Inhibition of the cdk5/p25 fragment formation may explain the antiapoptotic effects of melatonin in an experimental model of Parkinson’s disease

Abstract: In this study, the effects of melatonin on MPP⁺-treated cerebellar granule neurons (CGNs) in culture were investigated. Results showed that MPP⁺ treatment significantly decreased cell viability and increased the apoptotic cell population at 24 and 48 hr. Calpain and caspase-3 activation was also determined, with results showing a strong increase in calpain (74%) and caspase 3 activity (70%), as measured by z-spectrin cleavage and fluorometric and colorimetric analysis, respectively. There are several studies suggesting that the activation of the cdk5/p35 pathway at its cleavage to cdk5/p25 may play a role in neuronal cell death in neurodegenerative diseases. Moreover, these studies indicate that this cleavage is mediated by calpains, and that MPP⁺ prompted an increase in cdk5 expression, as well as the cleavage of p35–p25, in a time-dependent manner. 1 mm Melatonin not only reduced the neurotoxic effects of MPP⁺ on cell viability, but also prevented apoptosis mediated by this Parkinsonian toxin in CGNs. 1 mm Melatonin reduced cdk5 expression, as well as the cleavage of p35–p25. These data indicate that melatonin possesses some neuro-protective properties against MPP⁺-induced apoptosis. Moreover, these data suggest that the calpain/cdk5 signaling cascade has a potential role in the MPP⁺-mediated apoptotic process in CGNs.

Introduction

Parkinson’s disease (PD) is the second most frequently diagnosed neuro-degenerative disorder and is characterized by rigidity, slowness in voluntary movements, and postural instability [1]. The symptoms of PD result from a loss of dopaminergic neurons in the substantia nigra pars compacta [1, 2]. Currently, the primary drug treatment against this disease is the administration of l-dopa and inhibitors of l-dopa decarboxylase, the aim being to increase brain levels of dopamine. Other treatments involve the administration of dopaminergic agonist and MAO-B inhibitors [2].

The mechanism underlying dopaminergic neuronal death in this disease remains unknown. While the exact cause or mechanism of neuronal loss remains unknown, the use of neurotoxins to reproduce this disease in laboratory animals may facilitate our understanding of the mechanisms involved [3]. Thus, the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropridine (MPTP) and its metabolite 1-methyl-4-phenylpyridinium ion (MPP⁺) are useful tools for inducing PD in experimental animal models, as well as in humans [4–9]. Furthermore, the utilization of MPP⁺ clarifies understand the general role of mitochondria, and specifically the inhibition of the mitochondrial complex I in neuronal cell death [10–12]. In this neuronal degenerative process, following the inhibition of complex I, a well-known dramatic increase in oxidative stress occurs which is neurotoxic for neurons [7, 8]. Furthermore, this mitochondrial alteration induces a release of such pro-apoptotic proteins as cytochrome c (Cyt c), apoptosis inducing factor, and Smac/diablo which regulate the apoptotic process in caspase-dependent or -independent pathways [4].

The first approach to achieve a neuronal antiapoptotic action employed antioxidants. Several studies have demonstrated that antioxidants such as vitamin E, vitamin C, melatonin, and defereroxamine exert neuro-protective effects in vitro, as well as in animals models of PD. Interestingly, a potential antioxidant drug for the treatment of PD currently in phase II trial is coenzyme Q10 [7, 8]. These data confirm the importance of oxidative stress in the pathogenesis of this disease. Previous studies have demonstrated that MPP⁺-induced apoptosis is mediated through the release of Cyt c and by the activation of caspase-3 [13, 14]. However, the role of caspase activation in the neuronal
process remains less clear. Moreover, while several authors have suggested that caspase inhibitors offer substantial neuro-protection in this particular model of PD, others failed to find any neuro-protective effects [13–15].

Recently, a potentially new mechanism, namely cdk5 activation, has been postulated to play a role in PD [15, 16]. A study conducted by Smith et al. [16] demonstrated that following the administration of the neurotoxin MPTP, nigroestrial neuronal loss was prevented by flavopiridol, a pan-cyclin-dependent kinase inhibitor. Cdk5 is a cyclin-dependent kinase CDK that is not involved in cell-cycle regulation and whose primary role in neuronal demise is the phosphorylation of several substrates, including tau at the cytoplasmic level and myocyte enhancer factor (MEF-2) in the nucleus [17, 18]. In recent years, it has been postulated to play a significant role in Alzheimer’s disease because of its involvement in tau phosphorylation [18]. Thus, it appears that cdk5 may play an important role in the neurodegenerative process in several diseases in which cytoskeletal alterations are a prominent feature.

Melatonin, influences a number of physiological functions including sleep, reproduction, body temperature, and the regulation of cardiovascular function. Furthermore, alterations in melatonin levels have been described in several psychiatric and neurological disorders [19]. Indeed, some of the main functions attributed to melatonin include its role as a radical scavenger, and its indirect antioxidant properties [20, 21]. Previous studies suggested that melatonin could prevent neuronal cell death, as well as the damage induced by such neurotoxins as kainic acid [22], β-amyloid [23], 3-nitroproponic acid [24], and MPP+ [25–31]. Moreover, it has recently been demonstrated that melatonin inhibits the c-Jun-N terminal kinase signaling cascade, an important pathway responsible for executing the apoptotic process [32]. However, both the cellular in vitro mechanisms underlying neuronal cell degeneration as induced by cdk5 and the protective roles melatonin plays in the cell death signaling cascade have not been clearly demonstrated. Therefore, the aim of the present study was to examine in depth not only the neurotoxic effects MPP+ exerts on the cdk5 pathway, but also the melatonin-based prevention of neurotoxicity as induced by MPP+ in cerebellar granule neurons (CGNs).

Materials and methods

Cell cultures

Primary cultures of cerebellar granule cells were prepared from 7-day-old Sprague–Dawley rat pups according to the method published by Verdaguier et al. [33]. Meninges-free cerebella were trypsinized and treated with DNase. Cells were adjusted to 8 × 10^5 cells/mL and plated on poly-L-lysine coated at a density 320,000 cells/cm^2. Cultures were grown in Eagle’s medium (Eagle’s basal medium, BME; Gibco, Life Technologies, Paisley, UK) containing 10% FCS ( Gibco, Life Technologies), 2 mM L-glutamine, 0.1 mg/mL gentamicin, and 25 mM KCl. Cytosine arabinoside (10 μM) was added 16–18 hr after plating to inhibit the growth of non-neuronal cells. Cultures prepared by this method exhibited granule neuron enrichment of more than 95%.

Treatment of CGNs and viability assays

Cerebellar granule neurons were used after 7–10 days in vitro culture. Melatonin was added at the same time as MPP+ (200 μM) to investigate its effects. To assess the loss of cell viability, we used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] method. MTT was added to the cells at a final concentration of 250 μM and incubated for 1 hr, allowing the reduction in MTT to produce a dark blue formazan product. Media were then removed, and cells were treated with dimethylsulfoxide. The production of formazan was measured by the absorbency change at 595 nm using a microplate reader (BioRad, Laboratories Inc., Hercules, CA, USA). Viability results are expressed as percentages. The absorbancy measured from nontreated cells was taken to be 100%.

Analysis of apoptosis by flow cytometry

Apoptosis was measured following 48 hr of MPP+ treatment. Briefly, culture medium was removed, with the cells then collected from culture plates by pipetting and washed with phosphate-buffered saline (PBS). Flow cytometer experiments were carried out using an Epics XL flow cytometer with propidium iodide (PI, 10 μg/mL) added 30 min before. The instrument was set up in the standard configuration: excitation of the sample was conducted using a 488 nm air-cooled argon-ion laser at 15 mW power as a standard. Forward scatter (FSC), side scatter (SSC), and PI red (620 nm) fluorescence values were then acquired. Optical alignment was based on the optimized signal from 10 nm fluorescent beads (Immunocheck, Epics Division, Miami, FL, USA). Time was used as a control to stabilize the instrument while red fluorescence was projected onto a 1024 monoparametrical histogram. Aggregates were excluded while single cells were gated by their area versus the peak signal fluorescence.

Detection of apoptotic nuclei by propidium iodide staining

The PI staining was used to detect morphological evidence of apoptosis. CGNs were grown on glass coverslips following MPP+ treatment alone, or in the presence of melatonin 1 mM. Subsequently, cells were fixed in 4% paraformaldehyde/PBS solution pH 7.4 for 1 hr at room temperature. After washing with PBS, cells were incubated for 3 min with a solution of PI in PBS (10 μg/mL). Coverslips were mounted in Mowiol® 4-88. Stained cells were visualized under UV illumination using the 20× objective and their digitized images then captured.

Treatment of apoptotic cells resulted in shrunk, brightly fluorescent, apoptotic nuclei exhibiting high fluorescence and condensed chromatin compared with non-apoptotic cells. Apoptotic cells were scored by counting at least 500 cells in each sample over three separate experiments.
Melatonin prevents MPP⁺-induced apoptosis

Immunodetection: Western-blot and immunocytochemistry analysis

Aliquots of cell homogenates, containing 30 μg (cdk5, p35/ p25, α-tubulin) or 5 μg (α-spectrin) of protein per sample, were analyzed by Western blot. Briefly, samples were placed in sample buffer [0.5 M Tris-HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue] and denatured by boiling at 95-100°C for 5 min. Samples were then separated by electrophoresis on 10% acrylamide gels, with proteins subsequently transferred to polyvinylidene fluoride sheets (ImmobilonTM-P; Millipore Corp., Bedford, MA, USA) using a transblot apparatus (BioRad). Membranes were blocked overnight with 5% nonfat milk dissolved in TBS-T buffer (Tris 50 mM; NaCl 1.5%; Tween 20, 0.05%, pH 7.5). They were then incubated with primary monoclonal antibodies against α-spectrin (1:5000, Chemicon, MAB1622), cdk5 (sc-6247), p35/p25 (sc-820), and β-tubulin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After 90 min, blots were washed thoroughly in TBS-T buffer and incubated for 1 hr with a peroxidase-conjugated IgG antibody (Amer- sham Corp., Arlington Heights, IL, USA). Immunoreactive protein was visualized using a chemiluminescence-based detection kit following the manufacturer’s instructions (ECL kit; Amersham Corp.). Digital images were taken with a ChemiDoc XRS (BioRad), which permits semi-quantitation of band intensity. Protein load was periodically monitored by phenol red staining the blot membrane or via immunodetection of α-tubulin.

For immunocytochemistry experiments, CGNs were grown on sterile coverslips. After stimulation, cells were washed twice in PBS and fixed in 4% paraformaldehyde/PBS, pH 7.4 for 1 hr at room temperature. They were preincubated for 30 min in PBS containing 0.3% Triton X-100 and 30% normal horse serum at room temperature. The cultures were immunostained with antibodies specific for cdk5 and p25 followed by rhodamine-conjugated anti-rabbit IgG or anti-mouse IgG (1:200). Subsequently, coverslips were thoroughly washed and mounted in Mowiol® 4-88 and cells were then imaged using fluorescence microscopy at 100× oil immersion objective (Nikon Eclipse, Tokyo, Japan).

Measurement of caspase and calpain activities

Caspase-3 activity was determined by using the colorimetric substrate Ac-DEVD-p-nitroaniline, a synthetic substrate of caspase-3. Briefly, cells were collected from each treatment by scraping and were lysed on ice in cell lysis buffer (50 mM Hepes, 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA, pH 7.4) for 30 min. Fifty micrograms of cell homogenates were then incubated with 200 μM of colorimetric substrate in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mM EDTA, pH 7.4) in 96-well plates at 37°C for 24 hr. Absorbance of the cleaved product was measured at 405 nm using a microplate reader (BioRad). Results were expressed as the relative enzymatic activity per microgram of protein.

Calpain sample activity was measured by using the same commercial kit (Medical and Biological Laboratories Co Ltd, Nagoya, Japan) as was used to calculate caspase 3 activity. Briefly, fluorometric calpain substrate Ac-LLY-AFC was utilized according to the supplier’s instructions: 50 μg/μL of protein was incubated with 200 μM of fluorometric substrate in assay buffer (5 in 96-well plates) at 37°C for 24 hr. The negative control consisted of the calpain inhibitor Z-LLY-FMK. Fluorescence intensity of liberated AFC was monitored with a microplate spectro-fluorometer (Victor 3) using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Calpain activity was expressed as the relative enzymatic activity per microgram of protein.

Statistical analysis

Data are given as the mean ± S.E.M. of at least four experiments involving four to six independent cultures. In all experiments, data were analyzed by ANOVA followed by post hoc Tukey–Kramer multiple comparisons tests. P-values lower than 0.05 were considered significant.

Results

Consistent with previous studies, we demonstrated that MPP⁺, an inhibitor of mitochondrial complex I, induced...
neuronal cell death in CGNs. The viability of CGNs was assayed using the MTT method both 24 and 48 hr after MPP⁺ treatment (Fig. 1). MPP⁺-induced CGN death increased in a dose-dependent manner (Fig. 1). We successfully showed that treatment of CGNs with different concentrations of melatonin (over a range of 100 µM–1 mM) induced increases in neuronal cell viability as measured by the MTT method. Furthermore, we found that the viability of cells treated with 200 µM MPP⁺ over 48 hr was restored from 35% to 70% through treatment with 1 mM melatonin, and in a statistically significant manner (P < 0.01).

Subsequently, experiments were conducted to evaluate the anti-apoptotic properties of melatonin in CGNs in this in vitro model of PD. Changes in the neuronal features of apoptosis were assessed via two methods: (a) measuring DNA fragmentation by flow cytometry; and (b) analyzing the condensed nuclear fluorescence and counting by microscopy. Flow cytometry revealed differences between results obtained 24 and 48 hr following MPP⁺ treatment. Maximal apoptotic increase was detected at 48 hr after MPP⁺ treatment (Fig. 1). Analysis of DNA fluorescence by flow-cytometry (sub-diploid fluorescent PI peak) indicated that melatonin was capable of almost completely counteracting the 200 µM MPP⁺-induced apoptosis in neurons (Fig. 2). These data were corroborated by microscopic studies, whereby following MPP⁺ treatment, we noted a dramatic increase in condensed cell nuclei that was prevented by treatment with 1 mM melatonin.

Studies on cysteine proteases, calpain, and caspase 3, using the cleavage of the non-erythroid α-spectrin known as α-fodrin, demonstrated that MPP⁺ (200 µM) treatment of CGNs for 24 hr under our experimental conditions activates calpains and caspase 3 apoptotic routes. However, this increase was detected up to 12 hr following MPP⁺ treatment, as the 120 kDa fragment (indicative of caspase 3 activity) was very slight, although detectable with the Chemidoc System (BioRad). These results were confirmed with the use of specific fluorometric substrate in the case of calpain, or chromogenic substrate for that of caspase 3 (Fig. 3). We observed that the increase in both cysteine proteases had increased 35% 24 hr after treatment.

Using Western-blot and immunocytochemistry techniques, we evaluated those changes implicated in cdk5 expression following MPP⁺ (200 µM) treatment of CGNs. Our data demonstrated that the addition of MPP⁺ to CGNs prompted an increase in cdk5 expression 24 hr later that continued even 48 hr after treatment (Fig. 4A–C). In addition, we examined both the cleavage of p35 and the formation of the p25 fragment, as previous studies suggested that these processes were responsible for the induction of apoptosis in neurons. In fact, our Western-blot data demonstrate not only that MPP⁺ treatment evoked a time-dependent cleavage of p35–p25 (Fig. 4B,C), but also that the latter may constitute a route involved in MPP⁺-mediated apoptosis. Finally, treatment with melatonin 1 mM prevented formation of the cdk5/p25 fragment (Fig. 5A,B).

**Discussion**

Although current pharmacological treatment of PD can alleviate its symptoms over a limited time, it cannot halt the neuro-degeneration of nigrostriatal neurons. Therefore,
new pharmacological treatments are necessary to effect real protection of damaged neurons. Significant data from in vivo and in vitro studies suggest that antioxidant drugs are potential candidates, either alone or in concert with other drugs [1, 5]. Indeed, vitamin E reportedly guards against MPP+-mediated apoptosis in CGNs by inhibiting the intrinsic apoptotic pathway, and therefore, the activation of the caspase-3 pathway [8]. Furthermore, antioxidants such as coenzyme Q10, vitamin C, deferoxamine, and nitric oxide synthase inhibitors demonstrated neuro-protective properties in CGNs in vitro [7, 8]. Melatonin, a potent free radical scavenger, regulates the activity and expression of antioxidant enzymes [26–31]. Many previous studies have shown that melatonin protects the brain against a broad spectrum of injuries caused by agents or neurological injuries including MPTP [31], kainic acid [23], and nitroproponic acid [24]. These studies indicate that oxidative stress is a key feature of the neuro-degenerative process. Because of the efficacy of antioxidant drugs in neuronal cell cultures, clinical trials have been conducted to evaluate the potential application of these drugs in the treatment of PD. Coenzyme Q10 appears to be the antioxidant drug with the greatest potential application for treatment of PD in humans [34].

In the present study, we evaluated a potentially new drug for the treatment of PD, demonstrating long-term (48 hr) cell death and apoptosis following MPP+-inhibited melatonin treatment in CGNs. Moreover, our results are consistent with the majority of previously published data including our findings on the initial step of the PD process, in which mitochondrial perturbation induces alterations in mitochondrial respiration, oxidative stress, and drops in ATP levels [3, 4, 6, 7, 10, 11]. In the second step, mitochondria alterations generate the release of pro-apoptotic stimuli from the mitochondria to the nuclei [4]. It has thus been postulated that oxidative stress may constitute the initiating event driving neuronal cell death. Nonetheless, the pathways responsible for neuronal apoptosis are not yet fully understood.
Previous studies conducted by Gonzalez-Polo et al. [8] suggest a molecular apoptotic pathway through which the activation of some intrinsic pathway occurs and which effects the release of Cyt c from the mitochondria. It is well known that inhibition of complex I mitochondria is the principal target implicated in MPP+ neurotoxicity. In the present study, however, we examined the potential roles of the cysteine proteases calpain and caspase in the CGNs following exposure to MPP+. To this end, we evaluated the cleavage of the nonerythroid α-spectrin known as α-fodrin, one of the most well-characterized calpain substrates. In fact, calpain activation generates a characteristic fragment 145 kDa, while caspase activation generates a 120 kDa fragment. Our results demonstrate, consistent with those of Leist et al. [35], that calpain activation plays a prominent role in apoptosis as mediated by the inhibition of complex I.

CDKs have been implicated in the pathogenesis of several neurodegenerative disorders including Parkinson’s and Alzheimer’s diseases, as well as amyotrophic lateral sclerosis [37–39]. There is, therefore, increasing interest in the role of CDKs in the modulation of neuronal apoptosis via re-entry in the cell cycle. The main difference between cdk5 and the other CDKs is that the former exhibits a neuronal localization and is not implicated in cell-cycle progression. Moreover, cdk5 has also been implicated in both neurite outgrowth and neuronal differentiation [36, 37]. Indeed, previous studies have demonstrated that cdk5/p25, in tandem with GSK3β, is involved in the pathogenesis of Alzheimer’s disease via hyper-phosphorylation of tau.
and neurofilaments. In fact, the latter disrupts the physiological functions of cytoskeletal proteins, thereby inducing cell death [38–40].

Because MPP⁺ induced an increase in calpain activity, we investigated its potential target cdk5, as previous studies have demonstrated that the latter is not only activated by this cysteine protease, but is also involved in the process of neuronal cell death. In this way, calpain is the endogenous protease responsible for converting p35–p25 and increasing cdk5 activity [36–40]. Although numerous studies have described the neuro-protective properties of melatonin against a model of PD, few have demonstrated any antiapoptotic effects stemming from this antioxidant drug. Our results demonstrate that melatonin abolishes the formation of a cdk5/p25 fragment following treatment with MPP⁺. Moreover, these data could contribute to a better understanding of the apoptotic route in this PD model, and to a more detailed description of melatonin’s neuro-protective properties.

Nonetheless, one of the functions of cdk5 is the regulation of neurotransmission; specifically, cdk5 has been shown to play a role in dopaminergic neurotransmission [41]. Moreover, a recent study demonstrated that cdk5 regulates dopamine release in the striatum. It is therefore reasonable to speculate that, apart from its antioxidative and neuro-protective properties, melatonin may offer another potentially beneficial mechanism: the regulated release of neurotransmitters [41, 42].

In conclusion, the data from the present study indicate that melatonin provides strong neuro-protective and anti-apoptotic properties for CGNs against MPP⁺-induced neurotoxicity. In addition, calpain activation via MPP⁺ is implicated in the mechanisms of neurotoxicity. Although it has been postulated that melatonin exerts its neuro-protective effects through increases in complex I activation and inhibition of oxidative stress production, we have described in the present study a potential pathway involved in MPP⁺ neurotoxicity stemming from calpain activation and the cleavage of cdk5/p35 to cdk5/p25. Moreover, our results indicate that melatonin modulates the effects ultimately leading to neuronal death, and thus its role as a neuro-protective drug warrants further study.

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