EXPERIMENTAL CELL RESEARCH 332 (2015) 60–66

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Research Article

Differential regulation of caspase-2 in MPP$^+$-induced apoptosis in primary cortical neurons

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ARTICLE INFORMATION

Article Chronology:
Received 1 September 2014
Received in revised form
15 December 2014
Accepted 21 January 2015
Available online 30 January 2015

Keywords:
Parkinson's disease
MPP$^+$
Apoptosis
Primary cortical neurons
Caspase-2
Mitochondria

ABSTRACT

Parkinson's disease (PD), among the most common neurodegenerative diseases worldwide for which there is no cure, is characterized as progressive dopaminergic neuron loss in the substantia nigra through an unknown mechanism. Administering 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) causes neuronal cell death and Parkinsonism in humans. Commonly used in animal models of PD, MPTP can metabolize to 1-methyl-4-phenylpyridinium (MPP$^+$); however, the detailed mechanism through which MPP$^+$ causes neuronal cell death remains undetermined. Previous reports have indicated those knockout mice with Bcl-2 associated protein X (Bax) or caspase-2, two mitochondrial outer membrane permeabilization inducers, are resistant to MPTP administration, suggesting that mitochondria are involved in MPP$^+$-triggered apoptosis. Our previous study showed that MPP$^+$-triggered apoptosis can be distinguished from spontaneous apoptosis of primary cortical neurons. In the present study, we verified the involvement of mitochondria in MPP$^+$-induced and spontaneous apoptosis in cortical neurons through confocal microscope analysis. We demonstrated that caspase-2 activation is specific to MPP$^+$-induced apoptosis and occurs before Bax translocation to the mitochondria. Caspase-2 activation is one of the few early molecular events identified in PD models.

Introduction

Parkinson's disease (PD) is among the most common neurodegenerative disorders, affecting more than 6 million people worldwide [1]; however, no cure has been discovered. PD is classified as a motor neuron defect caused by progressive loss of dopaminergic neurons in the substantia nigra pars compacta and other monoaminergic neurons in the cerebral cortex [2,3]; the

Abbreviations: PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MPP$^+$, 1-methyl-4-phenylpyridinium; MOMP, mitochondrial outer membrane permeabilization; PCD, programmed cell death pathway; Bax, Bcl-2 associated protein X; JNK, stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase; DAPI, 4′-6-diamidino-2-phenylindole; Smac, second mitochondria-derived activator of caspase; AIF, apoptosis inducing factor

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http://dx.doi.org/10.1016/j.yexcr.2015.01.011
0014-4827/

progressive loss of neurons are involved in cell death caused by apoptosis, autophagic cell death, or programmed necrosis [4,5]. The major symptoms of PD are resting tremor, slowness of voluntary movement, rigidity, and postural instability [6]. Knowledge of how the neurodegenerative process occurs in dopaminergic neurons has been derived primarily from neurotoxin-induced animal models of PD [6,7].

1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), one of the most extensively characterized and widely used toxin in animal models of PD, is a byproduct of meperidine analog synthesis and exerts potent heroin-like effects that can induce Parkinsonism in humans. After administration, MPTP can penetrate the blood–brain barrier and be metabolized to 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B in glial cells. Once MPP^+ is transmitted to neurons by dopaminergic transporters, it impairs mitochondria complex I, subsequently increases free radicals, and finally induces oxidative stress in the mitochondria, initiating a programmed cell death (PCD) pathway [8]. However, the mechanism through which PCD occurs after MPP^+ impairs mitochondria complex I remain unclear [9]. PCD, also known as apoptosis, is characterized by membrane blebbing, shrinkage of the cell body, nuclear condensation, and DNA fragmentation. Induction of Bcl-2 associated protein X (Bax) and mitochondrial outer membrane permeabilization (MOMP) has been suggested to result in cytochrome *c* release into the cytosol of the ventral midbrain in an MPTP mouse model [10,11]; this suggestion is consistent with the observation that Bax knockout mice were more resistant to MPTP neurotoxicity than were wild type mice [11]. Caspases are a family of cysteine-specific protease associated with the initiator or effector of apoptosis [12]. Caspase-2 induces MOMP through cleavage and activation of Bid [13]. Capase-2 knockout mice exhibited greater resistance to MPTP-induced neurotoxicity than did wild type mice [14]. In addition, previous studies have reported that caspase-2 was involved in MPP^+-induced cell death in primary cultures of mesencephalic dopaminergic neurons and the MN9D mouse dopaminergic cell line [15,16]. This evidence indicates that the mitochondria-mediated apoptosis pathway is involved in MPTP-induced neurotoxicity. Caspase-2 is one of the less extensively characterized members in the caspase family; its role in PD and its relationship with Bax in MPP^+-induced neuronal cell death require further investigation.

Currently, there are no effective treatments for PD. Administering a dopamine agonist (L-dopa) combined with other enzyme inhibitors, such as the MAO-B inhibitor and peripheral decarboxylase inhibitors, to patients with PD is a symptomatic approach for ameliorating the motor deficits associated with PD [6]. Furthermore, long-term treatment with L-dopa was reported to lead to dyskinesia in patients with young-onset PD [17]. Because there is no effective treatment, a treatment that reduces neuronal degeneration is urgently required.

Our research team established a primary cortical neuron culture system to investigate the molecular mechanism of MPP^+-induced neuronal cell death [18]. In this culture system, we observed that two types of caspase-3-mediated apoptosis contributed to total cell death: MPP^+-triggered apoptosis and basal-level apoptosis, which spontaneously occurs in MPP^+-untreated long-term culture cells. The regulation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) activity in these two apoptotic responses differs [18]. In the present study, we characterized the molecular mechanism of mitochondria-mediated apoptosis in these two apoptotic responses.

Materials and methods

Animals

ICR mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and maintained in the Animal Experimentation Center of Tzu-Chi University. Two female mice were placed with one male mouse at 5:00 p.m., and the female mice were separated from the male mice the next morning. The pregnant mice were examined using vaginal plugs and sacrificed 13.5 days after gestation. All procedures involving animal manipulation were conducted according to the national guidelines of the Animal Protection Act (Taiwan) and approved by the Institutional Animal Care and Use Committee of Tzu-Chi University.

Primary cortical neuronal cell cultures

Cortical neurons were isolated from ICR mice embryos 13.5 days after gestation as described previously [18]. Neuronal cells were seeded on 96-well dishes (6×10^4 cells/well) for a viability assay, on 24-well dishes (1.5×10^5 cells/well) for an immunocytochemistry assay, and on 100 mm culture dishes (6.5×10^6 cells/well) for protein extraction. Culture dishes 100 mm in diameter were precoated with polyethylenimine (1 mg/mL, Sigma) overnight at 37 °C. Neuronal cells placed in 24-well and 96-well culture dishes were seeded on a 5×5-mm^2 piece of glass, which was precoated with poly-D-lysine (50 μg/mL, Sigma) for 30 min at 37 °C. Cells were cultured in Dulbecco's Modified Eagle's Medium for 4 h before attachment. Neurobasal (Gibco BRL) supplemented with 2% B27 (Gibco BRL) and 10% fetal calf serum (Biological Industries) was used to culture primary cortical neurons.

Cell viability

The cortical neurons were cultured on 96-well dishes for 6 days (days *in vitro*, DIV 6) and then treated with 20 μM MPP^+ for various time periods (6, 18, 24, 36, or 48 h). Untreated cortical neurons cultured for the same time periods were used as controls. For a caspase-2 inhibition assay, the DIV 6 cortical neurons were treated with 50 μM z-VDVAD-fmk (Calbiochem) for 1 h at 37 °C, and some of the neurons were then treated with 20 μM MPP^+ for various time periods (6, 24, or 48 h). Untreated caspase-2 inhibitor neurons at the same time points were used as controls. After the cell were washed with phosphate-buffered saline (PBS) once, the level of viable cells was observed and photographed using an inverted microscope (Axiovert 40 CFL, Carl Zeiss, Gottingen, Germany) and analyzed using the WST-1 (4-[3- (4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate) kit according to the manufacturer's instructions (Roche Diagnostics). WST-1, a tetraxolium salt (slightly red), can be cleaved to formazan (dark red) by mitochondrial dehydrogenases. The absorbance of the formazan at 440 nm is correlated with the number of viable cells.