ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 9 & 10, 2005

Forum Original Research Communication

Protective Effect of Melatonin on Rotenone plus Ca^{2+}-Induced Mitochondrial Oxidative Stress and PC12 Cell Death

SOLANGE C. SOUSA and ROGER F. CASTILHO

ABSTRACT

Chronic systemic inhibition of mitochondrial respiratory chain complex I by rotenone causes nigrostriatal dopaminergic degeneration in rats, producing an *in vivo* experimental model of Parkinson's disease. We recently showed that micromolar Ca^{2+} concentrations strongly stimulate the release of reactive oxygen species in rotenone-treated isolated rat brain mitochondria. In the present work, we show that the natural antioxidant melatonin inhibits Ca^{2+} plus rotenone-induced oxidative stress in isolated rat brain mitochondria. In addition, the Ca^{2+} ionophore A23187 strongly potentiates rotenone-induced death of intact cultured pheochromocytoma (PC12) cells, in a mechanism sensitive to melatonin. Moreover, melatonin inhibits the detection of reactive oxygen species release in PC12 cells treated with rotenone plus A23187. Melatonin does not alter free Ca^{2+} concentrations or the inhibitory effect of rotenone on mitochondrial complex I. We conclude that micromolar Ca^{2+} concentrations stimulate neuronal cell death induced by mitochondrial complex I inhibition in a mechanism involving oxidative stress, preventable by the antioxidant melatonin. *Antioxid. Redox Signal.* 7, 1110–1116.

INTRODUCTION

MANY NEUROLOGICAL DISORDERS have been associated with mitochondrial oxidative stress (for reviews, see 7, 17, 31), a situation that can be the outcome of either a failure of mitochondrial antioxidant systems or an increased generation of reactive oxygen species (ROS). The main sites of mitochondrial ROS generation have been identified as electron transport chain components complex I (NADH:ubiquinone oxidoreductase) and complex III (ubiquinol:cytochrome *c* oxidoreductase) (2, 12, 21, 24, 45, 46). The rate of electron transport through these complexes, which is largely determined by the mitochondrial inner membrane potential, plays an important role in controlling mitochondrial ROS production (2, 21, 24, 41, 45, 46).

Recent reports by Greenamyre's group (9, 39) and others (2, 48; but see 22) demonstrated that chronic and systemic treatment of rats with rotenone, a classical respiratory chain complex I inhibitor (18), leads to anatomical, neurochemical, behavioral, and neuropathological features of Parkinson's disease. The anatomical findings are characterized by fibrillar cytoplasmic inclusions in nigral neurons and selective nigrostriatal dopaminergic degeneration (9). Also, Parkinson's disease patients typically present partial inhibition (20–40%) of respiratory chain complex I activity (26, 36), further supporting the idea that chronic treatment of rodents with rotenone provides an interesting and relevant experimental model to study neuropathological events in this disorder. Mitochondrial ROS production secondary to a partial inhibition of complex I is believed to have a major role in rotenone-induced nigrostriatal dopaminergic degeneration (9, 38). In fact, rotenone increases brain mitochondrial ROS generation at complex I when respiration is supported by NADH-linked substrates (6, 21, 24, 40, 46), although it decreases succinate oxidation-stimulated ROS generation by blocking reverse electron transfer to complex I from complex II through ubiquinone (44).

We (40) and others (42) have shown that micromolar Ca^{2+} concentrations increase rotenone-induced mitochondrial oxidative stress. This finding may be relevant to situations of excitotoxicity in neurons with a partial inhibition of complex I. Under this situation, elevated Ca^{2+} influx to the cytosol will enhance mitochondrial ROS release and may result in cellular injury. Considering

Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, Brazil.

this perspective, the present report examines the effect of Ca^{2+} plus rotenone on pheochromocytoma (PC12) cell redox state and viability. In addition, the effect of melatonin, a natural compound with antioxidant properties, is tested on rotenone plus Ca^{2+}-induced mitochondrial oxidative stress and PC12 cell death.

MATERIALS AND METHODS

Isolation of rat forebrain mitochondria

Forebrain mitochondria were isolated, as described by Rosenthal *et al.* (34) with minor modifications, from female Wistar rats weighing 200–250 g. Rats were killed by decapitation, and their brains rapidly removed (within 1 min) and put into 10 ml of ice-cold "isolation buffer" containing 225 m*M* mannitol, 75 m*M* sucrose, 1 m*M* K^+-EGTA, 0.1% bovine serum albumin (free fatty acid), 10 m*M* K^+-HEPES (pH 7.2), and 5 mg of protease. The cerebellum and underlying structures were removed, and the remaining material was used as the forebrain. The tissue was cut into small pieces using surgical scissors and extensively washed. The tissue was then manually homogenized in a Dounce homogenizer using both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2,000 *g* in a Beckman JA 20 rotor. After centrifugation, the supernatant was recentrifuged for 8 min at 12,000 *g*. The pellet was resuspended in 20 ml of isolation buffer containing 80 µl of 10% digitonin and recentrifuged for 8 min at 12,000 *g*. The supernatant was discarded, and the final pellet gently washed and resuspended in isolation buffer devoid of EGTA, at an approximate protein concentration of 30–40 mg/ml. The respiratory control ratio (state 3/state 4 respiratory rate) was over 7.0, measured using pyruvate plus malate as substrates.

Standard incubation medium for isolated mitochondria

The experiments with isolated mitochondria were carried out at 30°C, with continuous magnetic stirring, in a standard reaction medium containing 100 m*M* sucrose, 65 m*M* KCl, 10 m*M* K^+-HEPES buffer (pH 7.2), 50 µ*M* EGTA, 1 m*M* P_i, 2 m*M* Mg^{2+}, 5 m*M* malate, 10 m*M* pyruvate, 200 µ*M* ATP, and 1 µg/ml oligomycin. Other additions are indicated in the figure legends. The results shown are representative of a series of at least three experiments, using different mitochondrial preparations. The results were reproduced within 10% of variation.

Oxygen uptake measurements

Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) in 1.3 ml of standard reaction medium, in a sealed glass cuvette equipped with a magnetic stirrer.

Determination of free Ca^{2+} concentrations in the reaction medium

Variations in the concentration of free Ca^{2+} concentrations were followed by measuring the changes in the absorbance spectrum of arsenazo III (40 µ*M*), using an SLM Aminco DW2000 spectrophotometer (SLM Instruments, Inc., Urbana, IL, U.S.A.) set at the 675–685-nm wavelength pair (35).

PC12 cell cultures

Cultured PC12 cells (37) were continuously maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose) supplemented with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Because PC12 cells present doubling times of 48–72 h, cells were passaged each 3 days and plated in 75-cm^2 tissue culture flasks at an initial density of 125,000 cells/cm^2. For experiments, PC12 cells were plated on 35-mm culture dishes at an initial density of 125,000 cells/cm^2, and the experimental incubations were conducted after 18–24 h. The experiments to evaluate PC12 cell death and ROS production were conducted in DMEM (4.5 g/L glucose) at 37°C and 5% CO_2, without horse serum, fetal bovine serum, antibiotics, or phenol red.

Measurements of cytoplasmic free Ca^{2+} concentration

PC12 cells plated on 35-mm culture dishes were loaded with 5 µ*M* Fluo-3 acetoxymethyl ester for 50 min in DMEM containing 0.1% bovine serum albumin, and 5 µ*M* pluronic acid at 37ºC and 5% CO_2, without horse serum, fetal bovine serum, antibiotics, or phenol red. PC12 cells were washed twice and resuspended in 2 ml of DMEM containing 20 m*M* Na^+-HEPES buffer (pH 7.2), without phenol red. Intracellular Ca^{2+} measurements were conducted in an F-4500 Hitachi spectrofluorometer (Hitachi Ltd., Tokyo, Japan) equipped with a magnetic stirrer, at excitation and emission wavelengths of 488 nm and 526 nm, respectively (30). Calibration was performed by sequential addition of 0.1% digitonin and 10 m*M* EGTA. The results shown are representative of a series of three independent experiments.

Estimation of ROS release

The production of ROS [hydrogen peroxide (H_2O_2)] by mitochondrial suspensions and PC12 cell cultures was determined spectrofluorometrically in an F-4500 Hitachi spectrofluorometer, using 0.025 µ*M* horseradish peroxidase (HRP) plus 30 µ*M* Amplex Red at 563 nm for excitation and 587 nm for emission (47). Prior to ROS determination, PC12 cell cultures were incubated for 1 h in the presence of rotenone, A23187, and/or melatonin, as described in the figure legend. Subsequently, Amplex Red and HRP were added to the incubation medium, and the fluorescence of the supernatant was determined after 20 min. The rate of H_2O_2 release detected under control conditions in mitochondrial suspensions and PC12 cultured cells was 245 ± 26 and 4.79 ± 0.42 pmol of H_2O_2/min/mg, respectively.

Alternatively, mitochondrial ROS release was measured using the membrane-permeable fluorescent dye dichlorodihydrofluorescein diacetate (H_2-DCFDA; 1 µ*M*) (25, 40). The fluorescence of dichlorofluorescein (DCF), the product of H_2-DCF oxidation, was determined at 488 nm for excitation and 525 nm for emission.

Estimation of PC12 cell death

Lactate dehydrogenase (LDH), which is release from dying cells with permeabilized membranes into the medium, was de-

termined using a commercially available kit (Doles, Goiana, GO, Brazil), based on LDH-catalyzed formation of pyruvate and NADH from lactate and NAD^+. Using this kit, the amount of color formed (at 510 nm) in the assay is proportional to the number of lysed cells. Each experiment was repeated three times, and data are expressed as percentage of the LDH release detected in control samples lysed with 1% Triton X-100.

Chemicals

Most chemicals, including A23187, arsenazo III, ATP, butylated hydroxytoluene, digitonin, ebselen, glutathione, HEPES, HRP, malic acid, melatonin (*N*-acetyl-5-methoxytryptamine), pyruvic acid, oligomycin, protease (P-8038), and rotenone (R-8875, minimum 95%), were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Amplex Red, Fluo-3 acetoxymethyl ester, H_2-DCFDA, and pluronic acid were purchased from Molecular Probes (Eugene, OR, U.S.A.). Rotenone was prepared at a stock concentration of 1 m*M* in 100% ethanol. Melatonin was prepared at a stock concentration of 0.5 *M* in dimethyl sulfoxide (DMSO).

RESULTS

Effect of melatonin on ROS release by isolated rat brain mitochondria

The results in Fig. 1 show that the addition of Ca^{2+} to isolated rat brain mitochondria treated with rotenone increased ROS detection (lines b), when ROS release was measured either using Amplex Red (A) or H_2-DCFDA (B). These results are in line with recent reports (40, 42) indicating a synergistic stimulatory effect of micromolar Ca^{2+} concentrations and respiratory chain complex I inhibition on mitochondrial ROS release. The presence of 50 μ*M* melatonin resulted in a partial inhibition of ROS detection (lines c), suggesting that this compound has an important antioxidant effect under our experimental conditions. This concentration of melatonin did not impair mitochondrial function, as estimated by the respiratory control ratio (data not shown). Melatonin (50 μ*M*) did not inhibit ROS detection observed under control conditions (lines f). It is important to note that DCF fluorescence may increase non-linearly due to self-catalyzed oxidation of H_2-DCFDA.

The experiments in Fig. 2 were conducted to verify if the protective effect of melatonin could be related to free Ca^{2+} chelation from the incubation medium or due to prevention of the inhibitory effect of rotenone on respiratory chain complex I. Measurements of free Ca^{2+} concentrations in the micromolar range were conducted using the metallochromic indicator arsenazo III, and show that melatonin does not change free Ca^{2+} concentrations (Fig. 2A). The results in Fig. 2B show mitochondrial oxygen consumption supported by NADH-linked substrates under phosphorylating conditions (state 3) in the presence of different rotenone concentrations. The presence of 50 μ*M* melatonin did not avoid the inhibition of mitochondrial oxygen consumption promoted by rotenone, suggesting that the melatonin effects observed in Fig. 1 occur downstream of complex I inhibition by rotenone.

Effect of melatonin on PC12 cell death

To test the effect of complex I inhibition and increased cytosolic Ca^{2+} concentrations on cell viability, we used PC12 cells. These rat pheochromocytoma cells are able to produce dopamine when differentiated in culture (37). To increase cytosolic Ca^{2+}, PC12 cells were treated with the Ca^{2+} ionophore A23187, which promotes $Ca^{2+}/2H^+$ exchange through membranes (32). Using Fluo-3-loaded PC12 cells in suspension, we verified that A23187 promoted a fast increase in cytosolic Ca^{2+} concentrations, with saturation of the fluorescence signal of this dye in less than 2 min (Fig. 3, line a). Thus, free cytosolic Ca^{2+} concentrations, initially at 60–80 n*M*, increased to levels above 1.5 μ*M* in the presence of A23187.

When incubated in the presence of either rotenone or A23187 for 5 h, only a small fraction of PC12 cells died, as estimated by LDH release (Fig. 4). However, rotenone and A23187, when present simultaneously, induced a high level of cell death, indicating a synergistic effect of increased cytosolic Ca^{2+} concentration and complex I inhibition on PC12 cell death. Melatonin shows a dose-dependent inhibitory effect on rotenone plus A23187-induced cell death. Concentrations of

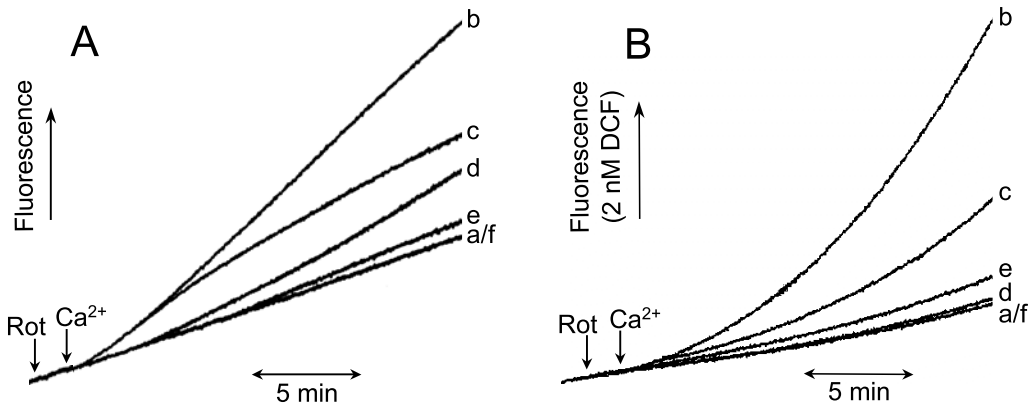

FIG. 1. Melatonin inhibits rotenone plus Ca^{2+}-induced increase in mitochondrial ROS release. (**A**) Isolated forebrain mitochondria (0.5 mg/ml) were incubated in standard reaction medium containing 30 μ*M* Amplex Red plus 0.025 μ*M* HRP. (**B**) Brain mitochondria (0.5 mg/ml) were incubated in standard reaction medium containing 1 μ*M* H_2-DCFDA. Rotenone (50 n*M*; lines b, c, and d) and/or 120 μ*M* Ca^{2+} (lines b, c, and e) were added where indicated by the arrows. Lines c and f represent experiments conducted in the presence of 50 μ*M* melatonin. Lines a and f represent control experiments without the addition of rotenone and Ca^{2+}.

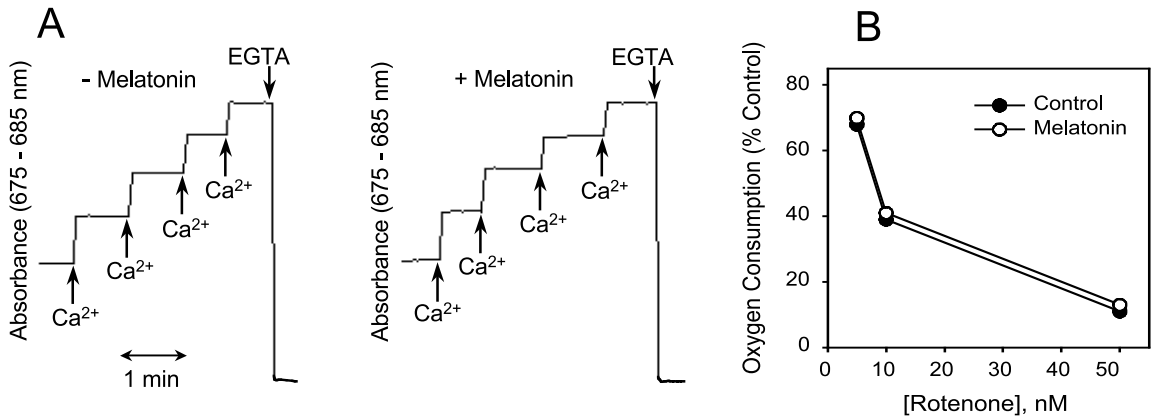

FIG. 2. Melatonin has no influence on free Ca^{2+} concentrations and mitochondrial complex I inhibition promoted by rotenone. (**A**) Free Ca^{2+} concentrations were determined in the absence of brain mitochondria, using arsenazo III, in the absence or presence of 50 μ*M* melatonin, as shown. Ca^{2+} (four serial additions of 10 μ*M*) and 500 μ*M* EGTA were added where indicated by the arrows. (**B**) Brain mitochondria (0.5 mg/ml) were incubated in standard reaction medium containing 1 m*M* ADP, without ATP and oligomycin, in the absence (●) or presence (○) of 50 μ*M* melatonin. Oxygen consumption rates were measured in the presence of different concentrations of rotenone, as indicated on the abscissa.

melatonin higher than 50 μ*M* did not show a further protective effect, and promoted decreases in mitochondrial respiratory control ratios (data not shown). Moreover, the protective effect of melatonin was not influenced by melatonin receptor antagonists prazosin and luzindole (Fig. 4) (19, 20), indicating that the protective effect of melatonin is not mediated by activation of melatonin receptors. Melatonin (50 μ*M*) did not inhibit PC12 cell death under control conditions (data not shown). In addition, 0.01% DMSO, used to dissolve melatonin, did not significantly inhibit PC12 cell death in the presence of A23187 plus rotenone (38.2 ± 0.38% of the total LDH release). Butylated hydroxytoluene (10 μ*M*) or ebselen (5 μ*M*) plus glutathione (200 μ*M*) had no significant inhibitory effect on A23187 plus rotenone-induced PC12 cell death (data not shown), probably due to different properties of these antioxidants when compared with melatonin. While butylated hydroxytoluene is highly lipophilic, inhibiting preferentially lipid peroxidation, glutathione plus ebselen removes H_2O_2 from the extracellular space.

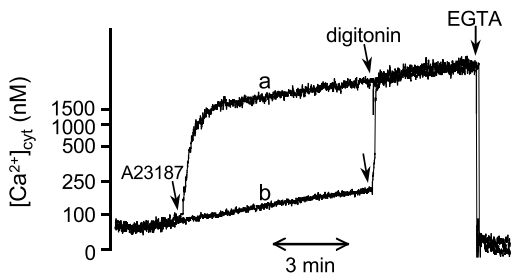

FIG. 3. Ca^{2+} ionophore A23187 increases cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) in PC12 cells. Fluo-3-loaded PC12 cells in suspension were incubated in DMEM containing 20 m*M* Na^+-HEPES buffer (pH 7.2), without phenol red. A23187 (8 μ*M*) (line a), 0.1% digitonin (lines a and b) and 10 m*M* EGTA (lines a and b), were added where indicated by the arrows.

Effect of melatonin on ROS release by PC12 cells

To verify the effect of melatonin on ROS release by PC12 cells, H_2O_2 measurements were conducted in the extracellular medium using Amplex Red. Samples were taken after 1 h of incubation, *i.e.,* before cells start to die. The presence of rotenone and A23187 increased H_2O_2 release by PC12 cells fourfold. This effect was prevented by the presence of 50 μ*M* melatonin (Fig. 5). Melatonin (50 μ*M*) did not inhibit ROS release by PC12 cells under control conditions (data not shown). In addition, 0.01% DMSO, the solvent of melatonin, did not significantly inhibit ROS release by PC12 cells in the presence of A23187 plus rotenone (31.5 ± 5.2 arbitrary fluorescence units).

DISCUSSION

Recently, we (40) and others (42) demonstrated that treatment of isolated brain mitochondria with rotenone and micromolar Ca^{2+} concentrations results in increased mitochondrial ROS production. In the present work, we found that melatonin, a naturally occurring compound with antioxidant properties (for recent reviews, see 4, 23, 33, 43), strongly inhibits ROS release by rotenone-poisoned isolated brain mitochondria treated with Ca^{2+} (Fig. 1). A similar inhibitory effect of melatonin on ROS release was observed in PC12 cells treated with rotenone and the Ca^{2+} ionophore A23187, used to increase the cytosolic free Ca^{2+} concentrations (Figs. 3 and 5). The inhibitory effect of melatonin on PC12 cell ROS release was correlated with protection against cell death (Fig. 4). Considering that the protective effects of melatonin were not sensitive to melatonin receptor antagonists, and that these effects were also observed with isolated mitochondria, we conclude that melatonin is acting as an antioxidant under our experimental conditions. In fact, melatonin has been extensively reported to scavange ROS, including hydroxyl radicals, H_2O_2, nitric oxide, peroxynitrite anions, singlet oxygen, and oxoferryl-derived species (4, 33, 43). However, melatonin has poor reactivity

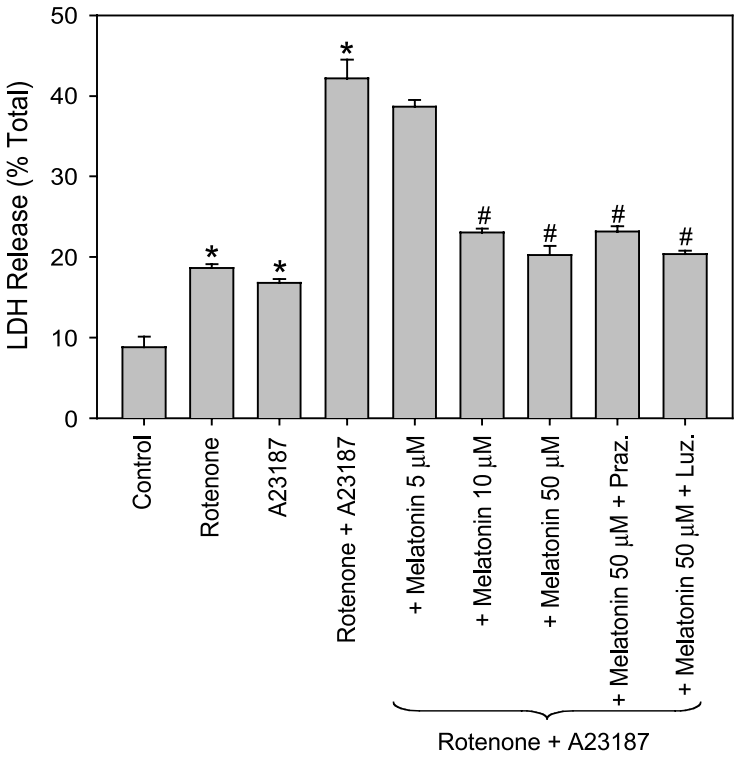

FIG. 4. Melatonin inhibits rotenone plus A23187-induced PC12 cell death. PC12 cells were incubated for 5 h at 37°C in the presence of 500 n*M* rotenone, 8 µ*M* Ca^{2+} ionophore A23187, 5–50 µ*M* melatonin, 10 µ*M* prazosin (Praz.), and/or 10 µ*M* luzindole (Luz.), as indicated on the abscissa. The activity of LDH released due to cell death was determined in the extracellular medium as described in Materials and Methods. Values represent averages of three experiments (± SEM). $*p < 0.01$, *post-hoc* Bonferroni/Dunn's test compared with control; $^{\#}p < 0.01$, *post-hoc* Bonferroni/Dunn's test compared with rotenone plus A23187.

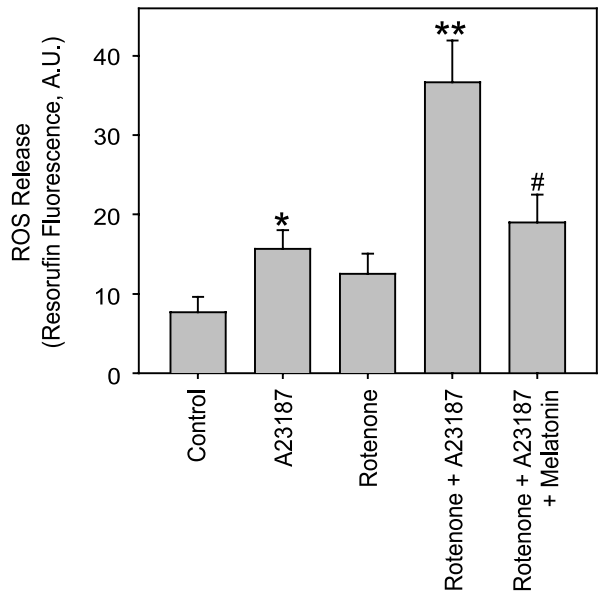

FIG. 5. Melatonin inhibits detection of ROS release by PC12 cells in the presence of rotenone plus A23187. PC12 cells were incubated for 1 h at 37°C in the presence of 500 n*M* rotenone, 8 µ*M* Ca^{2+} ionophore A23187, and/or 50 µ*M* melatonin, as indicated on the abscissa. The fluorescence of resorufin, the product of Amplex Red oxidation, was determined in the extracellular medium as described in Materials and Methods. Values represent averages of five experiments (± SEM). $*p < 0.05$, *post-hoc* Bonferroni/Dunn's test compared with control; $**p < 0.01$, *post-hoc* Bonferroni/Dunn's test compared with control; $\#p < 0.01$, *post-hoc* Bonferroni/Dunn's test compared with rotenone plus A23187. A.U., arbitrary units.

with superoxide anions and peroxyl radicals (4, 5). The indole moiety of the melatonin molecule is probably the reactive center for most of the oxygen and nitrogen reactive species it removes (4). It is still possible that, under our conditions, melatonin may also act by decreasing ROS generation.

The protective effects of melatonin found in this study are in line with similar results observed in experimental models of neurodegenerative disorders, including Parkinson's disease (1, 16, 29), Alzheimer's disease (28), and ischemia/reperfusion (14, 15). Furthermore, a recent communication by Betarbet and collaborators (10) reports a neuroprotective effect of melatonin on dopaminergic degeneration in adult rats chronically and systemically treated with rotenone.

Figure 6 summarizes a proposed model by which a partial respiratory chain complex I inhibition may contribute to neurodegeneration under pathological situations, such as in Parkinson's disease. A partial complex I inhibition decreases mitochondrial ability to generate ATP (Fig. 6, downward arrow), which may directly contribute to cell death by compromising intracellular homeostatic mechanisms. However, a small decrease in complex I activity may be not sufficient to explain neuronal cell death only by energy deprivation, and the toxic effect of this inhibition seems to be mainly mediated by an increased production of superoxide at the mitochondrial respiratory chain (forward arrow) (6). Interestingly, a subminimal energy deprivation promoted by partial complex I may increase the susceptibility to excitotoxicity (upward arrow) (3, 8, 11, 13), resulting in increased cytosolic free Ca^{2+} concentrations. Under this condition, complex I inhibition will potentiate the oxidative stress that accompanies excitotoxicity (8, 13) (forward arrows) and may induce cell death. Melatonin inhibits cell death in a highly efficient manner by preventing the end effect of these last two routes, namely, accumulation of mitochondri-

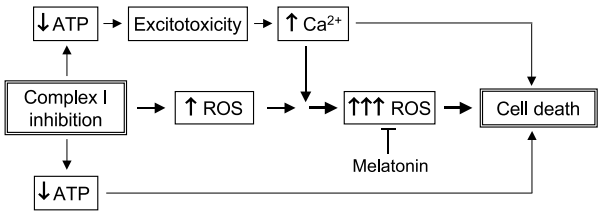

FIG. 6. Proposed model for neuronal cell death promoted by mitochondrial respiratory chain complex I inhibition. Inhibition of respiratory chain complex I activity results in energy deprivation (↓ ATP) and increased ROS production (↑ ROS). Energy deprivation may induce excitotoxicity with increased intracellular Ca^{2+} influx (↑ Ca^{2+}). When complex I is (partially) inhibited, increased cytoplasmic Ca^{2+} concentrations further stimulate mitochondrial ROS release. The antioxidant melatonin can prevent complex I inhibition-induced cell death by inhibiting the resulting oxidative stress.

ally generated ROS. Our findings and the model presented in Fig. 6 are in accordance with those of Marey-Semper and collaborators (27), who show a synergistic effect of *N*-methyl-D-aspartate receptor activation and energetic metabolism impairment by rotenone on toxicity to cultured mesencephalic dopaminergic neurons.

In conclusion, the results of the present study indicate that energetic metabolism impairment by complex I inhibition and high cytosolic Ca^{2+} concentrations act synergistically in neuronal degeneration, in a mechanism involving oxidative stress. Under these conditions, the use of antioxidants such as melatonin may be useful to prevent or inhibit cell death.

ACKNOWLEDGMENTS

The authors thank Dr. Alicia Kowaltowski for critical reading of the manuscript and Elisangela Silva and Edilene Siqueira for technical support. This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). S.C. Sousa is supported by an FAPESP fellowship.

ABBREVIATIONS

DCF, dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; H_2-DCFDA, dichlorodihydrofluorescein diacetate; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; PC12, pheochromocytoma; ROS, reactive oxygen species.

REFERENCES

1. Absi E, Ayala A, Machado A, and Parrado J. Protective effect of melatonin against the 1-methyl-4-phenylpyridinium-induced inhibition of complex I of the mitochondrial respiratory chain. *J Pineal Res* 29: 40–47, 2000.
2. Alam M and Schmidt WJ. Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. *Behav Brain Res* 136: 317–324, 2002.
3. Albin RL and Greenamyre JT. Alternative excitotoxic hypotheses. *Neurology* 42: 733–738, 1992.
4. Allegra M, Reiter RJ, Tan DX, Gentile C, Tesoriere L, and Livrea MA. The chemistry of melatonin's interaction with reactive species. *J Pineal Res* 34: 1–10, 2003.
5. Antunes F, Barclay LR, Ingold KU, King M, Norris JQ, Scaiano JC, and Xi F. On the antioxidant activity of melatonin. *Free Radic Biol Med* 26: 117–128, 1999.
6. Barrientos A and Moraes CT. Titrating the effects of mitochondrial complex I impairment in the cell physiology. *J Biol Chem* 274: 16188–16197, 1999.
7. Beal MF. Mitochondria, free radicals, and neurodegeneration. *Curr Opin Neurobiol* 6: 661–666, 1996.
8. Beal MF. Excitotoxicity and nitric oxide in Parkinson's disease pathogenesis. *Ann Neurol* 44(Suppl 1): S110-S114, 1998.
9. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, and Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* 3: 1301–1306, 2000.
10. Betarbet R, Sherer TB, Taylor G, Na HM, and Greenamyre JT. Melatonin protects against rotenone-induced parkinsonian symptoms. Program no. 754.9. 2004 Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online.
11. Blandini F, Nappi G, and Greenamyre JT. Subthalamic infusion of an NMDA antagonist prevents basal ganglia metabolic changes and nigral degeneration in a rodent model of Parkinson's disease. *Ann Neurol* 49: 525–529, 2001.
12. Cadenas E, Boveris A, Ragan CI, and Stoppani AO. Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase from beef-heart mitochondria. *Arch Biochem Biophys* 180: 248–257, 1977.
13. Castilho RF, Hansson O, Ward MW, Budd SL, and Nicholls DG. Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells. *J Neurosci* 18: 10277–10286, 1998.
14. Cho S, Joh TH, Baik HH, Dibinis C, and Volpe BT. Melatonin administration protects CA1 hippocampal neurons after transient forebrain ischemia in rats. *Brain Res* 755: 335–338, 1997.
15. Cuzzocrea S, Costantino G, Gitto E, Mazzon E, Fulia F, Serraino I, Cordaro S, Barberi I, De Sarro A, and Caputi AP. Protective effects of melatonin in ischemic brain injury. *J Pineal Res* 29: 217–227, 2000.
16. Dabbeni-Sala F, Di Santo S, Franceschini D, Skaper SD, and Giusti P. Melatonin protects against 6-OHDA-induced neurotoxicity in rats: a role for mitochondrial complex I activity. *FASEB J* 15: 164–170, 2001.
17. Dawson TM and Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. *Science* 302: 819–822, 2003.
18. Degli Esposti M. Inhibitors of NADH-ubiquinone reductase: an overview. *Biochim Biophys Acta* 1364: 222–235, 1998.
19. Dubocovich ML. Luzindole (N-0774): a novel melatonin receptor antagonist. *J Pharmacol Exp Ther* 246: 902–910, 1988.
20. Dubocovich ML. Melatonin receptors: are there multiple subtypes? *Trends Pharmacol Sci* 16: 50–56, 1995.

21. Gyulkhandanyan AV and Pennefather PS. Shift in the localization of sites of hydrogen peroxide production in brain mitochondria by mitochondrial stress. *J Neurochem* 90: 405–421, 2004.
22. Lapointe N, St-Hilaire M, Martinoli MG, Blanchet J, Gould P, Rouillard C, and Cicchetti F. Rotenone induces non-specific central nervous system and systemic toxicity. *FASEB J* 18: 717–719, 2004.
23. Leon J, Acuna-Castroviejo D, Sainz RM, Mayo JC, Tan DX, and Reiter RJ. Melatonin and mitochondrial function. *Life Sci* 75: 765–790, 2004.
24. Liu Y, Fiskum G, and Schubert D. Generation of reactive oxygen species by the mitochondrial electron transport chain. *J Neurochem* 80: 780–787, 2002.
25. Maciel EN, Vercesi AE, and Castilho RF. Oxidative stress in Ca^{2+}-induced membrane permeability transition in brain mitochondria. *J Neurochem* 79: 1237–1245, 2001.
26. Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AH, and Marsden CD. Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. *Brain* 115: 333–342, 1992.
27. Marey-Semper I, Gelman M, and Levi-Strauss M. A selective toxicity toward cultured mesencephalic dopaminergic neurons is induced by the synergistic effects of energetic metabolism impairment and NMDA receptor activation. *J Neurosci* 15: 5912–5918, 1995.
28. Matsubara E, Bryant-Thomas T, Pacheco Quinto J, Henry TL, Poeggeler B, Herbert D, Cruz-Sanchez F, Chyan YJ, Smith MA, Perry G, Shoji M, Abe K, Leone A, Grundke-Ikbal I, Wilson GL, Ghiso J, Williams C, Refolo LM, Pappolla MA, Chain DG, and Neria E. Melatonin increases survival and inhibits oxidative and amyloid pathology in a transgenic model of Alzheimer's disease. *J Neurochem* 85: 1101–1118, 2003.
29. Mayo JC, Sainz RM, Uria H, Antolin I, Esteban MM, and Rodriguez C. Melatonin prevents apoptosis induced by 6-hydroxydopamine in neuronal cells: implications for Parkinson's disease. *J Pineal Res* 24: 179–192, 1998.
30. Minta A, Kao JP, and Tsien RY. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J Biol Chem* 264: 8171–8178, 1989.
31. Polster BM and Fiskum G. Mitochondrial mechanisms of neural cell apoptosis. *J Neurochem* 90: 1281–1289, 2004.
32. Reed PW and Lardy HA. A23187: a divalent cation ionophore. *J Biol Chem* 247: 6970–6977, 1972.
33. Reiter RJ, Tan DX, Mayo JC, Sainz RM, Leon J, and Czarnocki Z. Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. *Acta Biochim Pol* 50: 1129–1146, 2003.
34. Rosenthal RE, Hamud F, Fiskum G, Varghese PJ, and Sharpe S. Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. *J Cereb Blood Flow Metab* 7: 752–758, 1987.
35. Scarpa A. Measurements of cation transport with metallochromic indicators. *Methods Enzymol* 56: 301–338, 1979.
36. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, and Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem* 54: 823–827, 1990.
37. Shafer TJ and Atchison WD. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. *Neurotoxicology* 12: 473–492, 1991.
38. Sherer TB, Betarbet R, Testa CM, Seo BB, Richardson JR, Kim JH, Miller GW, Yagi T, Matsuno-Yagi A, and Greenamyre JT. Mechanism of toxicity in rotenone models of Parkinson's disease. *J Neurosci* 23: 10756–10764, 2003.
39. Sherer TB, Kim JH, Betarbet R, and Greenamyre JT. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. *Exp Neurol* 179: 9–16, 2003.
40. Sousa SC, Maciel EN, Vercesi AE, and Castilho RF. Ca^{2+}-induced oxidative stress in brain mitochondria treated with the respiratory chain inhibitor rotenone. *FEBS Lett* 543: 179–183, 2003.
41. Starkov AA and Fiskum G. Regulation of brain mitochondrial H_2O_2 production by membrane potential and NAD(P)H redox state. *J Neurochem* 86: 1101–1107, 2003.
42. Starkov AA, Polster BM, and Fiskum G. Regulation of hydrogen peroxide production by brain mitochondria by calcium and Bax. *J Neurochem* 83: 220–228, 2002.
43. Tan DX, Reiter RJ, Manchester LC, Yan MT, El-Sawi M, Sainz RM, Mayo JC, Kohen R, Allegra M, and Hardeland R. Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. *Curr Top Med Chem* 2: 181–197, 2002.
44. Turrens JF. Superoxide production by the mitochondrial respiratory chain. *Biosci Rep* 17: 3–8, 1997.
45. Turrens JF, Alexandre A, and Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 237: 408–414, 1985.
46. Votyakova TV and Reynolds IJ. $\Delta\Psi_m$-dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J Neurochem* 79: 266–277, 2001.
47. Zhou M, Diwu Z, Panchuk-Voloshina N, and Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem* 253: 162–168, 1997.
48. Zhu C, Vourc'h P, Fernagut PO, Fleming SM, Lacan S, Dicarlo CD, Seaman RL, and Chesselet MF. Variable effects of chronic subcutaneous administration of rotenone on striatal histology. *J Comp Neurol* 478: 418–426, 2004.

Address reprint requests to:
Roger F. Castilho, M.D., Ph.D.
Departmento de Patologia Clínica
Faculdade de Ciências Médicas
Universidade Estadual de Campinas
SP 13083-970
Campinas, Brazil

E-mail: roger@fcm.unicamp.br

Received for publication November 6, 2004; accepted March 8, 2005.

This article has been cited by:

1. Naveen Kumar Singhal, Garima Srivastava, Sonal Agrawal, Swatantra Kumar Jain, Mahendra Pratap Singh. 2011. Melatonin as a Neuroprotective Agent in the Rodent Models of Parkinson's Disease: Is it All Set to Irrefutable Clinical Translation?. *Molecular Neurobiology* . [CrossRef]
2. Alicia J. Kowaltowski, Nadja C. de Souza-Pinto, Roger F. Castilho, Anibal E. Vercesi. 2009. Mitochondria and reactive oxygen species. *Free Radical Biology and Medicine* **47**:4, 333-343. [CrossRef]
3. Saori Morota, Roland Månsson, Magnus J. Hansson, Kazuhiko Kasuya, Motohide Shimazu, Erika Hasegawa, Shigeru Yanagi, Akibumi Omi, Hiroyuki Uchino, Eskil Elmér. 2009. Evaluation of putative inhibitors of mitochondrial permeability transition for brain disorders — Specificity vs. toxicity. *Experimental Neurology* **218**:2, 353-362. [CrossRef]
4. Yan Sai, Junfeng Chen, Qiang Wu, Hiu Liu, Jiqing Zhao, Zhaojun Dong. 2009. Phosphorylated-ERK 1/2 and neuronal degeneration induced by rotenone in the hippocampus neurons. *Environmental Toxicology and Pharmacology* **27**:3, 366-372. [CrossRef]
5. Russel J. Reiter, Sergio D. Paredes, Ahmet Korkmaz, Mei-Jie Jou, Dun-Xian Tan. 2008. Melatonin combats molecular terrorism at the mitochondrial level. *Interdisciplinary Toxicology* **1**:2, 137-149. [CrossRef]
6. P RAVENSTIJN, M MERLINI, M HAMEETMAN, T MURRAY, M WARD, H LEWIS, G BALL, C MOTTART, C DEVILLEDEGOYET, T LEMARCHAND. 2008. The exploration of rotenone as a toxin for inducing Parkinson's disease in rats, for application in BBB transport and PK–PD experiments. *Journal of Pharmacological and Toxicological Methods* **57**:2, 114-130. [CrossRef]
7. L RE, M MAWSOUF, S MENENDEZ, O LEON, G SANCHEZ, F HERNANDEZ. 2008. Ozone Therapy: Clinical and Basic Evidence of Its Therapeutic Potential. *Archives of Medical Research* **39**:1, 17-26. [CrossRef]
8. Cristina Malagelada, Lloyd A. GreenePC12 Cells as a model for parkinson's disease research 375-387. [CrossRef]
9. Karuppagounder S. Saravanan, Kizhakke M. Sindhu, Kochupurackal P. Mohanakumar. 2007. Melatonin protects against rotenone-induced oxidative stress in a hemiparkinsonian rat model. *Journal of Pineal Research* **42**:3, 247-253. [CrossRef]
10. Corinne E. Griguer, Claudia R. Oliva, G. Yancey Gillespie, Eric Gobin, Pascale Marcorelles, G. Yancey Gillespie. 2006. Pharmacologic manipulations of mitochondrial membrane potential (##m) selectively in glioma cells. *Journal of Neuro-Oncology* **81**:1, 9-20. [CrossRef]
11. Janet M. Dubinsky . 2005. CNS Mitochondria in Neurodegenerative Disorders. *Antioxidants & Redox Signaling* **7**:9-10, 1089-1091. [Citation] [Full Text PDF] [Full Text PDF with Links]