Caspase Inhibitors Attenuate 1-Methyl-4-Phenylpyridinium Toxicity in Primary Cultures of Mesencephalic Dopaminergic Neurons

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Parkinson's disease is characterized by a loss of dopaminergic nigrostriatal neurons. This neuronal loss is mimicked by the neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺). MPP⁺ toxicity is mediated through inhibition of mitochondrial complex I, decreasing ATP production, and upregulation of oxygen radicals. There is evidence that the cell death induced by MPP⁺ is apoptotic and that inhibition of caspases may be neuroprotective. In primary cultures of rat mesencephalic dopaminergic neurons, MPP⁺ treatment decreased the number of surviving dopaminergic neurons in the cultures and the ability of the neurons to take up [³H]dopamine ([³H]DA). Caspase inhibition using the broad-spectrum inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) spared MPP⁺-treated dopaminergic neurons and increased somatic size. There was a partial restoration of neurite length in zVAD-fmk-treated cultures, but little restoration of [³H]DA uptake. Peptide inhibitors

of caspases 2, 3, and 9, but not of caspase 1, caused significant neuroprotection. Two novel caspase inhibitors were tested for neuroprotection, a broad spectrum inhibitor and a selective caspase 3 inhibitor; both inhibitors increased survival to >90% of control. No neuroprotection was observed with an inactive control compound. MPP⁺ treatment caused chromatin condensation in dopaminergic neurons and increased expression of activated caspase 3. Inhibition of caspases with either zVAD-fmk or a selective caspase 3 inhibitor decreased the number of apoptotic profiles, but not expression of the active caspase. We conclude that MPP⁺ toxicity in primary dopaminergic neurons involves activation of a pathway terminating in caspase 3 activation, but that other mechanisms may underlie the neurite loss.

Key words: Parkinson's disease; apoptosis; MPP⁺; caspase; neuroprotection; dopaminergic neurons

Parkinson's disease is a neurodegenerative condition characterized by rigidity and akinesia. A major pathological hallmark of Parkinson's disease is the degeneration of nigrostriatal dopaminergic neurons (Marsden, 1990), which is mimicked *in vivo* by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The toxicity of MPTP is mediated through the toxic metabolite, 1-methyl-4-phenylpyridinium (MPP⁺). The mechanism by which MPP⁺ kills dopaminergic neurons is unclear. MPP⁺ is known to inhibit mitochondrial complex I, decreasing cellular metabolism and increasing generation of oxygen radicals (Akaneya et al., 1995; Degli, 1998; Schapira, 1998). Evidence has emerged recently that MPP⁺ treatment may lead to apoptosis.

After MPTP or MPP⁺ treatment, apoptotic nuclei have been detected *in vivo* (Tatton and Kish, 1997) and *in vitro* (Mochizuki et al., 1994; Dodel et al., 1998; Eberhardt et al., 2000). Transgenic mice overexpressing anti-apoptotic Bcl-2 are resistant to MPP⁺ toxicity *in vitro* and MPTP toxicity *in vivo* (Offen et al., 1998; Yang et al., 1998). Inhibition of caspases, mediators of the apoptotic response, has been reported to prevent MPP⁺-mediated cell death *in vitro* (Du et al., 1997; Dodel et al., 1998). Mice overexpressing dominant negative caspase 1 have been shown to be resistant to MPTP toxicity *in vivo* (Klevenyi et al., 1999), and activation of caspases 3, 8, and 2 has been reported in the substantia nigra of MPTP-treated mice (Yang et al., 1998; Hart-

mann et al., 2001; Turmel et al., 2001). Both caspase inhibition and overexpression of inhibitor of apoptosis protein (IAP) have been shown to protect dopaminergic neurons from MPP⁺ *in vivo* and *in vitro* (Eberhardt et al., 2000).

Although these data indicate that MPP⁺ toxicity is mediated by caspase activation and subsequent apoptosis, reports conflict regarding the mechanism of MPP⁺ toxicity *in vitro* and the efficacy of caspase inhibition. Lotharius and coworkers (1999) found no evidence of phosphatidylserine externalization, a marker of apoptosis, after MPP⁺ treatment of mesencephalic neurons, and they reported that the toxicity was not inhibited by treatment with a broad-spectrum caspase inhibitor. Hartmann and coworkers (2001) reported that caspase inhibition potentiated MPP⁺-mediated cell death *in vitro* by increasing necrosis, unless neurons were maintained in elevated glucose levels.

Thus, the mechanism of MPP⁺ toxicity *in vitro*, and the role of caspases, is unclear. In this study we have tested a number of peptide caspase inhibitors for neuroprotective effects against MPP⁺ toxicity in rat mesencephalic dopaminergic neurons *in vitro*, together with two novel caspase inhibitors and an inactive analog. MPP⁺-treated dopaminergic neurons show apoptotic profiles and express activated caspase 3. Caspase inhibition restores the number of surviving dopaminergic neurons and increases somatic size and neurite length in these neurons but is less effective in restoring [³H]DA uptake. Broad-spectrum caspase inhibitors caused survival of dopaminergic neurons to >90% of untreated control, as did a novel caspase 3 inhibitor. These data suggest that the pathways activated by MPP⁺ in this culture system converge during caspase 3 activation and that

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inhibition of caspase 3 is sufficient to prevent the MPP⁺-mediated death of these neurons.

MATERIALS AND METHODS

Materials. Pregnant Sprague Dawley rats were purchased from Harlan Seralabs. DMEM, HBSS, and trypsin were purchased from Invitrogen (Paisley, UK). Fetal bovine serum (FBS), mazindol, antibiotic/antimycotic solution, Cy-3-conjugated goat anti-rabbit IgG, extravidin-FITC, and tetramethylrhodamine isothiocyanate-conjugated anti-rabbit IgG were purchased from Sigma-Aldrich Co. (Poole, UK). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (zDEVD-fmk), benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (zLEHD-fmk), benzyloxycarbonyl-Tyr-Val-Ala-Asp-chloromethylketone (zYVAD-cmk), and the FITC-FragEL apoptosis detection kit were all purchased from Calbiochem/Novabiochem (Nottingham, UK). Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). MPP⁺ iodide was purchased from RBI. Vectastain Elite ABC kits, Vector SG insoluble peroxidase substrate, and normal goat serum were obtained from Vector Laboratories (Peterborough, UK). Rabbit polyclonal anti-tyrosine hydroxylase (TH) antiserum was purchased from the Institut Jacques Boy (Reims, France). Mouse monoclonal anti-TH was purchased from Chemicon. Rabbit anti-cleaved caspase 3 was purchased from New England Biolabs. Sato serum substitute (Bottenstein and Sato, 1979) was made in-house (final concentration in medium: 4.3 mg/ml bovine serum albumin, 0.77 μ g/ml progesterone, 20 μ g/ml putrescine, 0.49 μ g/ml L-thyroxine, 0.048 μ g/ml selenium, and 0.42 μ g/ml tri-iodo-thyronine). All components of this serum substitute were purchased from Sigma-Aldridge Co. [³H]DA was purchased from Amersham Biosciences.

Mesencephalic cultures. The ventral mesencephalon was dissected from 14 d gestation Sprague Dawley rat embryos (Harlan Ltd.). Tissues were incubated with 0.25% trypsin in HBSS for 20 min at 37°C/5% CO₂, then mechanically dissociated using a flame-polished Pasteur pipette. For cell survival assays, cells were plated at a density of 200,000 cells per well onto poly-D-lysine-coated eight-well chamber slides (Invitrogen) in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution and incubated for 2 hr. This medium was then aspirated and replaced with DMEM supplemented with Sato serum substitute. Cultures were incubated for a further 5 d before experimental procedures.

Treatment with compounds. zVAD-fmk, zDEVD-fmk, zLEHD-fmk, and zYVAD-cmk were prepared in DMEM supplemented with Sato and added to the cultures 15 min before MPP⁺ exposure at concentrations ranging from 0.1 to 300 μ M. Each compound was added to four independent wells at each concentration tested. Control cultures were returned to DMEM/Sato in the absence of compounds. MPP⁺ iodide was prepared at a concentration of 110 μ M, then added directly to the medium in the wells to give a final concentration in each well of 10 μ M; control cultures were treated with tissue culture medium in the absence of MPP⁺. Cultures were incubated at 37°C/5% CO₂ for a further 48 hr, then were fixed using 4% paraformaldehyde in PBS and immunostained for TH.

Determination of TH-immunoreactive neuronal survival. To determine the number of surviving dopaminergic neurons, immunocytochemistry was performed using a rabbit polyclonal antibody raised against TH. Nonspecific binding sites were blocked using 10% normal goat serum in PBS, then primary antibody was added at 4°C overnight. The next day, the cells were washed and treated with biotin-conjugated goat anti-rabbit IgG for 1 hr, followed by peroxidase-conjugated avidin-biotin complex, both made up from the Vectastain Elite ABC kit according to the manufacturer's instructions. Staining was visualized using Vector SG insoluble peroxidase substrate according to the manufacturer's instructions. After staining, the gaskets were removed from the chamber slides, and the slides were mounted using aqueous mountant. Slides were blinded by another investigator before quantification of TH-immunoreactive cell survival.

To determine TH-immunoreactive cell survival, cells were observed under transmitted light on a Zeiss Axiovert inverted microscope using a 10 $\times$ objective. Counts were made of all the TH-immunoreactive cells present in each well. The culture conditions described here typically produce a yield of ~0.5–1% TH-immunoreactive cells, or ~1500 cells in a control well. For each compound tested, three independent experiments were performed, each consisting of four independent wells. Each compound was also tested in the absence of MPP⁺ to detect any nonspecific neuroprotective or toxic effects (data not shown).

[³H]DA uptake assays. Primary cultures of mesencephalic dopaminergic neurons were prepared as described above and plated at a density of

2.5×10^5 cells per well in poly-D-lysine-coated 48-well tissue culture clusters. Cultures were maintained for 5 d at 37°C/5%CO₂ in DMEM supplemented with Sato. After 5 d, medium was aspirated and replaced with either MPP⁺ at concentrations ranging from 0.01 to 100 μ M or with zVAD-fmk at concentrations ranging from 1 to 300 μ M in the presence of 1 or 10 μ M MPP⁺. In both cases compounds were prepared in DMEM/Sato. Four independent wells were treated for each condition in each experiment; three independent experiments were performed for each data point. Cultures were incubated for a further 48 hr, then [³H]DA uptake was evaluated.

To determine [³H]DA uptake, the medium was aspirated from each well and replaced with DMEM supplemented with 5.6 mM glucose, 1.3 mM EDTA, 0.2 mg/ml ascorbic acid, and 0.5 μ Ci/ml [³H]DA. Control cultures were treated with the above medium with the addition of the dopamine uptake blocker mazindol (10 μ M). Cultures were incubated for 30 min, then washed twice and lysed using 95% ethanol at 37°C for 30 min. Lysates were transferred to aqueous scintillant, and the activity was quantified. Results were expressed as percentage of untreated control culture response.

Visualization of apoptotic nuclei. For determination of apoptotic nuclei, cells were plated as described above into eight-well chamber slides. After 5 d *in vitro*, the medium was aspirated and replaced with DMEM/Sato or zVAD-fmk 300 μ M. Cultures were returned to the incubator for 15 min, after which MPP⁺ iodide was added as described above to give a final concentration in each well of 10 μ M. Control cultures were treated with DMEM/Sato only. Cultures were fixed using 4% paraformaldehyde at 24 and 48 hr after MPP⁺ exposure and immunostained for TH. This was followed by determination of apoptotic nuclei using the nuclear stain Hoechst 33342 to evaluate chromatin condensation.

Quantification of somatic area and neurite length. Microcomputer imaging device (MCID) image analysis (Brock University, Ontario, Canada) was used to evaluate the somatic area of TH-immunoreactive neurons. Area quantification was made from dopaminergic neurons in one experiment, from untreated control cultures, from cultures treated with 10 μ M MPP⁺ for 48 hr, and from cultures treated with 10 μ M MPP⁺ in the presence of 100 or 300 μ M zVAD-fmk. One hundred cells were measured from random fields of view throughout each of four wells for each treatment group. To quantify area in micrometers squared, the image analysis system was first calibrated in micrometers using a graticule. The area of immunostained soma were then established using the Autoscan tool. For each neuron, a control density was set outside the area of the stained soma; the stained area of the soma was then established. Neurites were excluded from each measurement. Mean areas for soma within each area were then established, and the results were presented as the mean area across four wells.

For neurite length measurements, MCID image analysis was used to quantify the length of the longest neurite for each of 100 TH-immunoreactive neurons in four wells per treatment group. The image analysis system was calibrated as described above. Neurite length measurements were taken from control cultures, cultures exposed to 10 μ M MPP⁺ for 48 hr, or cultures exposed to 10 μ M MPP⁺ in the presence of 300 μ M zVAD-fmk. To determine neurite length, a sample tool was used to draw manually along the length of the longest visible neurite. Results were expressed both as mean neurite length for each group and as a percentage of cells with only rudimentary processes; rudimentary processes were defined as being ≤ 10 μ m in length.

Statistical analyses. All statistical analyses that were performed used one-way ANOVA followed by Dunnett's test comparing all groups with cultures treated with 10 μ M MPP⁺ alone; for control MPP⁺ experiments, all groups were compared with untreated control results. Significance was reached at $p < 0.05$.

RESULTS

Toxic effects of MPP⁺ on dopaminergic neurons

MPP⁺ was added at concentrations ranging from 0.001 to 100 μ M to primary cultures of mesencephalic dopaminergic neurons (Fig. 1). Significant decreases in the number of TH-immunoreactive neurons were observed with MPP⁺ concentrations of 0.1 μ M and above. At 10 μ M, MPP⁺ reduced the number of surviving TH-immunoreactive neurons to ~50% of control (Fig. 1A), and this concentration was selected for further experiments. MPP⁺ was more potent at decreasing [³H]DA uptake than at decreasing the number of TH-immunoreactive neurons, reflecting the loss of

Figure 1. Effects of MPP⁺ on survival (*A*) and [³H]DA uptake (*B*) in primary cultures of mesencephalic dopaminergic neurons. MPP⁺ was added at concentrations ranging from 0.01 to 100 μM for 48 hr. Cultures were then either fixed and immunostained for TH, and the surviving TH-immunoreactive cells were counted, or [³H]DA uptake was assayed. Data shown in each case are the mean ± SEM of three independent experiments and are expressed as percentage of untreated control cultures (***p* < 0.01; established by one-way ANOVA followed by Dunnett's test). Representative photomicrographs of control (*C*) or 10 μM MPP⁺-treated (*D*) TH-immunoreactive neurons are shown. Cultures were treated for 48 hr, then fixed and immunostained for TH.

dopamine transporter sites on the neurite terminals. Again, significant decreases were observed at 0.1 μM MPP⁺ and above, but the response was decreased to ~20% of control with MPP⁺ concentrations of 1 μM and above. Photomicrographs of control cultures (Fig. 1*C*) and cultures treated with 10 μM MPP⁺ for 48 hr (Fig. 1*D*) show a loss of dopaminergic neurons in the MPP⁺-treated cultures. The cell bodies of the MPP⁺-treated TH-immunoreactive neurons are also smaller, and there are fewer neurites.

The broad-spectrum caspase inhibitor zVAD-fmk protects dopaminergic cell bodies against MPP⁺ toxicity but does not restore [³H]DA uptake

To determine the role of caspases in mediating toxicity of MPP⁺, we tested the broad-spectrum caspase inhibitor zVAD-fmk for

neuroprotective effects. Figure 2 shows the effects of zVAD-fmk on the toxicity induced by 10 μM MPP⁺. Treatment of cultures with 10 μM MPP⁺ resulted in a loss of ~50% TH-immunoreactive neurons in the cultures; zVAD-fmk treatment resulted in a concentration-dependent sparing of these neurons, with the maximal effect restoring dopaminergic neuronal number to >90% of control cultures (Fig. 2*A*). Photomicrographs of these cultures are shown in Figure 2*C–E*. Control cultures are shown in Figure 2*C*; Figure 2, *D* and *E*, shows cultures treated with 10 μM MPP⁺ and 300 μM zVAD-fmk plus 10 μM MPP⁺, respectively. The cultures treated with MPP⁺ alone have reduced numbers of dopaminergic neurons, and those surviving neurons have smaller cell bodies. Speckled staining is apparent around the neurons, which may reflect the remains of degenerated neurites. In the cultures

Figure 2. zVAD-fmk attenuates 10 μM MPP⁺ toxicity and increases somatic size in mesencephalic dopaminergic neurons. For survival quantification (*A*), cultures were exposed to 10 μM MPP⁺ for 48 hr in the presence of various zVAD-fmk concentrations. Cultures were then fixed and immunostained for TH. Slides were blinded, and TH-immunoreactive cells were counted. Data points shown are from three independent experiments, each consisting of four independent wells, and are expressed as percentage of untreated control cultures (**p* < 0.05, ***p* < 0.01; established by one-way ANOVA followed by Dunnett's test). Somatic size measurements (*B*) were made from each of four wells from one representative experiment. Random fields of view were visualized using MCID image analysis, and densitometry was used to establish the area occupied by the soma of TH-immunoreactive neurons. One hundred cells per well were measured for each data point. Photomicrographs of control (*C*), 10 μM MPP⁺-treated (*D*), and 10 μM MPP⁺- and 300 μM zVAD-fmk-treated (*E*) mesencephalic cultures are shown. Cultures were immunostained for TH, and representative photomicrographs were taken.

treated with both MPP⁺ and zVAD-fmk, there is a restoration of cell number; those neurons remaining have larger cell bodies, and a restoration of neurite number can also be seen, although some speckled staining is also apparent that may reflect a loss or remodeling of neurites.

Quantification of the somatic area of the dopaminergic neurons is shown in Figure 2*B*. Treatment with 10 μM MPP⁺ resulted in a significant decrease in the somatic area of the surviving TH-immunoreactive neurons. This decrease in somatic area was attenuated by treatment with zVAD-fmk at 100 and 300 μM. At 300 μM zVAD-fmk, the somatic area was significantly greater than that observed in control cultures. The effects of zVAD-fmk on MPP⁺-mediated neurite loss and the decrease in [³H]DA uptake

are shown in Figure 3. To establish whether caspase inhibition could increase [³H]DA uptake in MPP⁺-treated primary mesencephalic cultures, zVAD-fmk was coadministered with MPP⁺ concentrations of either 1 or 10 μM (Fig. 3*A*). zVAD-fmk was tested at concentrations ranging from 1 to 300 μM. The results for both MPP⁺ concentrations show a significant increase in [³H]DA uptake only with a zVAD-fmk concentration of 300 μM. The increase observed was relatively small in comparison with the increases observed with counts of TH-immunoreactive neurons, indicating that those neurons spared by caspase inhibition may be compromised in their ability to take up [³H]DA. This limited effect may be mediated by degeneration of neurites in the dopaminergic neurons. MPP⁺ treatment causes a marked decrease in

Figure 3. Effects of zVAD-fmk on [³H]DA uptake in primary mesencephalic dopaminergic neurons exposed to 1 or 10 μM MPP⁺ and effects of 300 μM zVAD-fmk treatment on neurite length of dopaminergic neurons. For [³H]DA uptake assays (Fig. 3A), cultures were exposed to 1 or 10 μM MPP⁺ in the presence of various concentrations of zVAD-fmk for 48 hr, and then the ability of the cells to take up [³H]DA was assayed. Each data point is the mean ± SEM of three independent experiments, each consisting of four independent wells, and is expressed as percentage of untreated control cultures (**p* < 0.05, ***p* < 0.01; established by one-way ANOVA followed by Dunnett's test). Neurite length measurements were made from TH-immunoreactive neurons in control cultures, cultures exposed to 10 μM MPP⁺ for 48 hr, and cultures treated for 48 hr with MPP⁺ and 300 μM zVAD-fmk. MCID image analysis was used to quantify the length of the longest neurite in each of 100 TH-immunoreactive neurons in four independent wells per treatment group. *B* shows the mean neurite length of TH-immunoreactive neurons. *C* shows the percentage of TH-immunoreactive cells in each treatment group with no, or only rudimentary, neurites; this was defined as a longest process of <10 μm in length.

neurite length, which is only partially restored by zVAD-fmk treatment (Fig. 3B). Similarly, MPP⁺ caused an increase in the percentage of neurons with no or rudimentary processes (Fig. 3C), and this was only partially restored by 300 μM zVAD-fmk. Thus, the limited effects of zVAD-fmk in restoring [³H]DA uptake are likely to be attributable to a degeneration of processes and thus of dopamine transporter sites.

Peptide inhibitors of caspases 2, 3, and 9, but not of caspase 1, partially protect dopaminergic neurons from MPP⁺ toxicity

The effects of a range of peptide inhibitors based on the preferred cleavage sites of specific caspases are shown in Figure 4. Four specific inhibitors were tested: zDEVD-fmk, zVDVAD-fmk,

zLEHD-fmk, and zYVAD-cmk. These inhibitors are based on the cleavage sites of caspases 3, 2, 9, and 1, respectively, and act by binding to and inhibiting the respective enzymes. Although zVDVAD-fmk is an inhibitor based on the preferred cleavage site for caspase 2, it is unlikely to be absolutely specific for caspase 2. The presence of an Asp residue in the P4 position of the inhibitor is a requirement for peptide inhibitors of caspases 3 and 7, and the VDVAD sequence has also been shown to inhibit these enzymes (Thornberry et al., 1997). Thus, the neuroprotection observed with this inhibitor may be attributable in part to an inhibition of caspase 3. Neither zLEHD-fmk nor zYVAD-cmk is likely to significantly inhibit caspase 3-like proteases; neither of these sequences has the required Asp in the P4 position. The

Figure 4. Effects of peptide caspase inhibitors on survival of TH-immunoreactive neurons after 10 μ M MPP⁺ treatment. Primary cultures of mesencephalic dopaminergic neurons were exposed to 10 μ M MPP⁺ for 48 hr in the presence of zDEVD-fmk (*A*), zLEHD-fmk (*B*), zVDVAD-fmk (*C*), or zYVAD-cmk (*D*). Cultures were fixed and immunostained for TH. Slides were then blinded, and the number of surviving TH-immunoreactive neurons was counted. Each data point represents the mean $\pm$ SEM of three independent experiments, each consisting of four independent wells, and is expressed as percentage of untreated control cultures (** p < 0.01; established by one-way ANOVA followed by Dunnett's test).

YVAD sequence is ~10,000-fold more selective for caspase 1 than for caspase 2, 3, or 7 and ~1000-fold more selective for caspase 1 than for caspase 9 (Garcia et al., 1998). The LEHD sequence does resemble the cleavage sites of caspases 4 and 5; thus there may be some inhibition of these caspases.

Concentration-dependent increases were observed with three of the inhibitors, zDEVD-fmk (caspase 3), zLEHD-fmk (caspase 9), and zVDVAD-fmk (caspase 2) (Fig. 5*A–C*), but no significant increases were observed with the caspase 1 inhibitor zYVAD-cmk (Fig. 5*D*). Significant increases in TH-immunoreactive cell number were observed with zLEHD-fmk and zVDVAD-fmk concentrations of 100 μ M and above. The caspase 3 inhibitor zDEVD-fmk caused significant increases in dopaminergic neuronal survival only at 300 μ M, whereas no significant increases were observed with zYVAD-cmk at any concentration tested.

Effects of novel caspase inhibitors on survival of mesencephalic dopaminergic neurons exposed to MPP⁺

Two novel inhibitors of caspases were tested for neuroprotective effects in dopaminergic neurons exposed to 10 μ M MPP⁺: M-920, a nonspecific inhibitor of caspases, and M-791, a selective caspase 3 inhibitor. M-725, an inactive analog of M-920, was also tested. These inhibitors are described in a model of sepsis by Hotchkiss and coworkers (2000). The results of these experiments are shown in Figure 5*A*. Both of the active caspase inhibitors caused significant increases in the number of surviving TH-immunoreactive neurons. Significant neuroprotection was observed with M-920 concentrations of 10 μ M and above; at concentrations of 10 μ M and above, the survival was similar to that observed in untreated control cultures. Treatment of dopaminergic neurons

Figure 5. Effects of novel caspase inhibitors on survival of dopaminergic neurons treated with MPP⁺. Two novel caspase inhibitors were tested for survival-promoting effects in primary cultures of dopaminergic neurons, M-920 and M-791, together with an inactive analog, M-725. Compounds were coadministered with 10 μ M MPP⁺ for 48 hr, and then surviving TH-immunoreactive cells were quantified. Results shown are the mean $\pm$ SEM of four independent wells per treatment group (M-791 response, ** p < 0.01; M-920 response, +++ p < 0.01; both established by one-way ANOVA followed by Dunnett's test).

with M-791 caused significant neuroprotection at concentrations of 1 μ M and above; the maximal response observed with this inhibitor increased the number of surviving TH-immunoreactive neurons to >90% of untreated control. The significant neuroprotective effects that were observed with M-791 at 1 μ M indicate that the neuroprotection is likely to be mediated by inhibition of caspase 3-like proteases. The survival response with this caspase 3 inhibitor is considerably higher than that observed with zDEVD-fmk, the peptidergic caspase 3 inhibitor, which might indicate limited cell permeability of the peptide caspase inhibitor. The magnitude of the survival effect of M-791 is equivalent to the effects observed with both zVAD-fmk and M-920, the broad spectrum caspase inhibitors. This indicates that inhibition of caspase 3 alone is sufficient to prevent almost all the toxicity of MPP⁺ in this culture system. When cultures were treated with the inactive compound M-725, no neuroprotective effects were observed at any of the concentrations tested.

With regard to the specificity of the inhibitors, M-920 is reported to have an IC₅₀ value of 0.002 μ M for caspase 3 in sepsis models and submicromolar IC₅₀ values for caspases 1, 4, 7, and 8. The IC₅₀ values for caspases 5 and 6 are 2 and 1.5 μ M, respectively. M-791 has an IC₅₀ value of 0.008 μ M for caspase 3 and 0.23 μ M for caspase 7 in the sepsis model; the IC₅₀ for caspase 8 is 4 μ M, and for other caspases it is in the mid-micromolar range (Hotchkiss et al., 2000). IC₅₀ values on a range of caspases and in two whole-cell *in vitro* models for these three compounds are shown in Table 1.

Table 1. IC₅₀ values for novel caspase inhibitors for various caspases, together with the IC₅₀ values obtained in whole-cell apoptosis assays in cerebellar granule neurons (CGN) and hNT-2 cells

	Cell free assays		
	M-920	M-791	M-725
Caspase			
1	0.01	11	15
2	>50	>450	
3	0.002	0.008	45
7	0.02	0.23	>100
8	0.04	4	68
9	>50	>450	
Cell type	Whole cell apoptosis IC ₅₀		
hNT2	0.12 μ M	0.49 μ M	
1° mCGN	0.2 μ M	1.0 μ M	

M-920 potently inhibits a number of members of the caspase family, whereas M-791 is a potent inhibitor of caspase 3 and also inhibits caspase 7. M-725 has little potency at any caspase tested. IC₅₀ values presented are micromolar.

MPP⁺ causes apoptotic features and activated caspase 3 expression in degenerating dopaminergic neurons; effects of caspase inhibition

Nuclear morphology was assessed in dopaminergic neurons after MPP⁺ exposure to determine whether the induced cell death was apoptotic. Photomicrographs of mesencephalic cultures stained for TH and counterstained with Hoechst 33342 to visualize nuclei are shown in Figure 6. TH-immunoreactive neurons are stained green, and Hoechst stained nuclei fluoresce blue. Double exposures were also taken to confirm localization of TH-immunoreactive cell nuclei. TH-immunoreactive neurons are shown in Figure 6, A, D, and F, Hoechst 33342-stained nuclei are shown in Figure 6, B, E, and H, and double-exposed images to show colocalization are shown in Figure 6, C, F, and I. Figure 6A–C shows control cultures. TH-immunoreactive neurons have large cell bodies and extensive neurites; the nuclear morphology of these neurons shows no chromatin condensation, illustrated by the yellow arrows. Figure 6D–F photomicrographs are of a field of view from cultures exposed to 10 μ M MPP⁺ for 48 hr. Within the field, a number of degenerating TH-immunoreactive neurons can be observed (white arrows). The nuclei of these neurons show chromatin condensation when stained with Hoechst 33342, a characteristic feature of apoptosis. Also within the well are a number of TH-immunoreactive neurons that do not appear to have degenerated; the nuclei of these neurons do not show chromatin condensation (yellow arrow). Figure 6G–I shows cultures exposed to 10 μ M MPP⁺ for 48 hr in the presence of 300 μ M zVAD-fmk. The TH-immunoreactive neurons within the culture do not appear to have degenerated, and their nuclei do not show chromatin condensation (yellow arrow). Also within each well, there is a population of cells that exhibit chromatin condensation but are not TH immunoreactive; these are highlighted by the red arrows. Such nuclei are observed in control, MPP⁺-treated, and MPP⁺- and zVAD-fmk-treated cultures. These profiles may reflect a population of non-dopaminergic cells in the culture that are undergoing cell death, perhaps as a result of a change in the medium on the cultures.

To visualize activated caspase 3 in dopaminergic neurons after MPP⁺ treatment, double-immunolabeling studies were performed using primary antibodies to activated caspase 3 and to TH. Cultures were grown for 5 d, then returned to culture

Figure 6. Colocalization of apoptotic nuclei with TH immunoreactivity in primary mesencephalic cultures after MPP⁺ exposure. Cultures were stained with a primary antibody raised against TH and visualized using FITC. Nuclei were visualized by counterstaining using Hoechst 33342. *A–C*, Control cultures; *D–F*, cultures exposed to 10 μ M MPP⁺ for 48 hr; *G–I*, cultures exposed to 10 μ M MPP⁺ in the presence of 300 μ M zVAD-fmk. Each of the photomicrographs within a condition is of the same field of view, stained with tyrosine hydroxylase (*A*, *D*, *G*) or Hoechst 33342 (*B*, *E*, *H*) or dual exposed to show colocalization (*C*, *F*, *I*). Apoptotic dopaminergic nuclei are shown by *white arrows* (and magnified in *E*, *inset*). Representative non-apoptotic dopaminergic nuclei are indicated by *yellow arrows*, and non-dopaminergic apoptotic nuclei by *red arrows*.

medium alone (Fig. 7*A–C*) or treated with 10 μ M MPP⁺ for 24 hr (Fig. 7*D–F*) or 48 hr (Fig. 7*G–I*). Figure 7, *A*, *D*, and *G*, shows TH immunoreactivity. Figure 7, *B*, *E*, and *F*, shows activated caspase 3 immunoreactivity in the same field of view, and Figure 7, *C*, *F*, and *G*, shows colocalization of caspase 3 with TH immunoreactivity.

In control cultures, a number of TH-immunoreactive neurons

can be observed (Fig. 7*A*), along with a population of cells expressing activated caspase 3 (Fig. 7*B*); however, there is little coexpression of activated caspase 3 with TH in these cultures (Fig. 7*C*), indicating that caspase 3 is not active in dopaminergic neurons. In cultures treated with MPP⁺ for 24 or 48 hr, however, a population of dopaminergic neurons that coexpress TH and caspase 3 is apparent (Fig. 7*F*, *I*). In all treatment groups, a

Figure 7. TH-immunoreactive neurons exposed to 10 μM MPP⁺ for 24 or 48 hr express activated caspase 3. Cultures were treated with MPP⁺ for the required time, then fixed and double immunostained using a monoclonal tyrosine hydroxylase antibody and a polyclonal antibody raised against active caspase 3. *A–C*, Control cultures; *D–F*, cultures treated with 10 μM MPP⁺ for 24 hr; *G–I*, cultures exposed to MPP⁺ for 48 hr. *A*, *D*, and *F* show immunostaining using an antibody to TH; *B*, *E*, and *H* show the same field of view stained with the activated caspase 3 antibody. Colocalization of these antibodies is shown in *C*, *F*, and *I*.

population of non-dopaminergic neurons that express activated caspase 3 is apparent, indicating that there is a population of cells within the cultures undergoing apoptosis; this is in accordance with the presence of apoptotic profiles in a population of non-dopaminergic neurons observed in Figure 6. Thus, MPP⁺ treatment of primary cultures of dopaminergic neurons for 24 or 48 hr causes activation of caspase 3 in these neurons.

To quantify the MPP⁺-induced caspase activation and chromatin condensation, a triple-labeling experiment was performed. Cultures were treated with MPP⁺ for 48 hr in the presence or absence of 300 μM zVAD-fmk or the caspase 3 inhibitor M-791, then fixed and double immunostained for active caspase 3 and TH. Nuclei were counterstained using Hoechst 33342, and quantification was performed. Ten fields of view containing at least

three TH-immunoreactive cells were quantified in each of three independent wells. The total number of nuclei was established, and the number of these that showed apoptotic features was established. The number of TH-immunoreactive neurons and the number of active caspase-3 neurons were also counted. Each field of view was quantified for the number of neurons coexpressing TH/active caspase 3 and TH/condensed chromatin. These data are shown in Figure 8. In Figure 8*A*, the number of apoptotic cells and the number of active caspase 3-immunoreactive cells in each treatment group are shown, expressed as a percentage of the total number of cells within the cultures. In control cultures there is a population of ~20% of cells that express apoptotic morphology, likely as a result of stress through changing the medium or a natural attrition of cells within the culture. There is a slight

Figure 8. Effects of MPP⁺ treatment in the presence and absence of caspase inhibition on the number of apoptotic profiles and active caspase 3 immunoreactive cells. Cells were double labeled for TH and active caspase 3, and the nuclei were counterstained with Hoechst 33342. Cells were visualized using a 20× objective, and the total number of nuclei, the number of apoptotic nuclei, the number of TH-immunoreactive cells, and the number of active caspase 3-immunoreactive cells were quantified, together with the number of cells coexpressing TH and apoptotic nuclei, and TH and active caspase 3. Ten fields of view were quantified in each of three wells per treatment group. Cultures that were quantified were untreated control cultures, cultures exposed to 10 μM MPP⁺ for 48 hr, or cultures exposed to 10 μM MPP⁺ for 48 hr in the presence of either 300 μM zVAD-fmk or 100 μM M-791. *A* shows the expression of apoptotic profiles and active caspase 3 as a percentage of the total cell population. *B* shows the cells coexpressing either activated caspase 3 or apoptotic profiles with TH expressed as a percentage of the total number of TH-immunoreactive cells.

increase in the number of apoptotic cells in the MPP⁺-treated group that is reduced by the caspase inhibitors. There is also a small population (<10%) of active caspase 3-immunoreactive cells within control cultures. This is increased by MPP⁺ treatment, but this increase is not reversed by the caspase inhibitors. Figure 8*B* shows the expression of apoptotic nuclei and active caspase 3 in TH-immunoreactive neurons. Approximately 10% of TH-immunoreactive neurons have apoptotic nuclei in control cultures; this is markedly increased by MPP⁺ treatment, which increases the number of apoptotic nuclei in the remaining TH-immunoreactive neurons to ~60%. Both of the caspase inhibitors that were tested completely reverse the increase in apoptotic nuclei induced by MPP⁺. When coexpression of TH and activated caspase 3 was examined, there was again a marked increase in the number of coexpressing cells, from ~10% in control cultures to ~50% in 10 μM MPP⁺-treated cultures. When MPP⁺ was coadministered with the caspase inhibitors, however, there was little decrease in the expression of activated caspase 3 in TH-immunoreactive neurons. This lack of decrease with the caspase inhibitors is likely attributable to the mode of action of the inhibitors, which bind to the cleavage site of the active caspase and prevent cleavage of cellular substrates rather than preventing formation of the active caspase from the inactive zymogen. Thus, in the inhibitor and MPP⁺-treated dopaminergic neurons, the caspase appears to be activated as in cultures treated with MPP⁺ alone, but inhibition prevents it from executing the apoptotic response; this leads to the decreased evidence of chromatin condensation and the increased neuronal number.

Not all MPP⁺-treated dopaminergic cells visualized expressed chromatin condensation or active caspase 3; this may reflect a population that has not yet effected the apoptotic response after MPP⁺ treatment. At 48 hr treatment, only ~50% of dopaminergic cells remain in the cultures compared with untreated controls. Dopaminergic cells expressing active caspase 3 were also present within the cultures at earlier time points. It is likely that these

cells that activate caspase 3 earlier in the time course undergo apoptosis and detach from the substratum, resulting in this decrease in numbers, and that the number of dopaminergic neurons counted with the active enzyme at 48 hr underestimates the number of neurons that express this over the total treatment period.

DISCUSSION

Although MPP⁺ is a commonly used model for selective dopaminergic neuronal cell death *in vitro* (Sanchez Ramos et al., 1986; Michel et al., 1989; Michel et al., 1990; Beck et al., 1991), reports conflict regarding the cell death mechanism. Apoptosis has been shown *in vivo* in the substantia nigra of MPTP-treated mice (Tatton and Kish, 1997; Eberhardt et al., 2000), depending on the dosing regimen used (Jackson-Lewis et al., 1995). *In vitro*, apoptosis has been demonstrated after MPP⁺ treatment in rat mesencephalic–striatal cocultures (Mochizuki et al., 1994), in dissociated cultures of cerebellar granule cells and mesencephalic dopaminergic neurons (Dipasquale et al., 1991; Du et al., 1997; Dodel et al., 1998), and in the SH-SY5Y neuronal cell line (Fall and Bennet, 1998). In contrast, however, Lotharius and coworkers (1999) found no evidence for apoptosis in MPP⁺-treated rat mesencephalic neurons. MPP⁺ treatment of dopaminergic MN9D cells also failed to produce evidence of apoptotic markers (Choi et al., 1999). The evidence for and against a role of apoptosis in MPP⁺ toxicity is reviewed by Nicotra and Parves (2000); it appears most likely that the differences in types of cell death observed by different groups are dependent on the severity of the insult or the culture conditions that are used.

Here we show that MPP⁺ treatment of primary dopaminergic neurons causes apoptosis and that caspase inhibition with zVAD-fmk prevents the MPP⁺-mediated loss of dopaminergic neurons. The number of surviving dopaminergic neurons in 10 μM MPP⁺-treated cultures decreased to ~50%, with zVAD-fmk restoring numbers to ~90%, confirming several previous reports. zVAD-

fmk has been reported to attenuate MPP⁺ toxicity in cerebellar granule neurons (Du et al., 1997) and mesencephalic dopaminergic neurons (Dodel et al., 1998; Eberhardt et al., 2000). In this study, zVAD-fmk increased the number of MPP⁺-treated dopaminergic neurons and the somatic size of these neurons after 48 hr; increased TH-immunoreactive cell number was observed up to 5 d after coadministration of the compounds (data not shown). zVAD-fmk was less effective at preventing the MPP⁺-mediated loss of [³H]DA uptake, with significant increases only at 300 μ M. Only a partial restoration of the neurite length of these neurons was observed, indicating that the dopamine transporter sites may not be spared. These data are similar to reported studies with both MPP⁺ (Eberhardt et al., 2000) and 6-OHDA (Von Coelln et al., 2001), in which little neurite or [³H]DA uptake restoration was observed with zVAD-fmk. Although these data and reports from other groups indicate an important role for caspases in mediating MPP⁺ toxicity, a number of groups have found conflicting effects. Lotharius et al. (1999) found no protection from MPP⁺ toxicity with another broad-spectrum caspase inhibitor, Boc-Asp-fmk, and zVAD-fmk did not protect dopaminergic MN9D cells from MPP⁺ toxicity (Choi et al., 1999). Hartmann et al. (2001) reported that MPP⁺ treatment induced apoptosis in primary dopaminergic neurons, but that caspase inhibition potentiated cell death by increasing necrosis, an effect that has been reported previously in other cell types (Lemaire et al., 1998); this effect was reversed if cultures were grown in elevated glucose. Thus, reports conflict regarding the efficacy of caspase inhibition in preventing MPP⁺ toxicity *in vitro*.

In this study, caspase inhibition clearly promotes survival of dopaminergic neurons. Caspases can be divided into three families on the basis of structure and function; these families typically are involved in the inflammatory response, caspase activation, and execution of apoptosis, respectively (for review, see Nicholson and Thornberry, 1997; Stennicke and Salvesen, 1998). To determine which specific caspases mediate the toxicity, peptide inhibitors of specific caspases were tested; partial neuroprotection was observed with inhibitors of caspases 2, 3, and 9, but not with an inhibitor of caspase 1. A novel caspase 3 inhibitor had neuroprotective effects equivalent to either zVAD-fmk or another broad-spectrum caspase inhibitor, M-920.

Coadministration of dopaminergic neurons with MPP⁺ and the selective caspase 3 inhibitor M-791 caused almost complete protection of TH-immunoreactive neurons *in vitro*. The protection obtained with this compound was similar to that obtained with either of the broad-spectrum caspase inhibitors tested, zVAD-fmk or M-920, and greater than with the peptide inhibitor zDEVD-fmk. That the effects were mediated by caspase inhibition is indicated by the lack of effect of M-725, a structural analog of M-920 lacking activity at caspases. These data provide compelling evidence that in dopaminergic neurons exposed to MPP⁺ *in vitro*, inhibition of caspase 3 alone is sufficient to protect the neurons. Inhibition of caspase 3 with M-791 also decreased to control levels the number of apoptotic dopaminergic cells, a response similar to that of zVAD-fmk. In contrast, neither of these inhibitors prevented an MPP⁺-mediated increase in the number of TH-immunoreactive cells expressing activated caspase 3. A likely explanation for this is that the inhibitors do not prevent cleavage and activation of the caspase zymogen but rather bind to the active site of the activated caspase to prevent substrate cleavage.

Caspase 3 is involved in the execution of apoptosis in a number of neuronal cell types after a range of insults. *In vivo*, caspase 3

inhibition attenuates damage after ischemia (Ma et al., 1998) and axotomy of retinal ganglion neurons (Kermer et al., 1998). *In vitro*, caspase 3 inhibition protects cerebellar granule neurons from K⁺ deprivation-induced apoptosis (Ni et al., 1997) and PC12 cells from 6-hydroxydopamine toxicity (Ochu et al., 1998; Lotharius et al., 1999). Caspase 3 is activated by a range of factors, including caspase 9. Caspase 9 is activated during release of cytochrome *c* from mitochondria; the released cytochrome *c* forms a complex with cytoplasmic APAF-1 and caspase 9 in the presence of ATP and activates caspase 9 (Liu et al., 1996; Zou et al., 1997). Activated caspase 9 then cleaves and activates caspase 3, leading to the apoptotic death of the cell (Li et al., 1997; Cai et al., 1998; Pan et al., 1998). Cytochrome *c* release into the cytoplasm of cerebellar granule cells has been shown after MPP⁺ treatment (Du et al., 1997). Inhibition of caspase 9 using zLEHD-fmk significantly increased survival of MPP⁺-treated TH-immunoreactive neurons, indicating that this pathway may indeed be activated in MPP⁺ toxicity.

Because the specificity of the caspase 2 inhibitor is suspect, it is possible that the effects observed with this inhibitor are mediated through inhibition of another caspase such as caspase 3. There is evidence *in vivo* that caspase 2 may be involved in MPTP toxicity; mice overexpressing Bcl-2 are resistant to MPTP toxicity, with decreased expression of active caspase 2 after MPTP treatment compared with wild-type animals (Yang et al., 1998). More selective inhibitors may allow further clarification of the role of this caspase.

No effects were observed with the caspase 1 inhibitor zYVAD-cmk, consistent with previous reports in primary dopaminergic neurons (Dodel et al., 1998). These data, however, conflict with studies in transgenic mice overexpressing dominant negative caspase 1 that were resistant to MPTP toxicity *in vivo* (Klevenyi et al., 1999), and caspase 1 activation was observed in the dopaminergic cell line SN4741 after MPP⁺ or oxidant treatment (Chun et al., 2001). Caspase 1 has been implicated as both a downstream target and an activator of caspase 8, and caspase 8 inhibition has been shown to be protective against MPTP toxicity in mice *in vivo*, although not *in vitro* (Hartmann et al., 2001). Both caspase 3 and caspase 8 are expressed in the substantia nigra of Parkinson's disease patients (Hartmann et al., 2000, 2001). An explanation for the apparent conflict of these results may be that MPP⁺ is capable of activating multiple caspase pathways depending on cellular conditions and that there may be a redundancy of function of some of these pathways under certain conditions.

The toxicity of MPP⁺ for dopaminergic neurons under these conditions appears to be mediated by pathways that converge on activation of caspase 3, and inhibition of caspase 3 is sufficient to spare at least the neuronal somata. The events before the caspase 3 activation are less clear. Caspase 9 inhibition provides partial neuroprotection, indicating that cytochrome *c* release from mitochondria might be important. MPP⁺ has been shown to open the mitochondrial permeability transition pore (PTP) *in vitro* (Casarino et al., 1999), although inhibition of the mitochondrial PTP using cyclosporin A does not protect SH-SY5Y cells (Fall and Bennett, 1998) or mesencephalic dopaminergic neurons (data not shown). Cyclosporin A, however, is toxic at higher concentrations, so any potential neuroprotective effects may be masked. Another route of cytochrome *c* release from mitochondria is through pores formed by pro-apoptotic members of the Bcl-2 family. Mice overexpressing Bcl-2 are resistant to MPTP toxicity *in vivo* and MPP⁺ toxicity *in vitro* (Offen et al., 1998; Yang et al., 1998), so this may be a possible mechanism underlying the cell

death. In addition to cytochrome *c*, other pro-apoptotic factors can be released from mitochondria in apoptosis, including second mitochondria-derived activator of caspase (SMAC)/direct IAP binding protein with low pI (DIABLO) and apoptosis-inducing factor (AIF). SMAC/DIABLO inhibits the activity of members of the IAP family, leading to caspase activation (Du et al., 2000; Verhagen et al., 2000). In this regard, it is interesting that adenoviral expression of X-chromosome-linked IAP protects nigral neurons from MPTP toxicity in mice *in vivo* (Eberhardt et al., 2000). No reports have been published as yet showing direct evidence for release of either SMAC/DIABLO or AIF. Further investigation may clarify the roles of mitochondrial factors in MPP⁺-induced apoptosis.

In conclusion, we show that caspase inhibition protects dopaminergic neurons from MPP⁺ toxicity *in vitro* and that the caspases 2, 3, and 9, but not caspase 1, are involved in the pathway. The pathways activated by MPP⁺ appear to converge on activation of caspase 3, because inhibition of caspase 3 alone is sufficient to fully protect cells from MPP⁺-mediated cell death. Thus, the caspase cascade, or factors upstream regulating caspase activation, are targets for neuroprotective strategies in models of Parkinson's disease.

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