Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo


Abstract: Melatonin displays antioxidant and free radical scavenger properties. Due to its ability with which it enters cells, these protective effects are manifested in all subcellular compartments. Recent studies suggest a role for melatonin in mitochondrial metabolism. To study the effects of melatonin on this organelle we used ruthenium red to induce mitochondrial damage and oxidative stress. The results show that melatonin (10 mg/kg i.p.) can increase the activity of the mitochondrial respiratory complexes I and IV after its administration in vivo in a time-dependent manner; these changes correlate well with the half-life of the indole in plasma. Melatonin administration also prevented the decrease in the activity of complexes I and IV due to ruthenium red (60 µg/kg i.p.) administration. At this dose, ruthenium red did not induce lipid peroxidation but it significantly reduced the activity of the antioxidative enzyme glutathione peroxidase, an effect also counteracted by melatonin. These results suggest that melatonin modulates mitochondrial respiratory activity, an effect that may account for some of the protective properties of the indoleamine. The mitochondria-modulating role of melatonin may be of physiological significance since it seems that the indoleamine is concentrated into normal mitochondria. The data also support a pharmacological use of melatonin in drug-induced mitochondrial damage in vivo.

Key words: cytotoxicity – electron transport chain – melatonin – mitochondria – oxidative stress – ruthenium red

Address reprint requests to: Dr D. Acuña Castroviejo, Departamento de Fisiología, Facultad de Medicina, Avda. de Madrid, 11, E-18012 Granada, Spain.
E-mail: dacuna@goliat.ugr.es
Received June 29, 1999; accepted August 26, 1999.

Introduction

It is now accepted that melatonin exerts a cell protective role due to its antioxidant and free radical scavenging properties [Acuña-Castroviejo et al., 1995; Reiter et al., 1997; Reiter, 1998; Reiter et al., 1998]. The indoleamine is a high electrophilic molecule and detoxifies electron-deficient reactive oxygen species by electron donation [Poeggeler et al., 1994]. When melatonin donates an electron it must itself becomes a radical, the indolyl cation radical, which may scavenge one of several other free radicals (Hardeland et al., 1993; Tan et al., 1998). Thus, during its nonenzymatic degradation melatonin minimally scavenges two radicals. This property of the indoleamine makes it an efficient antioxidant [Reiter et al., 1994, 1995], possibly being more potent than other known antioxidants under certain experimental conditions [Tan et al., 1993; Pieri et al., 1994; Marchiafava and Longoni, 1999]. By scavenging the highly toxic hydroxyl radical (\(^\cdot\)OH), melatonin prevents the initiating step in lipid peroxidation [Pieri et al., 1994; Pierrefiche and Laborit, 1995; Sewerynek et al., 1995] and protects DNA and cytosolic proteins against oxidative damage [Sewerynek et al., 1995; Reiter et al., 1998]. Many organs including the brain, gastrointestinal tract and liver are protected from oxidative damage by melatonin [Reiter et al., 1994, 1995, 1998]. The protective role of the pineal indoleamine against oxidative stress in vivo depends not only on its direct antioxidant role but also on its effects on enzymes related to the redox network of the organism. Melatonin stimulates the activity and mRNA expression of glutathione peroxidase and...
increases the availability of glutathione [Pablos et al., 1995]. The hormone reduces the activity of both constitutive and inducible nitric oxide synthase [Betahhi et al., 1998; León et al., 1998; Crespo et al., 1999], the enzymes involved in the production of the potentially toxic nitric oxide.

The inorganic polycationic complex, ruthenium red, is a potent noncompetitive inhibitor of the mitochondrial Ca\(^{2+}\) uniport uptake system and it has acquired many applications in the study of oxidative stress. Ca\(^{2+}\) is important for the activation of the mitochondrial enzymes pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, and NAD-linked isocitrate dehydrogenase. Thus, ruthenium red inactivates these Ca\(^{2+}\)-dependent dehydrogenases impairing both the electron transport chain and ATP synthesis [Johnston and Brand, 1987; McCormack and Denton, 1994]. Below micromolar range, ruthenium red catalyzes the degradation of H\(_2\)O\(_2\) to H\(_2\)O and the superoxide anion radical which may account, at least partially, for its antioxidant properties including inhibition of lipid peroxidation [Bernardi, 1992; Meinicke et al., 1998]. At micromolar concentrations ruthenium red is a potent cytotoxin acting as a Fenton-type reagent by substituting for Fe\(^{2+}\) in the process of degradation of deoxyribose by H\(_2\)O\(_2\) and generating methyl radicals by the redox cycling process involving Ru\(^{III}/IV\) interconversion in the presence of ascorbate or succinate [Meinicke et al., 1998]. These data suggest that ruthenium red may cause mitochondrial uncoupling and cellular oxidative stress with important toxic consequences to the cell [Panov and Scarpa, 1986; Beal, 1992].

Recent published data suggest that some of the cell protective effects of melatonin may be produced through its action on mitochondria. Thus, we used an in vivo model of cytotoxicity, i.e., the administration of ruthenium red to rats, to study the ability of melatonin to counteract the toxic effects of ruthenium red including oxidative stress and mitochondrial function impairment.

**Materials and methods**

**Reagents**

All reagents used were of the highest purity available. Sucrose, melatonin, ruthenium red, 3-(N-morpholino)propanesulfonic acid (MOPS), ethylenediaminetetraacetic acid (EDTA), EDTA-Na\(_2\), β-nicotinamide adenine dinucleotide reduced form (β-NADH), antimicine A, succinic acid, bovine serum albumin (BSA), cytochrome c, sodium borohydride, reduced glutathione (GSH), glutathione reductase from baker yeast, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), t-butyl hydroperoxide, tris(hydroxymethyl)aminomethane (Tris), and the chlorides and phosphates of various metals were purchased from Sigma Química (Spain). Sodium azide and potassium ferrocyanide were obtained from Merck (Spain). The Bioxytech LPO-586 Kit (Cayman Chemical, Ann Arbor, MI), was used for measuring the products of lipid peroxidation.

**Animals and drugs dose**

Male Wistar rats weighing 200–230 g were housed 3–4 per clear plastic cage in a light (14:10 hr, light:dark cycle) and temperature (22 ± 2°C) controlled room. Animals received tap water and standard pellet diet ad libitum and were killed between 11:00 and 12:00 hr.

Melatonin (10 mg/kg b.w., dissolved in 2.5% ethanol/saline) was intraperitoneally (i.p.) injected in a total volume of 200 μL. Ruthenium red (60 μg/kg b.w., dissolved in saline) was injected i.p. in a total volume of 200 μL. In previous experiments we did not find differences in the measured parameters after injecting animals with vehicle solutions of each drug tested, i.e., 200 μL of 2.5% ethanol/saline as a vehicle for melatonin or 200 μL saline as a vehicle for ruthenium red. Thus, as controls, we used animals injected i.p. with 200 μL saline. Ruthenium red was injected 10 min after melatonin administration. The injections were done between 09:00 and 10:00 hr.

Time-dependent effects of melatonin were done in two groups of animals (20 rats per group): (a) control group, receiving 200 μL vehicle, and (b) melatonin group injected with 200 μL melatonin. At 0, 30, 60, 120, and 180 min after melatonin or vehicle administration, animals were killed by cervical dislocation in groups of four rats per time point.

Time-dependent effects of melatonin were done in two groups of animals (20 rats per group): (a) control group, receiving 200 μL vehicle, and (b) melatonin group injected with 200 μL melatonin. At 0, 30, 60, 120, and 180 min after melatonin or vehicle administration, animals were killed by cervical dislocation in groups of four rats per time point.

To study the effects of melatonin and ruthenium red, animals were divided into four groups (eight animals per group): (1) melatonin group, injected with 200 μL melatonin plus 200 μL vehicle; (2) ruthenium red group, injected with 200 μL vehicle plus 200 μL ruthenium red; (3) melatonin + ruthenium red group, injected with 200 μL melatonin plus 200 μL ruthenium red; (4) control group, injected with 400 (200 + 200) μL vehicle. Animals were sacrificed by cervical dislocation 2 hr after injections and liver and brain tissues were immediately processed.
Mitochondria preparation

Mitochondria from rat brain and liver were isolated as described elsewhere [Hogeboom, 1955] with some modifications. Rats were sacrificed by cervical dislocation and both the brain and liver were quickly removed, placed on dry ice and processed immediately. All procedures were carried out at 0–4°C. Forebrain and liver were weighed, diced, diluted in 8 volumes of 250 mM sucrose, and homogenized with a Teflon pestle (Stuart Scientific, mod. SS2). After two centrifugations at 800g for 10 min, the supernatant was centrifuged at 8,500g for 10 min. The pellet was resuspended in 4 volumes of 250 mM sucrose, homogenized with a Polytron and centrifuged at 8,500g for 10 min. This step was repeated twice to obtain the mitochondrial pellet which was frozen to −80°C. Submitochondrial particles were prepared by freezing and thawing (twice) the mitochondrial pellets suspended in 250 mM sucrose and homogenized in a Polytron. Mitochondrial protein concentration was measured using BSA as standard [Lowry et al., 1951]. Electron microscopic studies were done to assess the purity and the breaking of the outer mitochondrial membrane. The procedure allowed for the separation of particulate subfraction of mitochondria containing the bulk of the respiratory chain components.

Measurements of complex I, II-III, and IV activity

Pellets containing submitochondrial fractions were thawed, suspended in 5 mL of the incubation medium corresponding to the complex to be measured, and homogenized in a Polytron. To determine complex I activity (NADH-CoQ oxidoreductase), submitochondrial particles (0.2–0.4 mg protein/mL) were incubated in 4 mL (2 min at 25°C) of a medium consisting of 120 mM KCl, 25 mM potassium-MOPS, 5 mM MgCl₂, 5 mM potassium-phosphate, and 0.5 mM EDTA-Tris, pH 7.2. The activity of the enzyme was spectrophotometrically measured in the supernatant (25 μL) using t-butylhydroperoxide as substrate [Jaskot et al., 1983]. The level of lipid peroxidation was determined in liver tissue as follows: soon after animals were killed, the thoraco-abdominal cavities were opened and rat tissues were perfused with ice-cold (4°C) saline solution passing through the heart to remove any iron excess that could be released from intracellular storage sites. Liver was removed and placed on dry ice. Liver (100 mg) was homogenized with a Stuart Scientific stirrer in ice-cold 20 mM Tris-ClH buffer, pH 7.4, to produce a 1/10 homogenate. The crude homogenate was centrifuged at 2,500g for 30 min at 4°C. Aliquots of the supernatant were either stored at −20°C for total protein determination [Lowry et al., 1951] or used to calculate level of lipid peroxidation (Bioxytech LPO-586 kit).

Statistical analyses

Data are expressed as means ± S.E.M. A one-way ANOVA analysis was done for effects of melatonin and ruthenium red doses over the totality of the studied parameters. Duncan’s test was used to assess the significance between groups.
Results

Fig. 1 shows the time-dependent effect of in vivo melatonin administration on the activity of complexes I (A) and IV (B) in mitochondria prepared from brain and liver tissues. The maximal response elicited by melatonin was obtained at 30 and 60 min in liver and brain mitochondria, respectively. The activity of complex I returned to control values after 120 and 180 min of melatonin administration in brain and liver mitochondria, respectively. Fig. 1B shows the activity of complex IV, which reached maximal values between 30 and 120 min before returning to control values after 120–180 min, after melatonin treatment in brain and liver mitochondria, respectively. These results demonstrate that in vivo melatonin administration is able to increase the activity of the respiratory chain complexes I and IV in both rat liver and brain, the effect being more potent in the former. There were no changes in the activity of these complexes in animals treated with vehicle. Also, the activity of the complexes II–III did not change after melatonin administration (data not shown).

Fig. 2 shows the inhibitory effect of ruthenium red administration on the activity of the respiratory chain complexes I (A) and IV (B) in liver and brain mitochondria. Melatonin administration was able to counteract the effects of ruthenium red, restoring the activity of complex I to control values in both tissues. Fig. 2A also shows the increasing effect of melatonin on complex I activity in liver mitochondria of control rats. However, in brain mitochondria the effect of melatonin on complex I activity of control animals did not occur (Fig. 2A). This was probably because the animals were killed 2 hr after melatonin injection and, at this time, the effect of melatonin on brain complex I activity had disappeared (Fig. 1A). Fig. 2B shows the inhibitory effect of ruthenium red administration on the activity of the respiratory chain complex IV in liver and brain mitochondria. Melatonin administration was also able to counteract the effects of ruthenium red, restoring the activity of complex IV to control values in both tissues. Fig. 2B also shows the lack of effect of melatonin on complex IV activity in brain mitochondria of control rats, probably due to the time at which the animals were killed, i.e., 2 hr after melatonin injection, when the effect of melatonin on brain complex IV activity had disappeared (Fig. 1B).

Melatonin was able to partially counteract the inhibitory effect of ruthenium red on GPx activity in rat liver (Fig. 3A). Fig. 3B shows that either melatonin or ruthenium red administration significantly decreases hepatic lipid peroxidation to a similar extent, whereas the injection of melatonin plus ruthenium red does not further decrease the degree of peroxidized lipid products in the liver.
Discussion

By studying the protective role of melatonin against ruthenium red cytotoxicity, we found unexpectedly that the indoleamine increased the activity of mitochondrial respiratory complexes I and IV. The results also show that this effect is produced soon after i.p. injection of melatonin and that the activity of mitochondrial complexes returned to control value 120–180 min after melatonin administration. Since the half-life of plasma melatonin is roughly 40 min [Gibbs, 1985], the time-dependent effects of melatonin on mitochondrial activity correlates well with the decay in plasma melatonin concentrations. Interestingly, the activities of complexes II and III were not affected by melatonin at any measured time. Our results also show a different patterns of activation of complexes I and IV in brain compared with those in the liver. Melatonin administration produced a maximal effect at 30 min in liver and at 60 min in brain mitochondria. Also, liver mitochondrial complexes I and IV are activated for a longer interval than those in the brain, which may depend on the route of melatonin administration. After its i.p. administration, the indoleamine quickly reaches the liver where it is in part metabolized to 6-hydroxymelatonin, an active metabolite on mitochondria [Pierrefiche et al., 1993; Praast et al., 1995]. Thus, the effects of melatonin plus 6-hydroxymelatonin may explain the longer duration of the melatonin effect in liver compared with that in brain mitochondria.

The administration of ruthenium red in vivo is a reliable model to study oxidative stress and the efficiency of antioxidant drugs, since it exerts toxic effects throughout the cell. Ruthenium red toxicity depends on its behavior as a non-specific blocker of ion channels and transporters in the plasma membranes [Meinicke et al., 1998], and on its ability to inhibit mitochondrial Ca\(^{2+}\) transport [Gunter and Pfeiffer, 1990]. For this reason we measured GPx activity and lipid peroxidation, since both are good markers of cellular oxidative damage [Reiter et al., 1994; Pablos et al., 1995; Sewerynek et al., 1995] and we also measured the activities of mitochondrial electron chain complexes as indexes of ruthenium red cytotoxicity.

Although ruthenium red may increase oxidative stress, our results showing a decrease in lipid peroxidation products agree with those reported elsewhere [Bernardi, 1992; Meinicke et al., 1998]. At low concentrations ruthenium red behaves as an antioxidant inducing a rapid H\(_2\)O\(_2\) consumption thereby reducing the production of reactive oxygen species [Meinicke et al., 1998]. However, at micromolar concentrations, ruthenium red behaves as a prooxidant acting as a Fenton-type reagent [Meinicke et al., 1998]. The prooxidant effects of ruthenium red include a decrease in reduced adenine nucleotides, which are needed as cofactors for glutathione reductase and other enzymes. The resulting lack in reduced glutathione decreases GPx activity leading to an increased generation of free radicals [Pierrefiche and Laborit, 1995]. These data presumably explain the reduction in GPx activity found after ruthenium red treatment in the present experiments. Melatonin partially restored GPx activity which was inhibited by ruthenium red and decreased basal levels of lipid peroxidation. These observations support the well-known antioxidant role of the indoleamine [Reiter et al., 1995, 1997; Reiter, 1998; Reiter et al., 1998].

Ruthenium red administration also reduced the activity of mitochondrial complexes I and IV 120 min after its i.p. administration to rats. The data agree with the proposed uncoupling role of the drug [Panov and Scarpa, 1986] due to the inhibition of Ca\(^{2+}\)-dependent mitochondrial matrix dehydrogenases [Denton and McCormack, 1985; Johnston and Brand, 1987; Hamsford, 1991]. The mitochondrial production of free radicals due to ruthenium red also contributes to respiratory inhibition [Schapira and Cooper,
1992; Sohal and Brunk, 1992). It has been suggested that the ability of ruthenium red to inhibit the peroxidation of lipids and its ability to reduce the glutathione antioxidant enzyme system, are independent actions of the drug [Meinicke et al., 1998]. The former depends on the ability of ruthenium red to block membrane sites for calcium and the subsequent inhibition of Ca\(^{2+}\)-dependent lipid peroxidation. The other effects are related to the mitochondrial Ca\(^{2+}\) uptake blockade by the drug.

Cyanide-induced seizures and lipid peroxidation in mice, effects related to the mitochondrial toxicity of cyanide, were found to be prevented by melatonin [Yamamoto and Tang, 1996]. Melatonin protects against peroxynitrite-induced suppression of mitochondrial respiration in cultured cells [Gilad et al., 1987]. In experimental Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydroxyphenylpyridine, a drug that decreases mitochondrial complex I activity, melatonin was also shown to be neuroprotective [Acuña-Castroviejo et al., 1997]. In our hands, ruthenium red-dependent mitochondrial toxicity was prevented by melatonin. These findings, along with the role of the indoleamine on normal mitochondria as shown here, provide a heretofore unobserved function for melatonin in maintaining mitochondrial homeostasis. Further studies on the mitochondrial effects of melatonin during neurodegenerative processes including aging, which involves mitochondrial overproduction of O\(_2^−\) and H\(_2\)O\(_2\) and subsequent respiratory chain dysfunction [Miquel and Fleming, 1984; Schapira and Cooper, 1992; Sohal and Brunk, 1992; Sen and Packer, 1996] may lead to a better understanding of the functional roles of melatonin.

The results of this study show that melatonin acts to modulate mitochondrial metabolism by stimulating the activity of complexes I and IV of the mitochondrial respiratory chain. Additionally, melatonin is able to prevent mitochondrial toxicity induced by ruthenium red. These effects were found after administration of a pharmacological dose of melatonin, i.e., 10 mg/kg b.w., and thus, a pharmacological use of the indoleamine on drug-induced cytotoxicity in vivo is suggested. Moreover, melatonin easily enters cells and it is concentrated in some subcellular compartments such as nucleus [Menendez-Pelaez and Reiter, 1993] and mitochondria (unpublished observations). Thus, the data also point toward a physiological mechanism explaining the protective role of melatonin described in different models of cytotoxicity.

Acknowledgments

This work was supported by grant SAF98-0156 (CICYT) and the Consejería de Educación, Junta de Andalucía through Grupo de InvestigaciónCTS-101. MM and GE are recipients of postdoctoral fellowships from the Universidad de Granada and FIS (Ministerio de Sanidad y Consumo), respectively.

Literature cited


LOWRY, O.H., N.J. ROSENBRUGH, A.L. FARR, R.J. RAN


