma at night, their values are not indicative of the amount of melatonin present in the cells, because high physiological values of melatonin have been found in differing tissues (even two or three orders of magnitude more than the circulating level) (Martín et al. 2002).

Other studies have shown the neuroprotective effect of melatonin: it can reduce brain damage and improves the general neurological state in rats affected by ischemia (Andrabi et al. 2004), and it is reported to have some antioxidant and immunostimulant effects at high concentrations, although pro-oxidant and immunosuppressant effects have also been described (Arendt 1996; Srinivasan et al. 2008; Büyükavcı et al. 2006, Zhang et al. 2011).

In conclusion, our aim is to establish some precise results concerning the interactions between melatonin and

isolated liver mitochondria, using undamaged organelles able to carry out their energy functions. This was done in order to ascertain whether the observed opposite results are due to tissue specificity, different incubation media, or different actions of melatonin between isolated and in situ mitochondria.

In this study, we analyse the effects of melatonin on bioenergetic mitochondrial parameters, its role in oxidative stress, and its possible function on the establishment of the MPT.

Materials and methods

Materials

Mouse monoclonal antibody anti-cytochrome C was purchased from Pharmingen, and rabbit polyclonal antibody anti-apoptosis-inducing factor (AIF) from Millipore. All other reagents were purchased from Sigma, except where indicated, and were of the highest purity commercially available.

Isolation of rat liver mitochondria

Rat liver mitochondria (RLM) were isolated by conventional differential centrifugation (Schneider and Hogeboom 1950) in a standard medium containing 250 mM sucrose, 5 mM Hepes (pH 7.4) and 1 mM EGTA; EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with bovine serum albumin as standard (Gornall et al. 1949).

These studies were performed in accordance with the guiding principles in the care and use of animals and were approved by the Italian Ministry of Health.

Standard incubation procedure for RLM

RLM (1 mg prot/ml) were incubated in a water-jacketed cell at 20°C. The standard medium contained 200 mM sucrose, 10 mM Hepes (pH 7.4), 5 mM succinate, 1.25 μM rotenone, 1 mM phosphate. Variations and/or other additions are described with individual experiments presented. Increasing phosphate concentration until 5 mM may amplify melatonin effect.

Determination of mitochondrial functions

Membrane potential ($\Delta\Psi$) was measured in an open, thermostatically controlled, stirred vessel, by monitoring the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a selective electrode, prepared according to published procedures (Kamo et al. 1979). The obtained values were corrected as reported by Jensen et al. (1986). ΔE value, the electrode potential derived from TPP^+ distribution, was added into Nernst equation in order to obtain the mitochondrial potential $\Delta\Psi$.

Oxygen uptake was measured by a Clark electrode (Yellow Spring Instruments, OH) in a closed vessel equipped with thermostatic control and magnetic stirrer. ATP synthesis was measured by the enzymatic method of Lemasters and Hackenbrock (1976).

Determination of mitochondrial swelling

Mitochondrial swelling was determined by measuring the apparent absorbance change in mitochondrial suspensions at 540 nm, on a Kontron Uvikon mod. 922 spectrophotometer equipped with thermostatic control.

Measurement of the redox level of RLM

Determination of protein sulfydryl groups and mitochondrial glutathione oxidation was carried out with mitochondrial suspensions from the various incubations used to determine mitochondrial swelling. In brief, at the end of incubation (12 min), the total suspension (1 mg/ml) was placed in Eppendorf 4515c tubes and centrifuged for 1 min at 12,000*g*; then the supernatant was discarded and the pellet used for both measurements. A sulfydryl group oxidation assay was performed after solubilization of the pellet with 1 mg of solubilization medium (10 mM EDTA, 0,2 M Tris, 1% SDS, pH 8.3), with 5,5′-dithio-bis-(2-nitrobenzoic acid) at 412 nm in a Kontron Uvikon Model 922 spectrophotometer, according to Santos et al. (1998).

Glutathione oxidation was assessed by deproteinization of the pellet with 3% metaphosphoric acid and subsequent centrifugation to separate the supernatant on which oxidised glutathione was determined by the method of Tietze (1969). The redox state of endogenous pyridine nucleotides was directly followed fluorometrically in a Shimadzu spectrofluorophotometer RF-5000, with excitation at 354 nm and emission at 462 nm.

The production of H_2O_2 in RLM was measured fluorometrically by the scopoletin method (Loschen et al. 1973) in a Shimadzu spectrofluorophotometer RF-5000, with excitation at 350 nm and emission at 460 nm.

Detection of cytochrome C and AIF release

Mitochondria (1 mg prot/ml) were incubated for the times indicated in the specific experiments at 20°C, in standard medium, with the appropriate additions. The reaction mixtures were then centrifuged at 13,000*g* for 10 min at 4°C to obtain mitochondrial pellets. The supernatant

fractions were concentrated five times with Pierce SDS-PAGE sample Prep kit (Thermo Scientific). Aliquots of 10 µl of the concentrated supernatants were subjected to SDS-PAGE with 15% (w/v) acrylamide gel for cytochrome C detection and 10% acrylamide gel for AIF. After WB, the nitrocellulose membranes were treated with the specific antibodies anti-cytochrome C (Pharmingen) or anti-AIF (Millipore), and analyzed by enhanced chemiluminescent detection (ECL, GE Healthcare).

Results

A peculiar characteristic of energy-transducing membranes, such as the inner mitochondrial membrane, is their capacity to use the transmembrane electrochemical gradient ($\Delta\mu_H^+$), formed during the process of electron transport along the respiratory chain, as a driving force for ATP synthesis.

Experimentally, this process is usually detected by determining the "respiratory control index" (RCI), which is the respiratory rate ratio "state 3/state 4". In particular, the active respiring state taking place during ATP synthesis is called "state 3", and the slower rate, after all the ADP has been phosphorylated to form ATP, is called "state 4". On this basis, mitochondrial respiration is controlled by the availability of ADP, phosphate, and an oxidizable substrate which can create an electron flow along the respiratory chain, with the consequent promotion of $\Delta\mu_H^+$.

The RCI indicates the effectiveness of the mitochondria in promoting oxidative phosphorylation and thus the tightness of the coupling between oxygen consumption and ATP production. Typical RCI values range from 3 to 10, varying with the substrate and the quality of the mitochondrial preparation. Obviously, high values indicate a better biochemical function.

The reported results (Fig. 2) indicate that RLM, incubated in standard medium with succinate as substrate, in the conditions reported in "Materials and methods", exhibit an RCI of 10. The presence of 10 µM melatonin causes the RCI to fall to 2.8. Higher concentrations (20 and 40 µM) further reduce the index until complete uncoupling (RCI = 1 with melatonin 80 µM) (Table in Fig. 2). Uncoupled respiration by FCCP is not affected by 80 µM melatonin because this compound, at the concentration used, is able to cause a complete uncoupling effect (Fig. 2).

Mitochondrial inner membrane integrity and function can be estimated by $\Delta\Psi$ determination, a value which indicates the membrane insulating capacity. $\Delta\Psi$ is one of two factors (the other being ΔpH) which constitute the $\Delta\mu_H^+$. During ATP synthesis, a drop in $\Delta\mu_H^+$ is experimentally appreciable by a transient decrease in $\Delta\Psi$. This is observable by adding ADP to the mitochondrial suspension containing phosphate, which induces an immediate and transitory decrease in $\Delta\Psi$ from 180 to 160 mV, then restored to the initial value (e.g. state 3/state 4 transition). In the presence of melatonin, a reduction in the $\Delta\Psi$ drop occurs, with only partial or negligible transient $\Delta\Psi$ restoration, both dose-dependent (Fig. 3). The subsequent results deal with the estimation of melatonin effects on membrane function, in the presence or absence of Ca^{2+}. Ca^{2+} allows the possible action of melatonin as an inducer or amplifier of the MPT to be measured. As Fig. 4a shows, melatonin alone, even at increasing concentrations up to 60 µM, does not cause any significant variations in $\Delta\Psi$ (about 180 mV), when compared with control without Ca^{2+}. The addition of 40 µM Ca^{2+} (Fig. 4b) causes a rapid and transitory decrease in $\Delta\Psi$, due to the entry of positive charges into the matrix, which is immediately equilibrated by the increase in oxygen consumption and H^+ ejection (Lötscher et al. 1980), with a partial restoration of $\Delta\Psi$ with a subsequent slight decrease (control). If melatonin is present in the incubation medium, after the transitory variation noted with the addition of Ca^{2+}, $\Delta\Psi$ undergoes a collapse, the rapidity and extent of which is dose-dependent (Fig. 4b). MPT inhibitors—cyclosporin A (CsA) which blocks the pore opening, the antioxidant dithioerythritol (DTE), Mg^{2+} which binds the adenine nucleotide translocator (AdNT) and prevents its conformational exchange, the alkylant *N*-ethylmaleimide (NEM) and the reactive oxygen species (ROS) scavenger spermine—prevent the $\Delta\Psi$ collapse caused by the contemporaneous presence of melatonin and Ca^{2+}. Instead, unexpectedly, bongkrekic acid (BKA), one of the most powerful inhibitors of transition pore opening, is almost completely ineffective (Fig. 4c). These results may indicate MPT induction, caused by the synergic action of melatonin and Ca^{2+}. However, in order to confirm this hypothesis, we evaluated whether mitochondria, incubated as described above, undergo colloid-osmotic swelling. The results (Fig. 5a) show that mitochondria, suspended in standard medium with 20 µM melatonin and 40 µM Ca^{2+}, exhibit a decrease in apparent absorbance of about 0.4 units if compared with control with Ca^{2+} (control). The use of melatonin or Ca^{2+} alone induces only a very low decrease in absorbance and a slight drop in $\Delta\Psi$. In addition, by substituting the sucrose medium with a KCl medium, according to previous observations (Hansson et al. 2010) a slight reduction of the swelling induced by melatonin has been found (Online Resources 1). Moreover, experiments evaluating melatonin effect on mitochondrial membrane potential and swelling have been performed also with glutamate/malate instead of succinate/rotenone without finding substantial differences (Online Resources 2 and 3).

In the presence of EGTA (a calcium chelant) or ruthenium red (RR) (an inhibitor of calcium transport), the

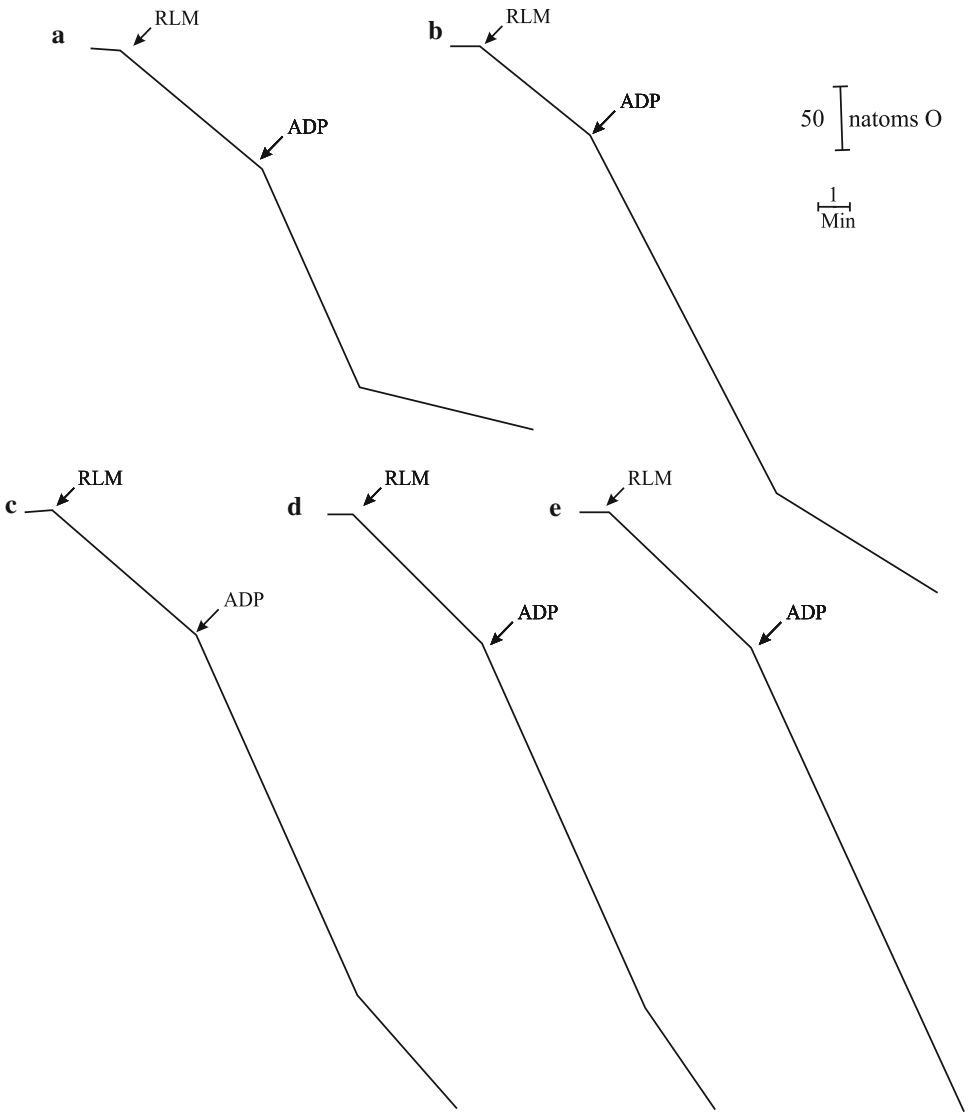

Fig. 2 Dose-dependent effect of melatonin on respiratory control index (RCI). RLM were incubated in standard medium in conditions indicated in "Materials and methods". 200 μM ADP was added where indicated. Melatonin (MLT) was present in medium at increasing concentrations, as indicated. *Insert table*: RCI values with ±SD of five experiments are reported, flux rates and P/O values. A typical experiment is reported; five other replicates gave almost identical results

Condition	RCI	V_3	V_4	P/O
(a) Control	10±0.01	80	8	1.7
(b) 10 μM MLT	2.8±0.02	68	24	1.1
(c) 20 μM MLT	1.9±0.02	72	36	0.9
(d) 40 μM MLT	1.4±0.03	68	52	0.7
(e) 80 μM MLT	1±0.01	-	-	-

decrease in absorbance is completely blocked (Fig. 5a). The effects of the other typical MPT inhibitors on mitochondrial swelling are shown in Fig. 5b. They all exhibit a preventive effect, except BKA—the same outcome shown in $\Delta\Psi$ detection.

The observation that the reductant DTE and the alkylant NEM prevent $\Delta\Psi$ collapse and mitochondrial swelling suggests that these events are caused by mitochondrial oxidative stress. The redox state of sulfydryl groups, glutathione and pyridine nucleotides was studied in order to assess this possibility. As Fig. 6a shows, the incubation of mitochondria with Ca^{2+} (control) or melatonin alone produces a decrease in the reduced state of thiols (SH groups) by 30 and 16%, respectively, while the simultaneous presence of the two agents produces a 48% decrease in reduced state. This reduction is clearly due to the formation of the same percentage of oxidised disulphides. NEM completely blocks the thiol oxidation induced by

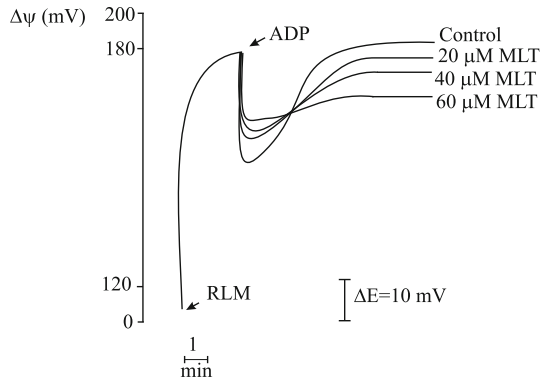

Fig. 3 Effect of melatonin on state4/state3 transition of ΔΨ. RLM were incubated in standard medium, in presence of 2 μM TPP^+ for ΔΨ measurement, in conditions indicated in "Materials and methods". 200 μM ADP was added where indicated. Melatonin (MLT) was present at indicated concentrations. Four other replicates showed similar results. Δ*E* electrode potential

both agents whereas, in the same condition, DTE causes only partial prevention. This different effect is most probably due to the fact that NEM can easily cross the membrane, whereas DTE is less permeable. A close analogy with sulfydryl group oxidation is observed with glutathione. Melatonin and Ca^{2+} perform the same oxidant action, both alone and together (Fig. 6b). In this case too, NEM exerts full protection, whereas the effect of DTE is only partial.

Figure 7 shows that mitochondria incubated in standard medium, in the absence of Ca^{2+}, do not show any variation in the redox level of pyridine nucleotides over time. The presence of Ca^{2+} (control) or melatonin alone causes some oxidation of the nucleotides, but both agents together induce rapid and more intense oxidation. Also in this case, CsA and NEM have a complete preventive effect on the oxidant action of melatonin and Ca^{2+}.

The results shown in Figs. 6 and 7, demonstrating that melatonin plus Ca^{2+} induces oxidative stress, led us to examine whether this effect was related to the production of ROS. The results (Fig. 8) show that melatonin, in the presence of Ca^{2+}, causes a large increase in H_2O_2, when compared with control in the absence of Ca^{2+}. It should also be noted Ca^{2+} alone produces a significant amount of hydrogen peroxide, as previously reported in the literature (Grijalba et al. 1999; Battaglia et al. 2010), whereas melatonin alone does not cause any production of ROS. A very recent paper reporting the induction of ROS production by melatonin in isolated mitochondria from human mesangial cells and mice kidney tissues is in agreement with our data (Zhang et al. 2011).

As MPT induction is often linked to apoptosis, due to the release of pro-apoptotic factors, the subsequent experiment examined this possibility. As Fig. 9 shows, a considerable loss of AIF and cytochrome C takes place when RLM are incubated with melatonin and Ca^{2+}. This is clear from the intensity of lane 4, as revealed by Western blotting of the supernatant fraction. Melatonin without Ca^{2+} has an almost negligible effect on cytochrome C release, but AIF is released in greater quantities (lane 3), although to a lesser extent than with Ca^{2+} (compare with lane 4). In any case, both amounts released in this condition (without Ca^{2+}) are similar to control (compare with lane 1). Ca^{2+} alone induces a considerable release of cytochrome C, whereas that of AIF is similar to control (lane 2). In this figure it is also shown that the mitochondrial fraction is compared with the protein content that is released during MPT induction (supernatant fraction).

This effect on cytochrome C is probably caused by attenuation of electrostatic interactions, responsible for the binding of this protein to the external surface of the inner mitochondrial membrane and also to possible induction of the permeability transition of the outer membrane. CsA effectively prevents the release caused by melatonin plus Ca^{2+} (lane 5), demonstrating that this effect is closely correlated with transition pore opening.

Discussion

As emphasised in the "Introduction", there are many data about the biological effects of melatonin, often in opposition and sometimes not supported by well-designed experimental approaches, especially in studies on mitochondrial function. In this regard, the main target of this study was to ascertain some essential points regarding the action of melatonin on the bioenergetic functions of mitochondria, in order to clarify whether melatonin may be considered toxic or beneficial to organelles.

The results shown in Figs. 2 and 3 clearly demonstrate that melatonin causes a collapse in the mitochondria bioenergetic in a dose-dependent fashion, as revealed by the decreased RCI and alterations in the state 4/state 3 transition at ΔΨ level. These results cannot clarify whether this damage is caused by specific lesions to membrane structures or direct interaction between melatonin and the ATP-synthase system. However, the lack of any change in the normal value of ΔΨ, which is 180 mV (Fig. 4a), observed without ADP addition, and the alterations in the state 4/state 3 transition evaluated on ΔΨ during ATP synthesis, suggest that melatonin acts at the level of ATP-synthase. The observation that the respiration rate in state 4 is gradually increased by enhanced melatonin concentration (Fig. 2 and table therein), without changing ΔΨ (Fig. 4a), also suggests stimulation of respiratory chain activity. The lack in the effect by high melatonin concentration on uncoupled respiration confirms that it does not inhibit the respiratory chain. Another important observation, which

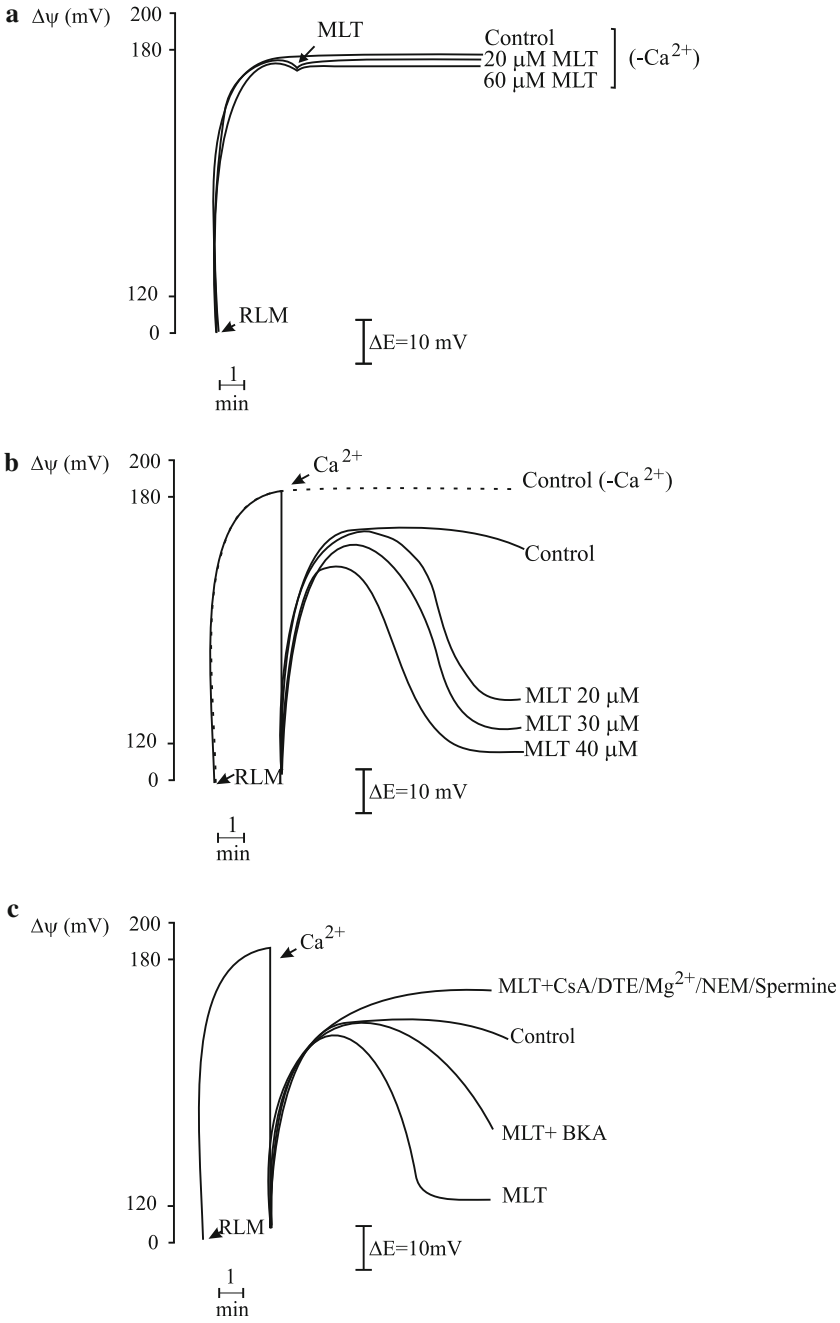

Fig. 4 Effect of melatonin on ΔΨ of RLM incubated in absence (**a**) or presence (**b**) of Ca^{2+}. Effect of MPT inhibitors on ΔΨ collapse induced by melatonin plus Ca^{2+} (**c**). RLM were incubated as in Fig. 3 and 40 μM Ca^{2+} was added to all curves except where indicated. **a**, **b** Melatonin (MLT) was present at concentrations reported on side of curves. **c** Melatonin was present at 20 μM. When present: 6 μM BKA, 1 μM CsA, 5 mM DTE, 1 mM Mg^{2+}, 10 μM NEM, 100 μM spermine. Four replicates gave comparable results. Δ*E* electrode potential

demonstrates the unequivocal action of melatonin on mitochondrial membrane permeability, regards its effect in the presence of Ca^{2+}. In this condition, melatonin induces dose-dependent ΔΨ collapse (Fig. 4b, c) and an increase in mitochondrial swelling (Fig. 5a, b), demonstrating that it behaves as a typical inducer or amplifier of the MPT.

This statement is further confirmed by inhibition by CsA, a specific inhibitor of MPT, and by the physiological MPT inhibitors, Mg^{2+} and spermine (Figs. 4c, 5b).

The dependence of this phenomenon on Ca^{2+} is demonstrated by the complete inhibition of mitochondrial swelling shown by EGTA and RR (Fig. 5a). Instead, the slight inhibition of BKA, another typical inhibitor of MPT, raises the debated problem regarding the identification of the main protein involved in pore opening. As previously reported, the proposal that the AdNT, which is inhibited by BKA, holding up ATP/ADP exchange, is responsible for pore opening, has been challenged with the proposal of other protein(s), including the phosphate carrier (Leung and Halestrap 2008; Leung et al. 2008). The inefficacy exhibited by BKA, together with the inhibition of MPT by NEM, which also inhibits the phosphate transporter,

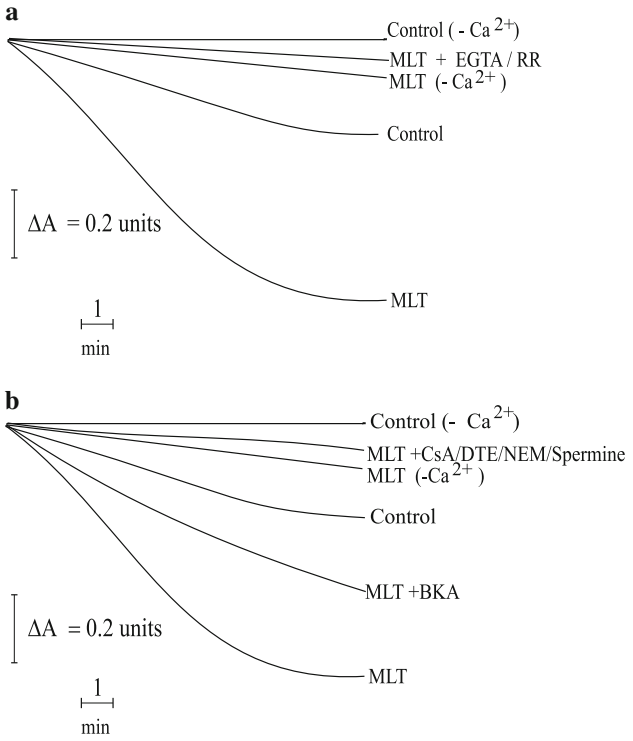

Fig. 5 Induction of mitochondrial swelling in the presence of Ca^{2+}. Effects of Ca^{2+} transport inhibitors (**a**) and MPT inhibitors (**b**). RLM were incubated in standard medium in presence of 20 μM melatonin (MLT) and 40 μM Ca^{2+}. When present: 1 μM CsA, 6 μM BKA, 5 mM DTE, 1 mM EGTA, 10 μM NEM, 1 μM RR, 100 μM spermine. Assays were performed at least four times with similar results. Downward deflections indicate mitochondrial swelling

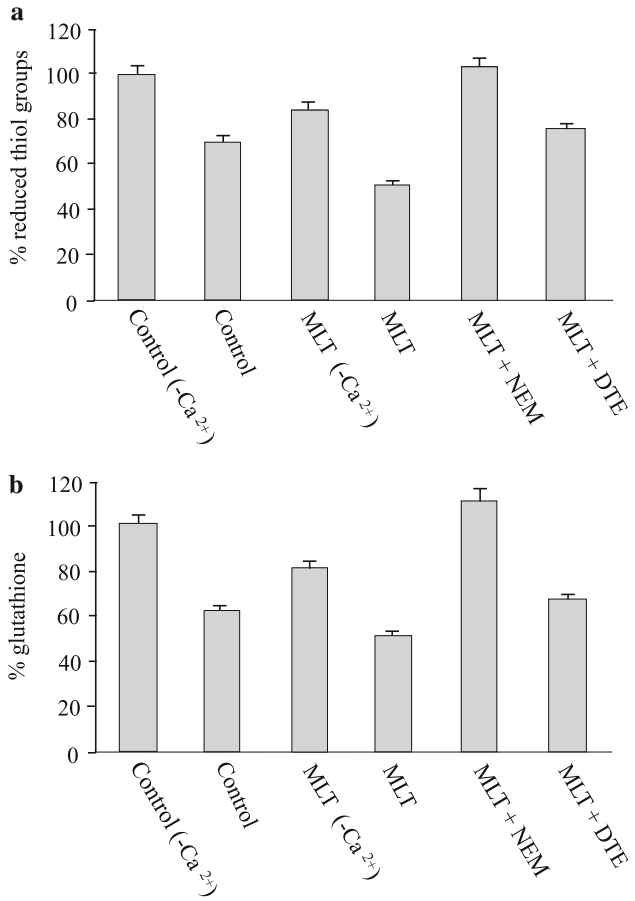

Fig. 6 Mitochondrial thiol (**a**) and glutathione (**b**) oxidation induced by melatonin. All incubations were carried out as indicated in "Materials and methods" in presence of 40 μM Ca^{2+}, except where indicated (-Ca^{2+}). When present: 20 μM melatonin (MLT), 5 mM DTE, 10 μM NEM. Data are expressed as percentage of thiol or glutathione reduction, and represent average ± mean SD from five independent experiments

supports this suggestion. In any case, the protective effect exerted by NEM and DTE on MPT is evidenced that the alkylation or reduction of critical thiol groups on AdNT, or phosphate carrier, prevents the opening of the transition pore and suggests the oxidant effect of melatonin on mitochondria. This proposal is strongly supported by oxidative stress, albeit slight, induced by the compound alone, as shown by the oxidation of thiols, glutathione and pyridine nucleotides (Figs. 6, 7).

In the presence of Ca^{2+} (necessary for MPT induction), a synergetic effect is exerted on oxidative stress, which is completely prevented—again by NEM, and partially by DTE (Figs. 6, 7). The mechanism by which melatonin induces oxidative stress cannot be deduced from the experimental data reported here. As Fig. 8 shows, melatonin and Ca^{2+}, incubated together, induce the production of a significant amount of H_2O_2. Ca^{2+} produces H_2O_2 by interacting with membrane cardiolipin with the result of membrane disorganization and consequent alteration of ubiquinone mobility leading to ROS generation (Grijalba et al. 1999). However, it should be noted that Ca^{2+}, alone, is able to produce the H_2O_2, whereas melatonin seems to be ineffective. It should also be recalled that H_2O_2 can only be detected outside the mitochondria and that the measured amount depends on the location of its generation, which may differ between melatonin and Ca^{2+} and, consequently, on the reaction rate with its targets and external diffusion rate. In other words, it is possible that melatonin, alone, can produce H_2O_2 but it can also very rapidly react or be transformed into other ROS, in which case it can no longer be detected.

It is also noteworthy that the effects of melatonin are similar to those exhibited in RLM by another biogenic amine, agmatine, which is oxidised in mitochondria by an amine oxidase (Cardillo et al. 2009), with production of ROS and opening of the transition pore (Agostinelli et al. 2009). Experiments are in progress in our laboratories to ascertain whether the action of melatonin is related to similar catabolism.

The oxidative stress induced by melatonin (Figs. 6, 7) and the consequent MPT induction in liver, in the light of

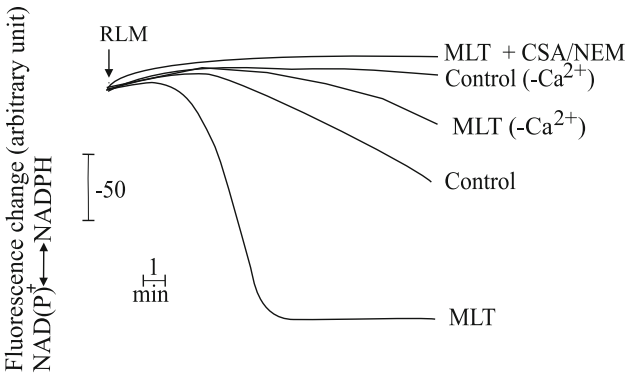

Fig. 7 Oxidation of pyridine nucleotides induced by melatonin. RLM were incubated in standard medium in presence of 40 μM Ca^{2+}, except where indicated (-Ca^{2+}). When present: 20 μM melatonin (MLT), 1 μM CsA, 10 μM NEM. Five additional assays exhibited same trend in fluorescence change

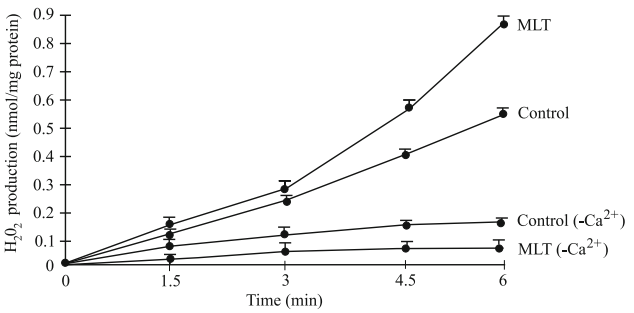

Fig. 8 Mitochondrial hydrogen peroxide production by Ca^{2+} and melatonin. Incubation conditions and reagent concentrations as in Fig. 7. Mean values from four experiments

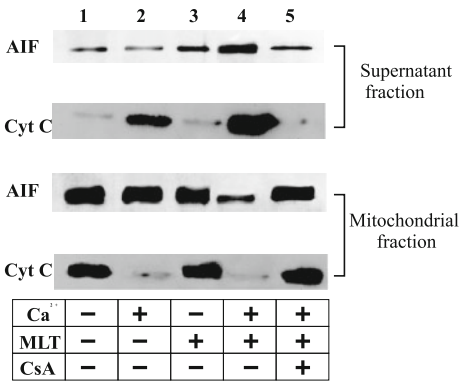

Fig. 9 Release of AIF and cytocrome C (Cyt C) induced by melatonin in presence of Ca^{2+} and cyclosporine A. Results of Western blotting of supernatant and mitochondrial fractions. Incubation conditions and reagent concentrations as in Fig. 7. *Lane 1* control (-Ca^{2+}), *lane 2* control, *lane 3* melatonin (MLT) (-Ca^{2+}), *lane 4* melatonin (MLT), *lane 5* melatonin (MLT) and CsA. Assays performed four times with almost identical results

the opposite results observed in RHM (Petrosillo et al. 2009), may be explained by considering that these authors used pyruvate plus malate as energizing substrates and tert-butylhydroperoxide (tBOOH) as MPT inducer. In these conditions, tBOOH produces the hydroxyl and peroxyl radicals responsible for the MPT induction and cytochrome C release, whereas melatonin protects by scavenging both radicals (Matuszak et al. 1997; Pieri et al. 1994). In our experiments with RLM, the substrate was succinate, which sends electrons to the respiratory chain through Complex II (succinate dehydrogenase) and ubiquinone. This complex contains heme b, which is not involved in electron transport but which serves as an electron sink to prevent leakage out of the respiratory chain (Yankovskaya et al. 2003).

The observed oxidative stress induced by melatonin alone, most probably responsible for pore opening in the presence of Ca^{2+}, is explained by considering that the indolamine favors the above leakage and hampers the function of heme b. In these conditions, regular electron flux from succinate toward ubiquinone would be inhibited and the semiquinone radical would be stabilized. Subsequently, the reaction of the radical with molecular oxygen would produce superoxide anion. One proposal is that this ROS, which cannot be scavenged by melatonin (Chan and Tang 1996), oxidises the critical sulfydryl groups responsible for pore opening (McStay et al. 2002). It is also assumed that H_2O_2 is generated in small amounts in the absence of Ca^{2+}, but it is not detectable as explained above. Instead, in the presence of Ca^{2+}, a large amount of H_2O_2 is generated, caused by pore opening which induces further oxidative stress (Fig. 8). This mechanism reported here would explain the different effect exhibited by melatonin in preventing MPT (Petrosillo et al. 2009) or in inducing it (this paper). In the first case melatonin scavenges both the hydroxyl and peroxyl radicals generated by tBOOH, without involving the electron flux triggered by pyruvate/malate. In the later, melatonin favors the generation of superoxide anion, a ROS that is not scavenged by melatonin itself (Chan and Tang 1996). In this case the electron flux is triggered by succinate and directly affected by the interaction with melatonin, which generates the ROS.

It should be noted that, although melatonin and Ca^{2+}, alone, induce oxidative stress, they are not able to induce the MPT (Fig. 5), which can only take place when both the agents are incubated together. This is because pore opening requires the interaction of Ca^{2+} with its specific site(s) located on AdNT or phosphate carrier (Leung and Halestrap 2008; Leung et al. 2008). Concomitantly, two specific thiols, located on the AdNT, have to be oxidised (Mcstay et al. 2002). In fact, Ca^{2+} alone can bind to the specific site, but ROS, most probably produced by itself, although giving rise to oxidative stress, are not able to react

with the specific thiol groups, perhaps because they are not produced near them. Conversely, melatonin induces oxidation of thiols but cannot induce the binding of Ca^{2+}. In both cases, the above necessary requirements for pore opening are not satisfied.

Observations of the ability of melatonin to cause the MPT through oxidative stress support the hypothesis that this hormone is potentially a pro-apoptotic agent. Figure 9, showing that mitochondria incubated with calcium and melatonin suffer a loss of cytochrome C and AIF, not only demonstrates this pro-apoptotic potential of melatonin, but is also indicative of the potential activation of both caspase-dependent and caspase-independent pathways.

These early results clearly demonstrate that, in the specific experimental conditions used, RLM treated with melatonin and calcium undergo oxidative stress, leading to the induction of MPT and the release of pro-apoptotic factors.

The observed opposite effects of melatonin in isolated mitochondria match previous observations of mitochondrial-mediated apoptosis in normal and cancer cells, as emphasized in a former review (Sainz et al. 2003). A very recent paper reports that melatonin protects human leucocytes against MPT induction and apoptosis. The authors suggest the same mechanism proposed for RHM (see above), i.e., that melatonin protects by scavenging hydroxyl and peroxyl radicals responsible for the oxidative stress leading to apoptosis (Espino et al. 2010). Another recent paper reports that, in human myeloid HL-60 cells, melatonin induces caspase activation accompanied by mitochondrial depolarization, induction of MPT, release of cytochrome C, and apoptosis (Bejarano et al. 2009). These findings match our results in RLM, although the authors do not report any effect regarding possible oxidative stress induced by melatonin. They suggest the involvement of the Bid/Bax signal, which cannot be considered in isolated RLM. However, Wölfler et al. (2001)reported that melatonin induces the formation of ROS in human leukemic Jurkat cells, leading to apoptosis. In this case, the mechanism of ROS generation is controversial, but their involvement and the lack of scavenging effect by melatonin are unequivocal. Both these studies confirm that melatonin only scavenges some forms of radicals (e.g., hydroxyl and peroxyl) whereas for others (superoxide anion and H_2O_2) it is ineffective. The indication that apoptosis occurs in Jurkat cells also strongly supports the mechanism proposed by us for MPT induction.

It is also reasonable to believe that these results, in a pathophysiological context and in cellular systems, may represent an important defence aspect for the organism, initiated by the action of melatonin.

As emphasized above, our results are preliminary and need to be completed and evaluated on more complex biological systems than isolated mitochondria, in particular hepatocytes and hepatoma cells, in order to ascertain the potential of melatonin as a pro-apoptotic and anticancer agent in these types of cells, as the results on isolated RLM seem to indicate.

Conflict of interest The authors declare that they have no conflict of interest.

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