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Effect of melatonin treatment on oxygen consumption by rat liver mitochondria

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Summary. The objective of this study was to examine the *in vivo* effect of melatonin on rat mitochondrial liver respiration. Two experiments were performed: For experiment 1, adult male rats received melatonin in the drinking water (16 or 50 μg/ml) or vehicle during 45 days. For experiment 2, rats received melatonin in the drinking water (50 μg/ml) for 45 days, or the same amount for 30 days followed by a 15 day-withdrawal period. At sacrifice, a liver mitochondrial fraction was prepared and oxygen consumption was measured polarographically in the presence of excess concentration of DL-3-β-hydroxybutyrate or L-succinate. Melatonin treatment decreased Krebs' cycle substrate-induced respiration significantly at both examined doses. The stimulation of mitochondrial respiration caused by excess concentration of substrate recovered after melatonin withdrawal. Basal state 4 respiration was not modified by melatonin. Melatonin, by curtailing overstimulation of cellular respiration caused by excess Krebs' cycle substrates, can protect the mitochondria from oxidative damage.

Keywords: Melatonin – Oxygen consumption – Free radicals – Liver mitochondria – β-hydroxybutyrate – Succinate

Introduction

The synthesis of ATP via the mitochondrial respiratory chain is the result of a proton potential generated by the electron transport chain (for review see Leon et al., 2005). Although ideally all the oxygen should be reduced to water via a 4-electron reduction reaction driven by complex IV, under normal conditions a certain, but relevant percentage of oxygen (depending on metabolic situation, 2–5%) can be reduced by 1–3 electrons only, yielding reactive oxygen species (ROS). This electron leakage can be increased by stimulating respiration, e.g., by adding supranormal quantities of Krebs' cycle substrates to mitochondrial preparations (Cadenas, 1989; Liochev and Fridovich, 2002). Oxidative stress may play an important role in the etiology and pathophysiology of several pathological conditions, such as inflammation, aging, diabetes, atherosclerosis and carcinogenesis among others.

There is considerable information on the effectiveness of melatonin to preserve mitochondrial homeostasis by reducing free radical generation and by safeguarding mitochondrial proton potential and ATP synthesis via stimulation of complex I and IV activities (Leon et al., 2005). Furthermore, melatonin is able to protect macromolecules in all parts of the cell from oxidative damage and especially in cell membranes due to its high lipid solubility and modest aqueous solubility, making them more resistant to the oxidative attack.

In a previous study we demonstrated that *in vitro* added melatonin was effective to prevent the increase in respiration caused by addition of excess amounts of Krebs' cycle substrates to rat liver mitochondrial preparations (Reyes Toso et al., 2003). The present study was carried out to verify if such an effect of melatonin is also seen *in vivo* after the administration of melatonin to rats in the drinking water for 45 days and to assess to what extent or whether such an effect is reversible after melatonin withdrawal.

Materials and methods

Animals and experimental design

Adult male Wistar rats (300–350 g) were kept under 12 h light/12 h dark photoperiods and controlled temperature (20–22 °C) and were given tap water and a commercial rat chow at libitum. The experiments were performed in accordance with the NIH guide for the Care and Use of Laboratory Animals. Melatonin (Elisium Lab., Buenos Aires, Argentina) dissolved in ethanol was added to the drinking water at a concentration

of 16 μg/ml or 50 μg/ml; the final ethanol concentration was 0.01% for both melatonin-treated and control rats. The water bottles were covered with aluminum foil and fresh solutions were prepared once weekly. Previously to the beginning of the experiments melatonin stability in water bottles was assayed during a period of one month (taken samples each week) by spectrophotometry. No significant degradation of melatonin was found in the period examined. Rats drank about 20 ml/day with 90–95% of this total daily water taken up during the dark period, nocturnal water consumption being similar in all the experimental groups. Circulating melatonin levels of about 2–6 ng/ml were obtained (Cardinali et al., 2004); these doses are clearly pharmacological in terms of circulating melatonin levels but not necessarily for some other fluids or tissues (Reiter and Tan, 2003).

Two experiments were performed. For experiment 1, rats were divided into 3 groups (n = 7–9/group) as follows: (1) control; (2) melatonin, low dose (16 μg/ml drinking water); melatonin, high dose (50 μg/ml of drinking water). After 45 days rats were killed and the liver was dissected out.

For experiment 2, rats were divided into 3 groups (n = 6–8/group) as follows: (1) control; (2) melatonin, 50 μg/ml drinking water for 45 days; (3) melatonin, 50 μg/ml drinking water for 30 days followed by a 15 day-withdrawal period. After 45 days rats were killed and the liver was dissected.

Oxygen consumption by liver mitochondria

Liver mitochondria was isolated by differential centrifugation as described in a previous report (Brignone et al., 1996). Liver samples (mean wt 4 g) were placed in an ice-cold medium containing 0.33 M sucrose plus 0.25 mM Tris – 0.25 mM EDTA buffer, pH 7.4 (9 ml/g of tissue) for the homogenization procedure and first centrifugation (750 *g* for 10 min). A 0.33 M sucrose plus 0.25 mM Tris buffer, pH 7.4 (2 ml/g of tissue) was used for the second and third high-speed centrifugations (7 000 *g*, 10 min). All centrifugations were performed at 4 °C. The final pellet of mitochondria was resuspended in the last buffer employed.

Oxygen consumption was measured polarographically in a Wilson Medical Electronics oxygraph at 30 °C. The reaction mixture (0.9 ml) contained 0.24 M sucrose, 34 mM KCl, 5 nM $MgCl_2$, 2 nM EDTA, 9 nM Tris-HCl, 6 mM KH_2PO_4-K_2HPO_4 and 1.4 mg of mitochondrial protein, pH 7.4 (10, 11). DL-3-β-hydroxybutyrate or L-succinate (5 mM) was used as substrate. Respiratory velocities at rest (state 4) and during rapid respiration (state 3) with available oxygen and high levels of substrate added to the reaction chamber of the oxygraph at the moment of the measurement were expressed as nanograms of oxygen atoms per min per mg of mitochondrial protein.

Statistical analysis

The statistical analysis of results was performed by using a one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls' test. Comparisons were considered significant at the 0.05 level.

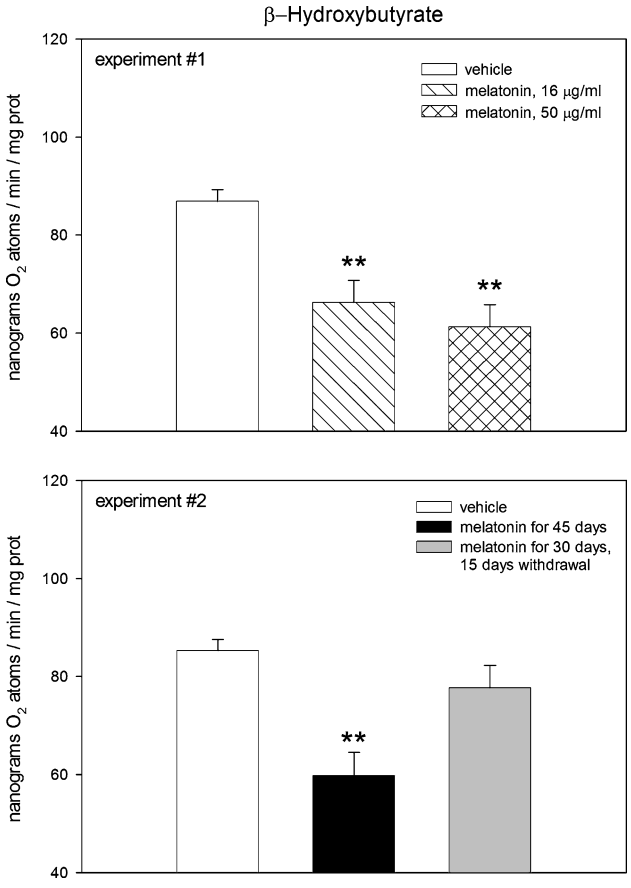

Fig. 1. Effect of melatonin treatment on oxygen consumption stimulation induced by excess (5 mM) β-hydroxybutyrate in rat liver mitochondria. Upper panel: Rats received melatonin in the drinking water (16 or 50 μg/ml) on vehicle during 45 days. Lower panel: Rats received melatonin in the drinking water (50 μg/ml) for 45 days, or the same amount for 30 days followed by a 15 day-withdrawal period. At sacrifice, a liver mitochondrial fraction was prepared and oxygen consumption was measured polarographically in the presence of β-hydroxybutyrate as described in Materials and methods. The means + SEM are shown (n = 7–9/group for experiment 1; 6–8/group for experiment 2). Asterisks designate significant differences as compared to the other two groups; $p < 0.01$, ANOVA, Student-Newman-Keuls' test

Results and discussion

Figures 1 and 2 summarize the results obtained. After 45 days of treatment with melatonin at any the two doses examined, the Krebs' cycle substrate-induced respiration decreased significantly (Figs. 1 and 2, upper panel). The capacity of β-hydroxybutyrate or succinate to augment mitochondrial respiration was restored after melatonin withdrawal (Figs. 1 and 2, lower panel). Basal state 4 respiration was not modified by melatonin (results not shown). Consequently, the respiratory control index followed closely the changes in state 3 mitochondrial respiration, with significant increases at melatonin concentrations 100 nM or higher.

When supranormal quantities of Krebs' cycle substrates are added to mitochondrial preparations the fraction of O_2 consumption attributed to free radical production increases, with the increased feasibility of oxidative damage of mitochondria (Cadenas, 1989; Liochev and Fridovich, 2002). Previous data indicated that melatonin is effective after acute adding *in vitro*, to decrease oxygen consumption by liver mitochondria under those conditions (Reyes Toso et al., 2003). The foregoing studies *in vivo* further

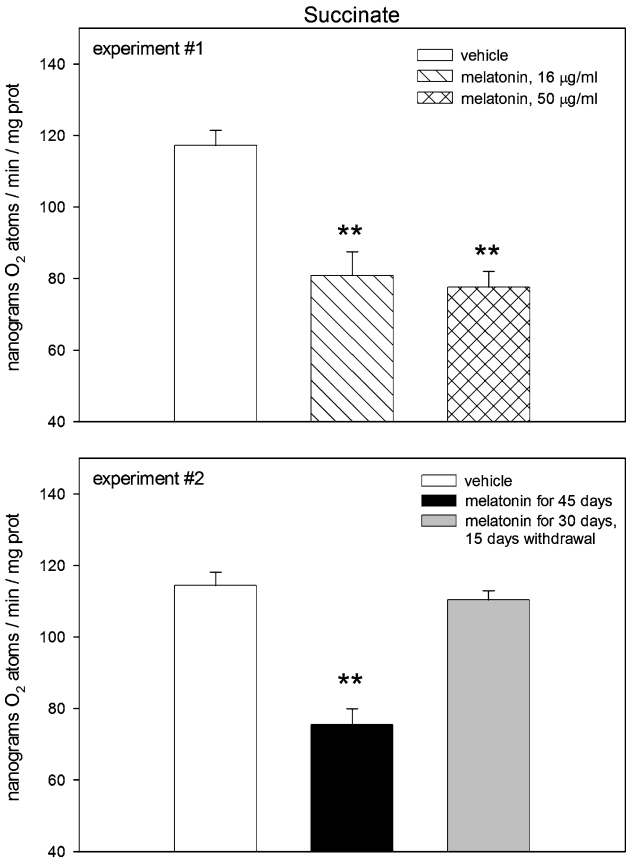

Fig. 2. Effect of melatonin treatment on oxygen consumption stimulation induced by excess (5 mM) succinate in rat liver mitochondria. For experimental details see legend to Fig. 1. Shown are the means + SEM (n = 7–9/group for experiment 1; 6–8/group for experiment 2). Asterisks designate significant differences as compared to the other two groups; $p < 0.01$, ANOVA, Student-Newman-Keuls' test

support the effect of melatonin and indicate that the activity of melatonin is reversible, fading after a withdrawal period of 15 days.

Melatonin has remarkable antioxidant properties (Reiter et al., 2005). It scavenges hydroxyl, carbonate and various organic radicals, peroxynitrite and other reactive nitrogen species. Melatonin also enhances the antioxidant potential of the cell by stimulating the synthesis of antioxidant enzymes like superoxide dismutase, glutathione peroxidase and glutathione reductase, and by augmenting glutathione levels. Moreover, the antioxidative efficiency of melatonin increased, due to the fact that the metabolites formed by free radical scavenging also act as free radical scavengers (e.g. cyclic 3-hydroxymelatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine and, with highest potency, N^1-acetyl-5-methoxykynuramine (Ressmeyer et al., 2003; Reiter et al., 2005). Thus, the interaction of melatonin with free radicals initiates an antioxidant cascade.

A long-term effect on mitochondrial function *in vivo* may be explained in different ways, either in terms of interactions with the electron flux or by induction of antioxidant enzymes which prevent damage to the respiratory chain. Moreover, changes in gene expression of components of complexes I and IV were observed and may also contribute to keep cellular respiration normal. It is important to stress that melatonin added *in vitro* did not affect basal mitochondrial respiration (Leon et al., 2005).

Mitochondria are not only the primary site of ROS generation but also the primary target of attack for them. Damage to the mitochondrial respiratory chain can cause breakdown of the proton potential, apoptosis or lead to further generation of free radicals maintaining a vicious cycle, which also may ultimately end up in cell death, either of the necrotic or apoptotic type (Leon et al., 2005). It is interesting to note that findings of several investigators point out that melatonin's neuroprotective role in Alzheimer's disease and Parkinsonism may be due to the direct and antioxidant role of melatonin in mitochondrial homeostasis (see Srinivasan et al., 2005).

Studies using a senescence-accelerated mouse strain investigated the effects of chronic administration of physiological doses of melatonin (2 μg/ml drinking water) on mitochondrial function (Okatani et al., 2002a, b; 2003). The studies demonstrated that the activities of complex I and IV in liver mitochondria from those mice show an age-related reduction causing an excessive free-radical generation combined with a less effective defense of mitochondria against oxidative stress (Okatani et al., 2002a). Since the reduced complex I activity is associated with a sign of enhanced electron leakage, melatonin's action in restoring complex I activity back to a normal level assumes significance in prevention of age associated degenerative changes.

Summarizing, the present results indicate that the increase in oxygen consumption, and consequently the high amounts of free radical production, seen in rat mitochondria after adding excess concentration of Krebs' cycle substrates is effectively curtailed by melatonin treatment. Collectively, previous and present results indicate melatonin efficacy to reduce oxygen radical-induced damage in the mitochondrial compartment.

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