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MPP+-induces PUMA- and p53-dependent, but ATF3-independent cell death

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HIGHLIGHTS

- PUMA and p53 mediate MPP+-induced toxicity.
- ATF3 does not mediate cell loss in response to MPP+.
- MPP+-induced UPR may represent a protective mechanism in response to protein damage.

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ABSTRACT

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons in the substantia nigra (SN) and depletion of striatal dopamine (DA), leading to a range of motor symptoms, including resting tremor, rigidity, bradykinesia and postural abnormalities. The neurotoxin (MPTP) and its active metabolite, 1-methyl-4-phenylpyridinium (MPP+), cause dopaminergic cell loss in a variety of animal species and produce symptoms similar to those seen in PD. Our lab has shown that MPP+ activates cell stress pathways, including the unfolded protein response (UPR) in mouse primary mesencephalic cultures. The BH3-only protein, PUMA (p53 upregulated mediator of apoptosis), has been shown to be activated in response to many cellular stresses, including endoplasmic reticulum (ER) stress and UPR, and to induce cell death. Therefore, we hypothesized that PUMA may mediate MPP+ toxicity. To test this hypothesis, we compared the response of primary mesencephalic cultures from wild-type and PUMA deficient (−/−) mice to MPP+. We also utilized cultures from p53 −/− and activating transcription factor 3 (ATF3) −/− mice to further elucidate the pathways involved. These studies revealed that PUMA and p53, but not ATF3, are required for MPP+-induced cell death, suggesting that UPR activation is parallel to the induction of MPP+-induced cell death.

1. Introduction

PD is the second most common neurodegenerative disorder and is characterized by the progressive loss of dopaminergic neurons in the SN and the resulting loss of dopaminergic innervation to the striatum. Although the molecular mechanisms underlying the pathogenesis of PD remain unclear, oxidative stress, mitochondrial dysfunction and ER stress have all been implicated in the etiology of this disorder (Dauer and Przedborski, 2003; Vila and Przedborski, 2004).

The neurotoxin, MPTP, and its active metabolite, MPP+, are used to model dopaminergic degeneration *in vivo* and *in vitro*, respectively (Blum et al., 2001). After intraperitoneal injection of MPTP, it crosses the blood–brain barrier and is rapidly converted to its active metabolite, MPP+, by monoamine oxidase B (MAOB) in glial cells (Markey et al., 1984). In *in vitro* systems, direct application of MPP+ induces cell death specifically in dopaminergic neurons due to its high affinity for the dopamine transporter (DAT) and other catecholamine uptake systems. Once inside the cell, MPP+ is sequestered into vesicles by the vesicular monoamine transporter 2 (VMAT2), displacing DA in the process (Staal and Sonsalla, 2000). The toxicity of MPP+ is likely due to its actions at cytosolic and mitochondrial sites of action rather than through the displacement of DA from vesicles to cytosol as dopamine deficient animals do not show altered MPTP toxicity (Hasbani et al., 2005).

MPTP- and MPP+-induced toxicity seems to involve activation of UPR and ubiquitin–proteasome system (UPS) dysfunction. UPR can be triggered by any or all of three different gatekeeper proteins: IRE1, PERK and ATF6 (Zhang and Kaufman, 2004, 2006). In cell lines and primary dopaminergic neurons, MPP+ activates UPR; however, which branches are upregulated varies by cell type (Holtz and O'Malley, 2003; Ryu et al., 2002). For example, in PC12 cells, both the PERK and IRE1 branches are activated, while in a CNS dopaminergic cell line, only the PERK pathway is activated. Whether this activation of UPR is protective or leads to cell death is not yet clear. Recent evidence suggests that it may be protective, as Xbp-1 overexpression protects against both MPTP *in vivo* and MPP+ *in vitro*

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(Sado et al., 2009) and ATF6α deletion accelerates MPTP toxicity (Egawa et al., 2011; Hashida et al., 2012).

Many studies have demonstrated the induction of at least some markers of apoptosis by MPTP and MPP^+ (Blum et al., 2001). However, whether MPTP and MPP^+ induce bona fide apoptosis or other forms of cell death depends on both the dosing paradigm and the cell type. For example, MPTP induces both caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage in the SN, if the toxin is given in small doses over five days (chronic model), but not if given in small doses within a single day (acute model) (Blum et al., 2001). In addition, chronic MPTP exposure leads to elevation of Bax mRNA and decreased Bcl-2 levels in the mouse SN (Vila et al., 2001). Using this same dosing paradigm, deletion of Bax is protective against MPTP (Vila et al., 2001). However, in primary dopaminergic neurons, Bax deletion did not protect against MPP^+ (O'Malley et al., 2003). Thus, exactly how MPTP/MPP+ leads to cell death in dopaminergic neurons is still unclear (Blum et al., 2001).

Many studies have suggested that prolonged and severe UPR can lead to cell death, possibly *via* apoptosis (Zhang and Kaufman 2004, 2006). Amongst other mechanisms, BH3-only proteins, such as PUMA, have been hypothesized to serve as links between ER stress pathways and apoptosis (Nakano and Vousden, 2001; Reimertz et al., 2003; Yu et al., 2001). We have previously demonstrated that PUMA mediates cell death induced by another parkinsonian mimetic, 6-hydroxydopamine (Bernstein et al., 2011). Therefore, we tested whether PUMA also mediates cell death in response to MPP^+ exposure. Here, we show that PUMA is required for MPP^+-induced cell death in primary mesencephalic cultures. However, further analysis using animals deficient in a key UPR pathway (ATF3) suggests that MPP^+-mediated cell death is independent of the upregulation of UPR. Instead, the DNA damage pathway (p53) may play a more direct role in mediating cell death in this model of PD.

2. Materials and methods

2.1. Animals

Animals were treated in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Wild-type C57/Bl6 mice were from Charles River Laboratories (Wilmington, MA). *Puma* knockout mice were previously generated and characterized (Jeffers et al., 2003). *ATF3* knockout mice were generated and provided by Dr. Tsonwin Hai (Ohio State University) (Hartman et al., 2004). *p53* knockout mice were provided by Dr. Helen Pwinica-Worms (Washington University Medical School) (Jacks et al., 1994).

2.2. Cell cultures and toxin treatment

For RT-PCR experiments, embryonic day 14 (E14) C57Bl/6 murine midbrains (Charles River Laboratories, Wilmington, MA, USA) were pooled and prepared as described previously (Lotharius et al., 1999). To assess survival of neurons after MPP^+ treatment, mice heterozygous for *Puma*, *ATF3* or *p53* were mated to produce wild type, heterozygous, and homozygous deficient embryos. Cultures were derived from individual pups and pups were individually genotyped using cortical tissue as described previously (Antenor-Dorsey and O'Malley, 2012; Bernstein et al., 2011). After seven days *in vitro* (DIV7), cells were treated with 1 μM MPP^+, a dose that produces 50% loss of dopaminergic neurons 48 h after treatment. Previous experiments from the lab have established this exposure paradigm (Antenor-Dorsey and O'Malley, 2012; Holtz and O'Malley, 2003; Kim-Han et al., 2011; Lotharius et al., 1999; O'Malley et al., 2003).

2.3. Reverse transcription PCR

Dissociated midbrain neurons were plated in 12-well plates and treated with MPP^+ at DIV7. Cultures were washed with PBS, total RNA was extracted (RNeasy Mini Kit; Qiagen, Valencia, CA) and then reverse transcribed (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Levels of *Puma* and *18S rRNA* were analyzed by semi-quantitative reverse transcription PCR (RT-PCR) using primers specific for the gene of interest. All reactions were performed in triplicate. PCR products were resolved with polyacrylamide gel electrophoresis, visualized (SYBR Safe DNA; Invitrogen) and imaged (Storm PhosphorImager; Molecular Dynamics, Piscataway, NJ). Band intensities were measured (ImageQuant; Amersham Biosciences, Piscataway, NJ) and *Puma* levels were normalized to 18S rRNA levels and then compared to levels in untreated samples. Data was pooled from 3 independent experiments. The following primer sequences were used:

18S rRNA (5′-GGGAACGCGTGCATTTATCAG-3′, 5′-CGCTATTGGAGCTGGAATTAC-3′)
PUMA (5′-ACGACCTCAACGCGCAGTA-3′, 5′-CTAGTTGGGCTCCATTTCTGG-3′).

2.4. Immunocytochemistry

Primary cultures were plated in 7 mm microwell plates (MatTek Corp., Ashland, MA). Cells were treated with MPP^+ and fixed with 4% paraformaldehyde in PBS after the appropriate incubation time. Cultures were stained with sheep polyclonal anti-tyrosine hydroxylase (TH) (Novus Biologicals, Littleton, CO) and Alexa488 α-sheep (Molecular Probes, Carlsbad, CA). Cultures were co-stained for NeuN where with mouse monoclonal α-NeuN (Chemicon, Billerica, MA). Cy3 α-mouse secondary antibody was purchased from Jackson Labs (Bar Harbor, ME). Cells were counted using unbiased stereological methods modified for use in a cell culture dish (Stereo Investigator, MicroBrightField, Williston, VT) (Antenor-Dorsey and O'Malley, 2012; Bernstein et al., 2011; Kim-Han et al., 2011). The estimated total number of TH neurons in the culture dish was calculated based on the following formula:

$$N = Q\text{-} \times \frac{1}{\text{ssf}} \times \frac{1}{\text{asf}},$$

where N is the estimate of the total number of cells, Q- is the number of objects counted, ssf is the section sampling fraction and asf is the area sampling fraction. Gundersen ($m = 1$) coefficients of error were less than 0.1. TH-positive neurite length was estimated by an unbiased stereological method (Petrimetrics, Stereo Investigator, MicroBrightField). Cell counts and neurite length were normalized to untreated control for each pup. Images were acquired by confocal microscopy (Olympus Fluoview 500, Olympus, Center Valley, PA) and processed in ImageJ.

2.5. Statistical analysis

GraphPad Prism software (San Diego, CA) was used for statistical analysis. All data were collected from a minimum of three independent experiments. The significance of effects between control and drug treatment conditions was determined by one-way ANOVA with Bonferroni Multiple Comparisons tests. The significance of effects between genotypes and drug treatment conditions was determined by two-way ANOVA with Bonferroni post-tests.

3. Results

3.1. MPP^+ induces upregulation of PUMA mRNA

Previously, we used an unbiased, bioinformatics approach to survey genes responding to MPP^+ treatment. This analysis did not detect changes in known BH3-only proteins; however, PUMA was not represented on the chip surveyed (Holtz and O'Malley, 2003). Since PUMA has been shown to be induced by ER stress and to trigger mitochondrial events leading to cell death in a variety of cell types, we sought to determine if PUMA is transcriptionally upregulated in response to MPP^+. Primary mesencephalic cultures were treated with MPP^+ and PUMA mRNA levels were analyzed by RT-PCR and normalized to 18S rRNA mRNA levels. Levels of PUMA mRNA were significantly increased by 12 h after treatment with MPP^+ (Fig. 1A and B). These results demonstrate that MPP^+ induces the upregulation of PUMA mRNA, suggesting that PUMA does play a role in MPP^+-mediated cell death.

3.2. Loss of PUMA protects against MPP^+ toxicity

To determine if PUMA plays an essential role in MPP^+ toxicity, primary mesencephalic cultures from PUMA +/+ and −/− mice were treated with or without MPP^+. After 48 h, cells were fixed and stained for TH, a marker of dopaminergic neurons, and NeuN, a marker of neuronal nuclei. TH-positive and NeuN-positive cells were counted by a non-biased stereological method and the percentage of TH-positive cells was calculated to determine the level of cell survival. In wild-type cultures, MPP^+ induced a 50% loss of TH neurons, while only about 20% of TH neurons were lost in PUMA −/− cultures (Fig. 1C and D). Neurite length was also estimated and loss of PUMA did not prevent loss of neurites (data not shown).