Melatonin protects against rotenone-induced cell injury via inhibition of Omi and Bax-mediated autophagy in Hela cells

Abstract: Parkinson’s disease is the second most common neurodegenerative disease, and environmental toxins such as rotenone play an important role in causing degeneration of dopaminergic neurons. Melatonin, a major secretory product of pineal, is recently reported to protect against rotenone-induced cell death in animal models. Yet, the mechanism involved in this protection needs to be elucidated. Here, we report that rotenone treatment (0–100 μM) decreased cell survival of Hela cells in a dose-dependent manner. At concentrations ranging from 0.1 to 100 μM, rotenone induced a dose-dependent increase in the expression of microtubule-associated protein 1 light chain 3 (LC3)-II, a protein associated with the autophagosomal membrane. Knockdown of Bax or Omi using shRNA inhibited 1 μM rotenone-induced autophagy. To determine whether melatonin would protect cells against rotenone-induced cell death and autophagy, we pretreated Hela cells with 250 μM melatonin for 24 hr in the presence of rotenone. Melatonin inhibited Bax expression and the release of the omi/HtrA2 into the cytoplasm induced by 1 μM rotenone. Melatonin 250 μM treatment also suppressed cell death induced by 0.1–100 μM rotenone and protected against the formation of LC3-II in cells exposed to 1 μM rotenone. This work demonstrates a novel role for melatonin as a neuroprotective agent against rotenone.

Introduction

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder affecting 2% of the population over the age of 65 [1]. As a slowly progressive condition, PD is characterized by slowness of movement, rigidity, and tremors. These symptoms result from a lack of dopamine because of the gradual degeneration of dopaminergic neurons in the substantia nigra pars compacta. Although mechanisms underlying the selective dopaminergic neuronal death are not well defined, mitochondrial dysfunction is considered a major contributor to this process [2–5]. Impaired mitochondrial function can cause excessive generation of reactive oxygen species (ROS), suppress energy production, and trigger cell death.

Autophagy is a cellular homeostatic process, which involves the sequestration of cytoplasmic material for bulk degradation by lysosomes. Recently, autophagy has emerged as an important player in the pathogenic process of PD [6–9]. However, whether activation of autophagy is beneficial or detrimental in PD is still not clear as both protective and detrimental effects have been reported [6, 10].

Rotenone is the most widely used toxin for modeling PD because it has been shown to induce PD-like symptoms in humans [11] and animal models [12, 13]. As a mitochondrial complex I inhibitor, rotenone can trigger intrinsic apoptosis in different cell lines [14, 15]. The intrinsic apoptotic pathway can be induced by the Bax translocation to mitochondria and the release of death-inducing proteins, e.g., HtrA2/omi, from the intermembrane space of mitochondria. Interestingly, Bax and Omi are also positive regulators of autophagy [16–18], suggesting a close interplay between the apoptotic and autophagic pathways. The role of autophagy is also complex in rotenone-induced PD models [19–21]. Rotenone has been reported to induce autophagic cell death through ROS generation [21]. On the other hand, induction of autophagy appears to protect against rotenone-induced cell death [10, 19, 20, 22].

Melatonin is the major hormonal product from pineal gland and has potential implications for prevention and treatment of neurodegenerative diseases because it can alleviate neuronal damage and halt disease progression in experimental models of various neurodegenerative diseases including AD, PD, and HD. Melatonin and its metabolites are potent ROS scavengers [23–27], which are known to be highly effective agents in reducing neuronal loss and neurophysiologic deficits associated with experimental PD [28, 29]. It has been reported that melatonin protects against PD-related toxins by preventing cytochrome c release, inhibiting Δψm depolarization and reducing ROS formation [28, 30, 31]. Furthermore, previous studies have found that melatonin protects against rotenone-induced cell death in animals [32].

In the present study, we investigated whether Bax and Omi activation is involved in rotenone-induced autophagy and whether melatonin protects against rotenone-induced...
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Materials and methods

Chemicals and reagents

Reagents were from the following sources: the rotenone and melatonin were purchased from Sigma-Aldrich (St Louis, MO, USA); RPMI-1640 and heat-inactivated fetal bovine serum were purchased from Invitrogen (Rockville, MD, USA); Mammalian cell extraction kit and Mitochondria/ Cytosol Fractionation Kit were purchased from BioVision (Palo Alto, CA, USA); the bicinechonic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA); the mouse monoclonal antibody β-action antibody was purchased from Multisciences (Shanghai, China); rabbit polyclonal antibody LC3 was purchased from Novus Biologicals (Littleton, CO, USA); rabbit monoclonal antibody HtrA2/Omi and mouse monoclonal antibody Bax were purchased from Abcam (Cambridge, UK); horseradish peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Amersham (Foster City, CA, USA); and chemiluminescence reactions were purchased from Milipore (Bedford, MA, USA).

Cell culture and transfection

Hela cells were cultured in RPMI-1640 (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen) in a humidified incubator (Thermo electron corporation, Waltham, MA, USA) containing 5% CO₂ atmosphere at 37°C.

Rotenone model and experimental groups

Experimental groups included group of normally cultured Hela cells (control), group of melatonin administration on normal Hela cells (melatonin), group of Hela cells undergoing rotenone (rotenone), and group of melatonin pre-treatment upon rotenone (melatonin + rotenone).

Assessment of cell viability by MTT

Cells were seeded in 96-well plates and were grown to 80% confluence before follow-up procedures. The cell viability of Hela cells subjected to different treatments was analyzed by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, 10 μL of the MTT labeling reagent at a final concentration of 0.5 mg/mL was added into each well at the time point before incubation in a humidified incubator at 37°C with 5% CO₂ for 4 hr to allow formation of purple formazan crystal. Subsequently, the formazan crystals were dissolved in dimethyl sulfoxide. Absorbance (A) value was measured at an absorbance wavelength of 570 nm in a microplate reader within 30 min.

Western blot analysis

Cells were harvested and lysed using Mammalian cell extraction kit (BioVision). The resulting lysates were subjected to Bradford protein assay to ensure equal protein loading. Protein was subjected to 8% or 12% SDS-polyacrylamide gel for electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). The membranes were blocked in Tris-buffered saline Tween-20 [50 mM Tris–HCl, 154 mM NaCl, 0.1% Tween-20 (pH 7.5)] containing 5% nonfat dry milk for 1 hr and then probed with different appropriately diluted antibodies (cat. no. NB100-2220, 1:5000 rabbit polyclonal antibody LC3; Novus Biologicals, cat. no.ab32092, 1:4000 mouse monoclonal antibody β-action, 1:2000 rabbit monoclonal antibody HtrA2/Omi; Abcam, cat. no.ab5714, 1:100 mouse monoclonal antibody Bax; Abcam) overnight at 4°C. After the primary antibody incubation, the membrane was washed and incubated with either HRP-conjugated anti-mouse IgG or anti-rabbit IgG for 1 hr at room temperature. Chemiluminescence reactions were conducted according to the manufacturer’s protocol. The intensity (INT × area) of each band was measured and analyzed with a Chemi Doc XRS imaging system (Bio-Rad, Hercules, CA, USA). Data are given as percentage of vehicle control and β-action was used as an internal control.

Mitochondrial and cytosolic fractions in hela cells

Subcellular fractionation was obtained using Mitochondria/Cytosol Fractionation Kit (BioVision). Briefly, cells were harvested by centrifugation at 600 g for 5 min at 4°C, washed with 10 ml of ice-cold phosphate buffered saline and centrifuged at 600 g for 5 min at 4°C, then removed supernatant. Cells were resuspended with 1.0 mL of 1× Cytosol Extraction Buffer Mix containing DL-dithiothreitol (DTT) and Protease Inhibitors and incubated on ice for 10 min, then homogenized in an ice-cold dounce tissue grinder. The cells were transferred to a 1.5-mL microcentrifuge tube, and centrifuged at 700 g in a microcentrifuge for 10 min at 4°C. The supernatant was transferred to a fresh 1.5-mL tube and centrifuged at 10,000 g in a microcentrifuge for 30 min at 4°C to generate supernatant (cytosol) and pellet (containing mitochondrial) fractions. The pellet was resuspended with 100 μL of the Mitochondrial Extraction Buffer Mix containing DTT and protease inhibitors vertex for 10 s and saved as Mitochondrial Fraction. Protein concentration was determined using the BCA protein assay kit (Pierce, USA).

RNA interference and stable cell line

Down-regulation of Bax, HtrA2/omi in Hela cells was achieved via short hair interfering RNA (shRNA). The target sequences were 5¢-GCCGGAACGATCAGACA CAT-3¢ for Bax-1 [nucleotides (nt) 456–477; accession no.NM_004324], 5¢-GCCTCAGAGTGCTCCACCAA-3¢ for Bax-2 [nucleotides (nt) 219–240; accession no.NM _004324], 5¢-GCCCAACAGCTCTGAGCAGAT-3¢ for Bax-3 [nucleotides (nt) 105–126; accession no.NM_004324]. The target sequences were 5¢-GCTGAACTACAGCTTCAT-3¢ for omi/HtrA2-1 [nucleotides [nt] 1725–1746; accession no.NM_013247], 5¢-CCAAAACATCTGTTGAGTACAT-3¢ for omi/HtrA2-2 [nucleotides [nt] 1466–1487; accession no.NM_013247].
ACTTCAT for omi/HtrA2-3 (nucleotides [nt] 1031–1052; accession no.NM_013247). A fluorescent siRNA oligonucleotide (DY-547 siGLO Lamin A/C; 20 nm; Dharmacon, Lafayette, IN, USA) was used to confirm that these conditions result in efficient delivery of siRNA to 95% ± 3.9 (S.D.) of cells. Nontargeted RNA oligonucleotide pool (Dharmacon) was used as a sham control.

Hela cells were transfected with Bax shRNA or Omi shRNA sequences using FuGENE HD transfection reagent (Roche, Mannheim, Germany) according to the manufacturer’s protocol. The following day, the standard media were replaced with media containing G418 (Invitrogen). The media were changed every 3 days until only colonies of stable cells were left. Colonies were picked and then maintained in the medium containing G418. Efficacy and specificity of protein knockdown were assessed by western blotting.

**Statistical analysis**

Statistical comparisons are made against the control using Student’s t-test. Experiments were performed in triplicate. For all graphs bars represent means ± S.E.M. The significance was taken when P-values were <0.05.

**Results**

In this study, we investigated the neurotoxic effect of rotenone in Hela cells and found that rotenone induced cell death and autophagy in a dose-dependent manner. Treatment cells with rotenone at 100 nm, 1, 10, and 100 μm for 24-h caused approximately 18%, 28%, 31%, and 33% reduction in cell viability, respectively (Fig. 1A). In parallel with the reduction in cell viability, the level of the microtubule-associated protein light chain 3 (LC3-II), an autophagosome protein that serve as a good indicator of autophagy, dose-dependently increased (Fig. 1B).

To test whether rotenone-induced autophagy is mediated by the Bax signaling pathway, Bax-shRNA stable cell line was established in the present study (Fig. 2A). An immunoblot of extracts from the cells transfected with Bax shRNA revealed that the cells transfected with Bax shRNA-1 had almost 50% reduction in Bax protein levels compared with cells transfected with shRNA negative control (Fig. 2B). In addition, the level of LC3-II was significantly lower in Bax-deficient cells compared with control cells when treated with 1 μm rotenone for 24 hr (193 ± 17% versus 152 ± 21%, P < 0.05) (Fig. 2C), indicating that Bax plays a significant role in rotenone-induced autophagy.

To investigate whether rotenone-induced autophagy is mediated by the Omi signaling pathway, Omi-shRNA stable cell line was established in the present study (Fig. 2D). An immunoblot of extracts from the cells transfected with Omi shRNA revealed that the cells transfected with Omi shRNA-2 had almost 70% reduction in Omi protein levels compared with cells transfected with shRNA negative control (Fig. 2E). In addition, the level of LC3-II was significantly lower in Omi-deficient cells compared with control cells when treated with 1 μm rotenone for 24 hr (179 ± 19% versus 120 ± 20%, P < 0.05) (Fig. 2F), indicating that Omi also plays a significant role in rotenone-induced autophagy.

To study whether melatonin can attenuate rotenone-induced Bax and Omi expression, we exposed cells to rotenone in the presence or absence of melatonin and then...
examined the Bax and Omi expression levels. As expected, exposure of Hela cells to rotenone resulted in a significant increase to 258 ± 15% \((P < 0.05)\) in the level of Bax protein expression and melatonin at 250 \(\mu M\) significantly attenuated rotenone-induced Bax expression (Fig. 3A). Similarly, rotenone significantly induced Omi expression. However, melatonin did not significantly alter the total expression of Omi (Fig. 3B). We further examined the release of Omi from mitochondria to cytoplasm. We found that melatonin significantly inhibited rotenone-induced Omi release (Fig. 3C).

Finally, to determine whether melatonin alone is toxic to Hela cells, different doses (62.5–500 \(\mu M\)) of melatonin were added to normal Hela cell cultures. There was no significant difference in cell viability between melatonin-treated group and vehicle-treated group (Fig. 4A). To determine whether melatonin can protect against rotenone-induced autophagy and cell death, Hela cells were exposed to 1 \(\mu M\) rotenone with or without a 3 hr pretreatment with different doses of melatonin. Melatonin at a concentration range of 250–500 \(\mu M\) significantly prevented rotenone-induced decrease in MTT values in a concentration-dependent manner (Fig. 4B). According to the concentration-effect trend of melatonin, melatonin at 250 \(\mu M\) was applied to all subsequent experiments. Treatment with rotenone significantly increased LC3-II levels, whereas pretreatment with melatonin significantly attenuated rotenone-induced LC3-II expression levels (Fig. 4C).

### Discussion

Autophagy has been well described in the substantia nigra neurons of PD patients [33] and dopaminergic cells of...
neurotoxin-induced PD models [34]. Likewise, we found that autophagy was evident in rotenone-treated HeLa cells as evidenced by elevated LC3-II levels. It is generally accepted that mitochondria-derived ROS are a major cause of rotenone-induced toxicity. In addition, ROS are important regulators of autophagy, and different levels of ROS elicit different autophagic responses [35]. For example, low concentrations of ROS stimulate autophagy to selectively remove oxidatively damaged mitochondria and reduce cell death [36]. In contrast, high concentrations of ROS enhance the permeability of lysosomal membranes and thus result in the release of lysosomal proteases which in turn contribute to the lysosomal degradation mechanism of autophagic cell death [37]. Consistent with this, we found that elevated LC3-II was accompanied by a concomitant reduction in cell viability, suggesting that autophagy is involved in rotenone-induced cell death. Indeed, inhibiting excessive ROS or increasing endogenous antioxidant enzymes have been reported to reduce rotenone-induced autophagic cell death [21, 22]. However, it should be noted that LC3-II is an autophagosomal marker and does not necessarily reflect the degree of autophagy. In fact, accumulation of immature autophagosomes, a marker for incomplete autophagy, has been detected in rotenone-treated cells. This may explain the beneficial effects of autophagy induction in some studies [10, 20].

The autophagic and apoptotic pathways are not mutually exclusive and these two pathways can be regulated by the same mechanisms under certain circumstances. The toxicity of rotenone is mainly associated with mitochondrial dysfunction. Interestingly, several mitochondrial proteins including Bax and Omi, despite their well-established roles in apoptosis, also participate in regulation of autophagy [17, 18]. To investigate whether Bax and Omi mediate rotenone-induced autophagic cell death, we developed stable Bax and Omi-deficient cell lines using ShRNA. We found that knockdown of Bax or Omi significantly inhibited rotenone-induced autophagy. Melatonin is potent antioxidant [23, 26, 38] and has been shown to reduce rotenone-induced toxicity [39] and aberrant Bax expression in neurotoxin models [40, 41]. Herein, we further tested whether melatonin can be a feasible treatment option for rotenone-induced autophagic cell death. Consistently, mel-
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Rotenone treatment induced autophagic cell death dependent of Bax and Omi release to cytoplasm. Furthermore, we demonstrated a novel role for melatonin in protecting against autophagic cell death via the Bax/Omi pathway. These findings provide additional information regarding the link between apoptosis and autophagy signaling, which could lead to the development of therapeutic strategies that exploit the neurotoxicity induced by environment toxins of PD.

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Author contributions

Conception and design of the experiments: ZP, LC, HZ, JZ.; Performing the experiments: HZ, JC, XL, CS.; Analysis of data: HZ, JC.; Drafting the manuscript: HZ, ZP.; Critical revision of the manuscript and approval of the article: ZP.