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INHIBITION OF CYCLIN-DEPENDENT KINASES IS NEUROPROTECTIVE IN 1-METHYL-4-PHENYLPYRIDINIUM-INDUCED APOPTOSIS IN NEURONS

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Abstract—The biochemical pathways involved in neuronal cell death in Parkinson's disease are not completely characterized. Mitochondrial dysfunction, specifically alteration of the mitochondrial complex I, is the primary target of the parkinsonian neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) induced apoptosis in neurons. In the present study, we examine the role of caspase-dependent and -independent routes in MPP^+-induced apoptosis in rat cerebellar granule neurons (CGNs). We show a distinct increase in the expression of the cell cycle proteins cyclin D, cyclin E, cdk2, cdk4 and the transcription factor E2F-1 following a MPP^+ treatment of CGNs. Flavopiridol (FLAV), a broad inhibitor of cyclin-dependent kinases (CDKs), attenuated the neurotoxic effects of MPP^+ and significantly attenuates apoptosis mediated by MPP^+ 200 μM. Likewise, the antioxidant vitamin E (vit E) increases neuronal cell viability and attenuates apoptosis induced by MPP^+. Moreover, the expression levels of cyclin D and E2F-1 induced by this parkinsonian neurotoxin were also attenuated by vit E. Since, the broad-spectrum caspase inhibitor zVAD-fmk did not attenuate MPP^+-induced apoptosis in CGNs, our data provide a caspase-independent mechanism mediated by neuronal reentry in the cell cycle and increased expression of the pro-apoptotic transcription factor E2F-1. Our results also suggest a potential role of oxidative stress in neuronal reentry in the cell cycle mediated by MPP^+. Finally, our data further support the therapeutic potential of flavopiridol, for the treatment of Parkinson's disease. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cyclin dependent kinases, apoptosis, cerebellar granule cells, flavopiridol, vitamin E.

Parkinson's disease (PD), along with Alzheimer's disease, is probably the most common neurodegenerative disorders affecting the human population in old age (Moore et al., 2005; Cookson, 2005). Several neurotoxins have been used to reproduce the disease and to study the potential mechanisms involved in PD. Thus, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin widely used in experimental neuroscience as a Parkinson's model in laboratory animals (Tretter et al., 2004; Crocker et al., 2003; Chee et al., 2005). Once administered MPTP is converted by glial cells into 1-methyl-4-phenylpyridinium (MPP^+), an inhibitor of the complex I mitochondrial respiratory chain that is responsible for neuronal toxicity (Gonzalez-Polo et al., 2004a). Therefore, the main pathway proposed to explain the mechanism involved in PD centers upon mitochondrial alteration, oxidative stress generation, and apoptosis probably caused by caspase-3 activation (Chee et al., 2005; Beal, 2004; Gonzalez-Polo et al., 2004a; Choi et al., 2001; Dodel et al., 1999; Du et al., 1997).

Furthermore, the hypothesis of reactive oxygen species (ROS) production is supported by *in vitro* studies showing that such antioxidants as vitamin E (vit E), coenzyme Q_{10}, and other compounds with scavenger properties exert neuroprotective properties in neuronal cell cultures (Gonzalez-Polo et al., 2004a; Beal 2004). Likewise, Gonzalez-Polo et al. (2001, 2003a,b, 2004a,b) recently demonstrated that cerebellar granule neurons (CGNs) undergoing MPP^+ treatment could induce apoptosis through mitochondrial alteration, caspase-3, activation and ROS generation. The same authors suggested that caspase activation, specifically of caspase-3, constitutes the last step and is the effector of this process. The role of caspase activation in MPP^+ has been supported by other studies showing that caspase inhibitors offer robust neuroprotection (Gonzalez-Polo et al., 2004a).

However, others authors have suggested that additional cysteine proteases could contribute to the apoptotic process mediated by complex I inhibition (Leist et al., 1998). Among them are previous studies indicating that calpains, which are cysteine proteases activated by calcium, may be involved in the apoptotic process mediated by complex I inhibition in neuronal cell preparations. Moreover, Crocker et al. (2003), in an excellent study adminis-

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Abbreviations: AIF, apoptosis-inducing factor; CGN, cerebellar granule neuron; FLAV, flavopiridol; H_2DCFDA, 2,7-dichlorodihydrofluorescein diacetate; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium; PBS, phosphate-buffered saline; PCNA, proliferating control nuclear antigen; PD, Parkinson's disease; PI, propidium iodide; ROS, reactive oxygen species; TBS-T, Tris 50 mM, NaCl 1.5%, Tween 20, 0.05%, pH 7.5; vit E, vitamin E; 3-MA, 3-methyladenine.

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tering calpain inhibitors in a mice model of PD, demonstrated the beneficial neuroprotective and behavioral effects of calpain inhibitors.

In an attempt to characterize the neuronal apoptotic mechanisms mediated by mitochondrial respiratory chain complex I inhibition, a caspase independent pathway has been recently suggested. Under this hypothesis MPP^+ induces a release of the apoptosis-inducing factor (AIF), which is regulated by calpain activation (Chu et al., 2005; Liou et al., 2005). Thus, these studies may explain why caspase inhibitors do not completely attenuate the apoptotic process mediated by inhibition of the mitochondrial complex I.

In the present study, therefore, we attempted to investigate the possible role of caspase-dependent and -independent routes in MPP^+-induced apoptosis in CGNs. In addition to the role cysteine proteases play in the apoptotic route orchestrated by MPP^+, recent studies suggest the potential role of cell cycle activation in neuronal apoptosis (Smith et al., 2004; Raina et al., 2004; Bowser and Smith, 2002; Giovanni et al., 1999; Copani et al., 2001a,b, 2002a,b). The hypothesis regarding the role of cell cycle reentry as a potential mechanism involved in neuronal cell death is based on studies performed in brains of Alzheimer's disease and PD patients where there is an increase in the expression of proteins involved in the cell cycle (Yang et al., 2001, 2003; Jordan-Sciutto et al., 2003). Subsequent studies performed in neuronal cell preparations using different neurotoxins such as kainic acid (an agonist of non-NMDA receptors), camptothecin (a DNA damaging agent), and β-amyloid (an *in vitro* model of AD), suggest that re-expression of cell cycle proteins could constitute a common pathway involved in the mechanisms underlying neuronal cell death (Giovanni et al., 1999; Copani et al., 2001a; Verdaguer et al., 2004). Although the exact mechanism by which neuronal reentry in the cell cycle induces apoptosis is not completely known, several studies suggest that the transcription factor E2F-1 could be the link (Jordan-Sciutto et al., 2003; Hou et al., 2000; Raina et al., 2001; Klein et al., 2002; Biswas et al., 2005; Stevens and La Thangue, 2003, 2004). Another interesting point lending support to the potential role of cell cycle reentry in neuronal apoptosis is that cyclin-dependent kinase inhibitors, mainly flavopiridol (FLAV), offer strong neuroprotective properties against several experimental apoptotic paradigms both "*in vitro*" and "*in vivo*" (Verdaguer et al., 2004; Di Giovanni et al., 2005; Cernak et al., 2005; Mirjany et al., 2002; Wu et al., 2004; P.D. Smith et al., 2003; Wang et al., 2002; Webber et al., 2005). Since a characteristic common to all neurodegenerative diseases is the generation of oxidative stress production, one of the aims of the present study was to determine a relationship between oxidative stress production and reentry in the cell cycle (Herrup et al., 2004; Langley and Ratan, 2004; Becker and Bonni 2004). To answer all of these questions in the present manuscript, we evaluated the neuroprotective effects of vit E and the broad-spectrum CDK inhibitor FLAV on MPP^+-induced apoptosis in CGNs. A study addressing the role of cell cycle reentry in the apoptotic route mediated by the parkinsonian neurotoxin MPP^+ may be of potential interest to the development of pharmacological treatments for PD.

EXPERIMENTAL PROCEDURES

Materials

Pharmacological agents used in this study include: MPP^+, rotenone, 3-methyladenine (3-MA), vit E, mimosine, roscovitine and propidium iodide (PI) from Sigma Chemical Co (St. Louis, MO, USA). zVAD-fmk was obtained from Bachem AG (Bubendorf, Switzerland), PD151746 from Calbiochem (Darmstadt, Germany), and cell culture media and fetal calf serum (FCS) from GIBCO (Life Technologies, Paisley, UK). FLAV was a gift from Aventis Inc.

Cell cultures salts, enzymes, Mowiol® 4–88 and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Other chemical reagents were of analytical quality and purchased from Sharlab (Barcelona, Spain).

Cell cultures

Primary cultures of CGNs were prepared from postnatal day 7 Sprague–Dawley rat pups as described previously (Verdaguer et al., 2002). Cells were dissociated in the presence of trypsin and DNase I and plated in poly-L-lysine (100 μg/ml)-coated dishes at a density of 8×10^5 cells/cm^2 in basal Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mg/ml gentamicin, 2 mM L-glutamine, and 25 mM KCl. Cytosine-D-arabinofuranoside (10 μM) was added always to the culture medium 24 h after plating to prevent the replication of non-neuronal cells. The cultures were maintained at 37 °C in a humidified incubator with 5% CO_2, 95% air and left undisturbed until experiments were performed. All procedures involving animals and their care were approved by the ethics committee of the University of Barcelona, and were conducted in accordance with national (Generalitat de Catalunya) and international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, DC, 1996). All efforts were made to minimize the number of animals used and their suffering.

Treatment of CGNs and viability assays

CGNs were used after 7–10 days of *in vitro* culture. Drugs (FLAV, zVADfmk, 3-MA, PD151746) were added at the same time as MPP^+ (200 μM) to determine their 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 h, allowing the reduction in MTT to produce a dark blue formazan product. Media were then removed, and cells were dissolved in dimethylsulfoxide. Formazan production was measured by the absorbency change at 595 nm using a microplate reader (BioRad Laboratories, Richmond, CA, USA). Viability results were expressed as percentages. The absorbency measured from non-treated cells was taken to be 100%.

Analysis of apoptosis by flow cytometry

Apoptosis was measured upon 48 h of MPP^+ treatment. Briefly, culture medium was removed, with the cells then collected from culture plates by pipetting and washing with PBS. Flow cytometer experiments were carried out using an Epics XL flow cytometer with PI (10 μg/ml) added 30 min beforehand. 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