Activation of c-Jun N-Terminal Protein Kinase Is a Common Mechanism Underlying Paraquat- and Rotenone-Induced Dopaminergic Cell Apoptosis

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Parkinson’s disease (PD) is characterized by selective loss of dopaminergic neurons in the substantia nigra of the brain. Although the underlying causes are not well characterized, epidemiological studies suggest an elevated risk of PD with occupational pesticide exposure. Here, we utilized pheochromocytoma (PC) 12 and SH-SY5Y cells as well as rat primary cultured dopaminergic neurons to investigate mechanisms for dopaminergic cell death induced by paraquat and rotenone, pesticides that are used to model PD in rodents. Both paraquat and rotenone induce selective loss of dopaminergic neurons in primary cultures. We discovered that paraquat induces apoptosis in PC12 cells but not in SH-SY5Y cells, while rotenone exposure causes apoptosis in SH-SY5Y cells but not in PC12 cells. The selective ability of paraquat and rotenone to induce apoptosis in different cell lines correlates with their ability to activate c-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinases. Furthermore, JNK and p38 are required for rotenone-induced apoptosis in SH-SY5Y cells (K. Newhouse et al., 2004, Toxicol. Sci. 79, 137–146) as well as primary neurons, and for paraquat-induced apoptosis in PC12 cells. However, JNK but not p38 plays a role in paraquat-induced loss of primary cultured dopaminergic neurons. Our data identify JNK activation as a common mechanism underlying dopaminergic cell death induced by both paraquat and rotenone in model cell lines and primary cultures.

Key Words: rotenone; paraquat; MAP kinases; dopaminergic neurons; apoptosis; PD.

Parkinson’s disease (PD) is the second most common aging-related neurodegenerative disorder and is characterized by selective loss of dopaminergic neurons in the substantia nigra pars compacta of the brain (Olanow and Tatton, 1999). Despite extensive investigation over the past two decades, the etiology of PD remains elusive. Mutations in several genes, including parkin and α-synuclein, have been linked to early onset, familial PD, suggesting a role for specific genes in the susceptibility to PD. However, the majority of PD cases are nonfamilial, and the cause of sporadic PD is largely unknown. Epidemiological studies have suggested a potential link between general pesticide exposure and increased risk for PD. For example, agricultural workers, especially those who work with pesticides including orchardists and pesticide applicators, are at increased risk for developing PD (Baldi et al., 2003; Engel et al., 2001; Gorell et al., 2004; Hertzman et al., 1990). Several studies have found an association between exposure to 1,1′-dimethyl-4,4′-bipyridinium dichloride (paraquat) and increased risk for PD (Hertzman et al., 1990; Liou et al., 1997). However, whether pesticides contribute to the etiology of idiopathic PD remains controversial, and more research is needed to establish a causal link.

Paraquat and rotenone are pesticides used worldwide (Ecobichon, 2001). Chronic administration of paraquat or rotenone to rodents in vivo induces many key features of PD, including motor deficits, loss of dopaminergic neurons, and the presence of α-synuclein–containing inclusion bodies (Betarbet et al., 2000; Brooks et al., 1999; Manning-Bog et al., 2002, 2003; McCormack and Di Monte, 2003; Sherer et al., 2003; Thiruchelvam et al., 2000, 2003). Paraquat also accelerates the formation of α-synuclein fibrils in vitro (Uversky et al., 2001). Thus, treatment of rodents or cultured cells with paraquat or rotenone provides useful models to study mechanisms of dopaminergic cell death associated with PD.

Rotenone is a potent inhibitor of mitochondrial complex I (Sherer et al., 2003). Paraquat is structurally similar to 1-methyl-4-phenylpyridinium (MPP+), a mitochondrial complex I inhibitor and metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Because of this structural similarity, it has been suggested that paraquat, like rotenone, may also inhibit mitochondrial complex I (Dawson and Dawson, 2003; Fukushima et al., 1994; Tawara et al., 1996). However, a recent study suggests that paraquat neurotoxicity is quite distinct from that of rotenone and MPTP (Richardson et al., 2005). The objective of this study was to directly compare cell death...
induced by paraquat and rotenone and to determine if cell death caused by these two agents is mechanistically related. The elucidation of cell death mechanisms common to different models of PD may lead to the identification of drug targets for treatment of the disease.

We utilized rat adrenal–derived pheochromocytoma (PC) 12 and human neuroblastoma SH-SY5Y cells in this study because these cells produce dopamine and have been used extensively as model “dopaminergic” cell lines for the study of PD (Hartley et al., 1994; Hsuan et al., 2006; Kang et al., 1997; Kitazawa et al., 2003; Newhouse et al., 2004; Salinas et al., 2003; Spina et al., 1992; Storch et al., 2000; Viswanath et al., 2001). Although the determination of apoptotic mechanisms derived from the study of model cell lines is clearly informative, it is also important to confirm major observations using real neurons from the central nervous system (CNS). Therefore, we carried out parallel experiments using primary dopaminergic neurons from embryonic day 14 (E14) rats.

To determine if rotenone and paraquat induce apoptosis through common signaling pathways, we specifically focused on the c-Jun N-terminal protein kinase (JNK) and the p38 mitogen-activated protein (MAP) kinases because these stress-activated MAP kinases have been implicated in several forms of neuronal apoptosis (Caughlan et al., 2004; Davis, 2000; Figueroa-Masot et al., 2001; Ghatan et al., 2000; Hetman and Xia, 2000; Ip and Davis, 1998; Namgung and Xia, 2000, 2001; Weston and Davis, 2002; Xia et al., 1995). Most notably, there is increasing evidence both in vitro and in vivo suggesting a role for these kinases in the pathogenesis of dopaminergic neuron death in PD models (Junn and Mouradian, 2001; Newhouse et al., 2004; Niso-Santano et al., 2006; Peng and Andersen, 2003; Peng et al., 2004; Saporito et al., 1999, 2000; Xia et al., 2001). Here, we report that although both rotenone and paraquat induce selective loss of primary cultured dopaminergic neurons, PC12 cells are only sensitive to paraquat while SH-SY5Y cells are sensitive to rotenone. Using pharmacological inhibitors and transient expression of dominant interfering constructs of the JNK or p38 signaling pathway, we demonstrate that activation of the JNK MAP kinase is a common mechanism required for both paraquat- and rotenone-induced apoptosis in cell lines (Newhouse et al., 2004) as well as in primary cultured dopaminergic neurons. This suggests the interesting possibility that the JNK pathway may be a useful drug target for treatment of PD.

**MATERIALS AND METHODS**

**Materials.** The following constructs have been described: dominant negative (dn) MAP kinase kinase 3 (MKK3) (pRc-RSV-Flag-MKK3 (Ala)), glutathione S transferase–activating transcription factor 2 (GST-ATF2) (Xia et al., 1995), and dn c-Jun (Rapp et al., 1994). The plasmid-encoding enhanced green fluorescent protein (eGFP) was purchased from Clontech (Palo Alto, CA). Lipofectamine 2000 and polyclonal anti-GFP antibody were purchased from Invitrogen (Carlsbad, CA). The inhibitors 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190) and 1,9-pyrazoloanthrone (SP600125) were purchased from Calbiochem (San Diego, CA). The anti-phospho-p38, anti-phospho-JNK, and anti-phospho-c-Jun antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-tyrosine hydroxylase (α-TH) antibody, anti-β-actin antibody, paraquat and rotenone were purchased from Sigma (St Louis, MO).

**Cell culture.** Rat adrenal–derived PC12 cells were maintained on rat tail collagen-coated 10-cm tissue culture dishes in Dulbeccoo’s Modified Eagle’s Medium (DMEM, Mediatech, Herndon, VA) containing 10% horse serum, 5% fetal bovine serum (FBS), 200 mM glucose (Gibco, Grand Island, NY), 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma) at 37°C and 7% CO2. Human neuroblastoma SH-SY5Y cells were maintained on poly-D-lysine (PDL, Sigma)—coated 10-cm tissue culture plates in DMEM-F12 (Gibco) supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10 mM N2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES, Sigma). Both PC12 and SH-SY5Y cells were plated on PDL-coated, 24-well or 35-mm plates the day before drug treatment or transfection at a density of 2 × 105 cells per well or 1.5 × 106 cells per plate, respectively. For [3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) metabolism or caspase assays, cells were plated onto 96-well plates at a density of 1.5 × 105 cells per well. Both PC12 and SH-SY5Y cells were obtained from American Type Culture Collection and passaged <15 times for the experiments.

To obtain differentiated PC12 cells, undifferentiated PC12 cells were plated on collagen-coated 10-cm tissue culture dishes at a density of 0.9 × 106 cells per plate for seven or more days (Xia et al., 1995). Cells were maintained in DMEM containing 50 ng/ml nerve growth factor (NGF) (Austral Biologicals, San Ramon, CA), 1% horse serum, 200 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C and 7% CO2. The day before treatment or transfection, the NGF-differentiated PC12 cells were replated on aclar coverslips (Electron Microscopy Sciences, Fort Washington, PA) placed in 35-mm plates. The coverslips and plates were coated with 50 μg/ml PDL and 5 μg/ml laminin (BD Bioscience, Bedford, MA); the seeding density was 0.85 × 106 cells/35-mm plate.

Primary ventral mesencephalic cultures were prepared from E14 Sprague-Dawley rats (Harlan, Indianapolis, IN) as described (Hsuan et al., 2006). Briefly, ventral mesencephalon was dissected in phosphate-buffered saline (PBS). Tissue was mechanically dissociated in DMEM (Gibco) growth media. Dissociated cells were then plated in 24-well plates containing aclar coverslips coated with PDL and laminin and seeded at a density of 8 × 105 cells per well. The cultures were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, and 30 mM glucose (Sigma) in a humidified incubator at 37°C containing 7% CO2. At 24 and 72 h, one-half of the media was removed and replaced with fresh media. On the fifth and seventh day-in-vitro (DIV), one-half of the culture media was replaced with DMEM-F12 supplemented with 1% N2 supplement (Invitrogen) and 10 μg/ml bovine serum albumin (BSA). Drug treatment was performed on DIV7.

**Drug treatment.** Paraquat was dissolved in deionized water, which served as the vehicle control. The incubation time for paraquat (0–500 μM) treatment ranged from 0 to 36 h as indicated in the figures and figure legends. Rotenone, SB202190, and SP60025 were dissolved in dimethyl sulfoxide, which was used as the vehicle control for these agents. Because rotenone is highly lipophilic and may bind to proteins in the serum, cells were transferred to low serum medium containing 0.5% FBS before rotenone treatment to prevent excessive retention of rotenone in the serum. Rotenone was used at 0–500 μM for 0–24 h as indicated in the figures and figure legends. SB202190 or SP60025, at a final concentration of 10 μM, was administered 1 h before rotenone or paraquat treatment. The final volume of vehicle or drug-containing solutions added to cell cultures did not exceed 1%. 

**Apoptosis assay and transferase biotin-dUTP nick-end labeling.** These assays were performed as previously described (Hetman et al., 1999; Hsuan et al., 2006). For cell lines, apoptosis was determined by nucleod condensation and/or fragmentation after staining the cells with the DNA dye Hoechst 33258. Briefly, cells were fixed in 4% paraformaldehyde (PFA)/4% sucrose for 30 min followed by staining with Hoechst 33258 (2.5 μg/ml in PBS containing 0.1%
Triton X-100) for 10 min. Cells were analyzed at a single-cell level using fluorescence microscopy. Cells with condensed or fragmented nuclei were scored as apoptotic, while those with uniformly stained nuclei were scored as healthy. A minimum of 2000 cells in randomly chosen fields were counted per data point. A reduction in mean number of cells per field was also used as an indicator of cell death (or decrease in cell viability) because apoptotic cells eventually detach from tissue culture plates. For these experiments, a minimum of 2000 cells per data point were counted and divided by the number of fields scored to yield mean number of cells per field.

For primary ventral mesencephalic cultures, all TH⁺ cells on each coverslip were counted. The total number of TH⁺ cells at the end of each experiment under control conditions was defined as 100% survival. The percent of survival in the general population was quantified as the percent of cells with uniformly stained nuclei (noncondensed, nonfragmented) after Hoechst staining to reveal nuclear morphology. Two thousand cells were counted per data point, and the mean survival at the end of each experiment under control conditions was defined as 100% survival. To obtain unbiased counting, all slides were coded and cells were scored blindly without prior knowledge of treatment for all cell lines and primary cultured neurons.

Terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) was performed using terminal deoxynucleotidyl transferase (TdT, Promega, Madison, WI) according to manufacturer’s suggestions. Briefly, cells were fixed with 4% PFA and permeabilized in PBS containing 0.1% Triton X-100 and 0.1% sodium citrate. Cells were then incubated with a TUNEL mixture containing 0.5 unit/ml of TdT, 0.2 nmol biotin-11-dUTP (Perkin-Elmer Life Sciences, Boston, MA), followed by streptavidin-fluorescein isothiocyanate (Sigma). TUNEL staining was monitored using fluorescence microscopy.

Western analysis. Cell lysates were prepared as described (Détjard et al., 1994; Figueroa-Masot et al., 2001). About 20–40 µg of protein lysates were used for Western analysis. After antibody incubation, blots were treated with enzynmic chemiluminesence reagent (GE Healthcare, Piscataway, NJ) and exposed to Blue Basic Autorad Film (ISC Bioexpress, Kaysville, UT). Images are representatives of three independent experiments. Fold induction was determined by densitometry analysis of scanned blots with Image J software (National Institutes of Health) and normalized to β-actin loading control. Reported data were derived from three independent Western blot experiments.

Kinase assay. In vitro p38 kinase assay was performed as described (Xia et al., 1995). Briefly, protein A sepharose (PAS) beads were conjugated with p38 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 2 h. To immunoprecipitate p38, 600 µg of protein lysates were added to PAS-p38 beads and mixed at 4°C overnight. The activity of p38 in the immune precipitates was quantitated by a kinase assay using recombinant GST-ATF2 (5 µg) and 32P-ATP (10 µCi) as the substrates.

Transient transfection of plasmid DNA. The day before transfection, undifferentiated PC12 cells were plated at a seeding density of 2 × 10⁵ cells per well of 24-well plates, and NGF-differentiated PC12 cells were plated at a density of 0.85 × 10⁶ cells per 35-mm plates. Cells were transiently transfected with 1–2 µg of total plasmid DNA using Lipofectamine 2000 as per manufacturer’s instruction. An expression vector for eGFP was cotransfected as a marker to identify transfected cells after immunostaining against GFP. The empty cloning vector pCDNA3 was used as a control. Cells were treated with drugs 24 h after transfection. To quantify apoptosis in transfected cells, at least 200 GFP⁺ cells were analyzed per determination in each experiment, or 1200 GFP⁺ cells per data point.

Immunocytochemistry. This was performed as previously described (Hsu et al., 2006). Briefly, cells were fixed with 4% PFA/4% sucrose at room temperature for 30 min and permeabilized with 0.5% Igepal CA 630 (Sigma) in PBS for 30 min. To block nonspecific binding, cells were treated for 2 h with 2.5% BSA, 5% BSA, and 5% goat serum in PBS/0.1% Triton X-100. Cells were then incubated with mouse α-TH monoclonal antibody (1:500) or anti-GFP polyclonal antibody (1:1000) overnight at 4°C. Secondary antibody (goat anti-mouse Alexa Fluor 488, or goat anti-rabbit Alexa Fluor 488, 1:500) was then added for 1 h followed by nuclear staining with Hoechst 33258 (2.5 µg/ml) for 10 min.

MTS metabolism assay. CellTiter 96 aqueous one solution cell proliferation assay (Promega) was used to assess MTS metabolism as an indicator of cell viability as per manufacturer’s instructions. This assay is based on the colorimetric conversion of the yellow MTS tetrazolium to the blue formazan product. This conversion is catalyzed by cellular mitochondrial dehydrogenases. Briefly, PC12 or SH-SY5Y cells were plated in 96-well plates at a density of 1.5 × 10⁴ cells/well the day before experiments. Cells were treated with paraquat or rotenone for 24 h, followed by incubation with MTS solution for 4 h. Absorption at 490 nm was quantified using a colorimetric plate reader.

Caspases 3/7 assay. Caspase-Glo 3/7 assay (Promega) was used as per manufacturer’s instructions. Briefly, PC12 cells were seeded at a density of 1.5 × 10⁴ cells/well in 96-well white-walled plates and incubated overnight. After 24 h of paraquat treatment, Caspase-Glo 3/7 reagent was added for 1.5 h. Caspase activity was analyzed using a luminometer and quantified as relative light units according to manufacturer’s instructions.

Statistical analysis. For all experiments using cell lines, data were results from at least three independent experiments, each with duplicate determinations (n ≥ 3). For Western analysis, blots from three independent experiments were used for densitometry quantification (n = 3). For primary cultured neurons, data were pooled from three independent experiments, each with at least duplicate determinants (n = 3). Statistical analysis of the data was performed using one-way ANOVA with statistically significant values representing an alpha level of 0.05 or below. Error bars represent SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not statistically significant (p > 0.05).

RESULTS

Pesticide-Induced Apoptosis in SH-SY5Y and PC12 Cells

To compare cell death mechanisms induced by rotenone and paraquat, we treated human dopaminergic SH-SY5Y neuroblastoma cells with these agents for 24 h and monitored cell viability. Although rotenone decreased the viability of SH-SY5Y cells using the MTS assay (Fig. 1A) and induced apoptosis (Figs. 1B and 1C), paraquat did not, even at concentrations as high as 500 µM (Figs. 1D and 1E). However, paraquat did reduce the viability of undifferentiated, rat PC12 cells measured by the MTS assay (Fig. 2A) and by the loss of cells (Fig. 2B). Furthermore, treatment of PC12 cells with paraquat induced nuclear condensation (Figs. 2C and 2D), positive TUNEL (Fig. 2C), and activation of caspases 3/7 (Fig. 2E). These data indicate that paraquat induces apoptotic cell death in PC12 cells but not in SH-SY5Y cells.

When PC12 cells were treated with rotenone under normal culture conditions, there was no significant increase in apoptosis even at concentrations as high as 500 µM (Fig. 3A). Since the normal culture media for PC12 cells contains 15% serum and rotenone is lipophilic, we were concerned that binding of rotenone to serum proteins may compromise its activity. To address this issue, PC12 cells were transferred into serum-reduced media (0.5% FBS), culture conditions similar to the rotenone treatment in SH-SY5Y cells in Figure 1. The cells were treated with 100 µM rotenone, a concentration that causes
significant apoptosis in SH-SY5Y cells (Fig. 1B). Although lowering the serum in the culture media in itself increased apoptosis, rotenone did not stimulate apoptosis in PC12 cells in serum-reduced media (Fig. 3B). The insensitivity of PC12 cells to rotenone in serum-reduced media was confirmed by measuring the number of surviving cells after exposure to rotenone (Fig. 3C).

PC12 cells can be differentiated by NGF into postmitotic, terminally differentiated cells that possess many properties of neurons. NGF-differentiated PC12 cells may be more similar to dopaminergic CNS neurons than undifferentiated, proliferating PC12 cells. Therefore, we treated PC12 cells with NGF for 7–10 days to induce differentiation (Fig. 4A) and examined the effect of rotenone and paraquat on apoptosis. NGF-differentiated PC12 cells were more sensitive to paraquat-induced apoptosis than undifferentiated cells (Figs. 4B and 4C). However, like undifferentiated PC12 cells, they were still resistant to rotenone (Figs. 4D and 4E). Collectively, data in Figures 1–4 indicate that SH-SY5Y, undifferentiated PC12, and NGF-differentiated PC12 cells have differential sensitivity to paraquat- and rotenone-induced apoptosis.

**The Sensitivity of PC12 Cells to Paraquat and SH-SY5Y Cells to Rotenone Correlates with Activation of the JNK and p38 MAP Kinases**

Because rotenone-induced apoptosis in SH-SY5Y cells is accompanied by activation of JNK and p38 MAP kinases, it is likely that differential sensitivity to rotenone may be mediated by these kinases. Further studies are needed to elucidate the molecular mechanisms underlying the differential sensitivity of these cell lines to rotenone and paraquat.

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**FIG. 1.** SH-SY5Y cells are sensitive to rotenone but not paraquat. SH-SY5Y cells were treated with 0–150 nM rotenone for 24 h (A–C). (A) Cell viability as measured by MTS assay. Data represent percent viability where colorimetric reading at 490 nm for vehicle control was set as 100% viability. (B) Cells were stained with the DNA dye Hoechst 33285 to visualize nuclear morphology. Cells with condensed or fragmented nuclei were scored as apoptotic using fluorescence microscopy. (C) Mean number of cells per field as an indicator of cell viability after rotenone treatment. (D) SH-SY5Y cells were treated with 0–500 μM paraquat for 24 h and analyzed as in (B). (E) Mean number of cells per field as an indicator of cell viability after paraquat treatment. Cells were treated as in (D). A total of 1.2 × 10^5 cells were analyzed per data point in panel A, and a minimum of 2000 cells were counted per data point for panels B–E. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not statistically significant (p > 0.05).
(Newhouse et al., 2004), we hypothesized that the selective toxicity of paraquat and rotenone in PC12 and SH-SY5Y cells may be due to differential activation of JNK or p38 in these cells. To test this hypothesis, p38 activation was monitored by Western analysis using an antibody that recognizes phosphorylated, active p38 (p-p38). Treatment with 200 μM paraquat increased phosphorylation of p38 in PC12 cells (Fig. 5A) but not in SH-SY5Y cells (Fig. 5B). Paraquat-induced p38 phosphorylation in PC12 cells was detectable within 6 h after treatment and persisted for at least 36 h. Consistent with the anti-p-p38 Western analysis data, paraquat treatment in PC12 cells activated the kinase activity of p38 toward a recombinant GST-ATF2 substrate measured by an immunoprecipitation-coupled kinase assay (Fig. 5C). These data suggest that paraquat activates p38 in PC12 but not in SH-SY5Y cells. In contrast, although rotenone activates p38 in SH-SY5Y cells (Newhouse et al., 2004), it had no effect on p38 phosphorylation in PC12 cells over time periods of 1–24 h (Fig. 5D).

c-Jun is a nuclear transcription factor and a downstream target of JNK. Paraquat also induced phosphorylation of c-Jun in PC12 but not in SH-SY5Y cells (Figs. 6A and 6B). c-Jun phosphorylation was detectable 2 h after paraquat treatment of PC12 cells and persisted for at least 36 h. Conversely, rotenone induced c-Jun phosphorylation in SH-SY5Y cells but not in PC12 cells (Figs. 6C and 6D). These data suggest that the selective sensitivity of PC12 and SH-SY5Y cells to paraquat or rotenone correlates with differential activation of the JNK and p38 MAP kinases in these cell lines.
Activation of JNK and p38 MAP Kinases Is Required for Paraquat-Induced Apoptosis in PC12 Cells

Rotenone-induced apoptosis in SH-SY5Y cells is inhibited by blocking activation of JNK or p38 MAP kinases (Newhouse et al., 2004). To determine if p38 is required for paraquat-induced apoptosis, undifferentiated PC12 cells were either transfected with dnMKK3 (Fig. 7A) or treated with a pharmacological inhibitor of p38, SB202190 (Fig. 7B), to inhibit p38 activation. MKK3 is an upstream activating kinase for p38. Expression of dnMKK3 or treatment with SB202190 significantly attenuated paraquat-induced apoptosis (Fig. 7C).

Similar results were obtained with NGF-differentiated PC12 cells. Inhibition of p38 signaling either by application of pharmacological inhibitor SB202190 (Fig. 8A) or by transient expression of dnMKK3 (Fig. 8B) inhibited paraquat-induced apoptosis. In addition, blocking JNK signaling by a JNK inhibitor, SP600125, (Fig. 8A) or by transient expression of dn-c-Jun (Fig. 8B) attenuated paraquat-induced apoptosis. These data suggest that paraquat-induced apoptosis in undifferentiated and NGF-differentiated PC12 cells requires activation of both JNK and p38 MAP kinases.

Paraquat and Rotenone Cause Selective Degeneration of TH⁺ Dopaminergic Neurons in Primary Mesencephalic Cultures

PD is characterized by selective loss of dopaminergic neurons. To determine if dopaminergic neurons are sensitive to paraquat and rotenone, we prepared primary mesencephalic cultures from E14 rats. This culture preparation contained approximately 92% MAP2⁺ neurons and 8% GFAP⁺ glial cells, a cellular composition similar to that reported in other studies (Cheung et al., 1997; Solomon et al., 1990). Dopaminergic neurons were identified by positive immunostaining for TH (Fig. 9A) and comprised approximately 1–3% of the total cell population. This is consistent with other reports (Gille et al., 2002; Hsuan et al., 2006). We routinely obtained about 50–200 TH⁺ cells per coverslip.

Rotenone induced a small but statistically significant loss of TH⁺ neurons at concentrations as low as 1nM (Fig. 9B). Treatment with 5nM rotenone for 24 h caused an almost complete loss of TH⁺ neurons. However, 1–5nM rotenone did not cause a significant amount of cell death in the general cellular population (Fig. 9C).

Similar results were obtained when primary mesencephalic cultures were treated with paraquat. Treatment with 2–20μM paraquat for 24 h caused a dose-dependent loss of dopaminergic neurons (Figs. 10A and 10B) indicating that primary dopaminergic neurons are more sensitive to paraquat than PC12 cells. While as little as 2μM paraquat induced a small but statistically significant loss of TH⁺ neurons, there was very little change in the percent survival of cells in the general population even after treatment with 100μM paraquat (Fig. 10C). Furthermore, the amount of apoptosis in the entire population, monitored by nuclear condensation and fragmentation, remained relatively constant at 5–6% throughout the treatment (data not shown). These data indicate that dopaminergic neurons are more sensitive to paraquat or rotenone treatment than other cells in the same culture preparation. Furthermore, primary dopaminergic
neurons are much more sensitive to paraquat- and rotenone-induced cell death than undifferentiated PC12 cells, differentiated PC12 cells, or SH-SY5Y cells.

Pesticide-Induced Dopaminergic Neuron Death in Primary Mesencephalic Neuron Cultures Requires JNK

The efficiency for transient transfection of primary cultured neurons is generally below 10% (Hetman et al., 1999; Wang et al., 2006; Xia et al., 1996). Furthermore, only 1–3% of the total cells in primary mesencephalic cultures are TH⁺ dopaminergic neurons, and the yield of total cells from each litter is generally low. Consequently, it was not technically feasible to inhibit JNK or p38 signaling in TH⁺ dopaminergic neurons by the transient transfection of dn-c-Jun or dnMKK3. Thus, we utilized pharmacological inhibitors to investigate the significance of p38 and JNK in dopaminergic neuron death in primary cultures. We pretreated neuron cultures with SB202190 or SP600125 to block p38 or JNK signaling, respectively. SP600125 but not SB202190 significantly inhibited paraquat-induced loss of TH⁺ neurons (Fig. 11A), indicating that JNK, but not p38, is required for paraquat-induced dopaminergic neuron death.

Although we have reported that both JNK and p38 are required for rotenone-induced apoptosis in SH-SY5Y cells (Newhouse et al., 2004), the role of these MAP kinases in rotenone-induced death of primary cultured dopaminergic neurons had not been evaluated. In contrast to paraquat treatment, blocking either p38 or JNK activity caused a significant increase in the number of surviving TH⁺ neurons after
FIG. 5. Effect of paraquat and rotenone on p38 MAP kinase activation. (A) Paraquat induces p38 phosphorylation in undifferentiated PC12 cells, indicative of p38 activation. Undifferentiated PC12 cells were treated with 200 μM paraquat for 0–36 h. Twenty micrograms of protein lysates were used for Western analysis using an antibody that recognizes phosphorylated p38 (p-p38) (top panel). β-Actin was used as a loading control. The intensity of the bands on Western blots was quantified and reported as fold induction (bottom panel). (B) Paraquat (200 μM for 0–24 h) does not induce p38 phosphorylation in SH-SY5Y cells. (C) Paraquat treatment activates the kinase activity of p38 in undifferentiated PC12 cells. PC12 cells were treated with 150 μM paraquat for 0 or 24 h. Total p38 protein was immunoprecipitated from the cell lysates and subjected to an in vitro kinase assay using GST-ATF2 and 32P-ATP as substrates (top panel). The same lysates were subjected to Western blotting for p-p38 (middle panel). β-Actin was used as a loading control (bottom panel). (D) Rotenone does not induce p38 phosphorylation in undifferentiated PC12 cells. PC12 cells were treated with either vehicle control or 100 nM rotenone for 0–24 h in media containing 0.5% serum.

FIG. 6. Effect of paraquat and rotenone on JNK activation. (A) Paraquat induces c-Jun phosphorylation in undifferentiated PC12 cells. PC12 cells were treated with 200 μM paraquat for 0–36 h. Twenty micrograms of protein lysates were used for Western analysis using an antibody that recognizes phosphorylated c-Jun (p-c-Jun) to monitor JNK activation (top panel). β-Actin was used as a loading control. The intensity of the bands on Western blots was normalized to β-actin and reported as fold induction (bottom panel). (B) Paraquat does not induce c-Jun phosphorylation in SH-SY5Y cells. (C) Rotenone does not induce c-Jun phosphorylation in undifferentiated PC12 cells. PC12 cells were treated with either vehicle or 100 nM rotenone for 0–24 h in media containing 0.5% serum. (D) Rotenone induces c-Jun phosphorylation in SH-SY5Y cells. Normalization of c-Jun (bottom panel) was performed as in panel A.
rotenone treatment (Fig. 11B). These data suggest that both p38 and JNK contribute to rotenone-induced dopaminergic neuron death in primary cultures.

**DISCUSSION**

The objectives of this study were to characterize signal transduction pathways underlying paraquat- and rotenone-induced dopaminergic cell death and to identify common molecular mechanisms. Using two model cell lines originating from different species and tissue as well as primary cultured dopaminergic neurons, we discovered that PC12 cells are sensitive to paraquat exposure and that paraquat-induced apoptosis requires activation of both the p38 and the JNK pathways. In contrast, rotenone did not induce cell death or activate p38 or JNK pathways in PC12 cells. Conversely, results were obtained with SH-SY5Y cells. While rotenone causes JNK- and p38-dependent apoptosis (Newhouse et al., 2004), SH-SY5Y cells were insensitive to paraquat treatment. We also discovered that rotenone and paraquat selectively induce cell death of dopaminergic neurons in primary mesencephalic cultures. JNK and p38 were both required for rotenone-induced dopaminergic neuron death. However, paraquat-induced loss of dopaminergic neurons was mediated primarily by JNK with little contribution from p38.
Our data showing that PC12 cells and primary dopaminergic neurons are sensitive to paraquat are consistent with other reports (Peng et al., 2004; Yang and Sun, 1998). We also found that PC12 cells are not as sensitive as SH-SY5Y cells to rotenone. Although other studies have suggested that rotenone causes cell death in PC12 cells, those studies used much higher concentration of rotenone (2 nM vs. 100 nM rotenone in our study) or treated cells for longer periods of time (48 vs. 24 h in our study) (Bal-Price and Brown, 2000; Hartley et al., 1994). In contrast to our data, another study reported 200 μM paraquat is cytotoxic to SH-SY5Y cells (Ding and Keller, 2001). However, Ding and Keller (2001) did not describe the cell culture conditions used, thus precluding a direct comparison of the two studies. It is possible that Ding and Keller (2001) used a cell culture method that rendered the cells more vulnerable to cellular insults. In addition, another study reported paraquat-induced SH-SY5Y cell death; however, the cell death effect was only observed after treatment with 500 μM paraquat for 48 h (Yang and Tiffany-Castiglioni, 2005), a much higher dose and longer treatment time than conditions used in our study. Consistent with our results, another paper reported no obvious cell death after SH-SY5Y cells were treated with 800 μM paraquat for 24 h (Fordel et al., 2006). Regardless, in the present study, we were able to measure the relative sensitivity of SH-SY5Y and PC12 cells to rotenone and paraquat under comparable conditions. Our data clearly demonstrate that SH-SY5Y cells are more sensitive to rotenone, while PC12 cells are more vulnerable to paraquat.
Furthermore, the differential sensitivity of PC12 and SH-SY5Y cells to paraquat is reflected in differential stimulation of the JNK pathway in these cells. Paraquat treatment of rodents may provide a useful model for PD because paraquat induces dopaminergic neuron degeneration and formation of α-synuclein-containing inclusion bodies in vivo (Manning-Bog et al., 2002, 2003; McCormack et al., 2002). However, it was still uncertain whether dopaminergic neurons are selectively sensitive to paraquat (Dauer and Przedborski, 2003; Uversky, 2004). Our in vitro data using cultured neurons demonstrate that paraquat induces selective loss of dopaminergic neurons over other cells in the same preparation, suggesting that paraquat toxicity may be selective for dopaminergic neurons.

Given that epidemiological evidence for an association between pesticide exposure and PD is incomplete, it is important to test this hypothesis biochemically using appropriate cellular and animal models. We have discovered that paraquat and rotenone, representing two different classes of pesticides, specifically target dopaminergic neurons in mixed primary cultures. Our data are consistent with epidemiological studies implicating pesticide exposure in increased PD risk.

The differential sensitivity of PC12 and SH-SY5Y cells to paraquat and rotenone is interesting. Inhibition of mitochondrial complex I is believed to be the primary mechanism for rotenone neurotoxicity. Although structurally similar to MPP⁺, the active metabolite of MPTP, paraquat may not directly affect the dopamine transporter (DAT) or mitochondrial complex I as does MPP⁺ (Richardson et al., 2005). However, other studies have demonstrated an interaction between paraquat and DAT (Ossowska et al., 2005; Yang and Tiffany-Castiglioni, 2005). Thus, the exact role of DAT in selective toxicity of paraquat to dopaminergic neurons remains elusive. The fact that PC12 and SH-SY5Y cells have opposite sensitivities to paraquat and rotenone supports the hypothesis that these two pesticides have distinct cellular targets.

Elucidating biochemical mechanisms underlying selective loss of dopaminergic neurons in PD is important for understanding the etiology of this disease and may identify new drug targets for treatment. Dopamine metabolism, either by auto-oxidation or through the action of monoamine oxidase, can generate reactive oxygen species thereby increasing the burden on dopaminergic neurons, leaving them more susceptible to other forms of stress. Interestingly, dopamine itself induces apoptosis in SH-SY5Y cells through p38 activation. This causes cytochrome C release from mitochondria and caspase activation (Junn and Mouradian, 2001). However, a recent study reports that exogenous dopamine administration to SK-N-MC cells does not exacerbate the toxicity of either paraquat or rotenone (Ramachandiran et al., 2007). It would be interesting to examine the relationship between oxidative stress produced by dopamine metabolism and that induced by rotenone or paraquat in dopaminergic neurons.

Glial cells have also been hypothesized to play an important role in selective dopaminergic neuron loss associated with various PD models. For example, astrocytes are important in MPTP-induced dopaminergic neuron death since they biotransform MPTP into the toxic metabolite MPP⁺ (Ransom et al., 1987). In addition, the presence of activated microglia in mixed primary cultures increases rotenone toxicity to dopaminergic neurons (Gao et al., 2002, 2003). Data reported here show that primary cultured neurons are more sensitive to paraquat- and rotenone-induced cell death than model cell lines. This is consistent with previous reports (Gonzalez-Polo et al., 2004; Hsuan et al., 2006) and supports the suggestion that glia cells, which are present in mixed primary cultures, may play a role in dopaminergic neuron death. However, more research is needed.

**FIG. 11.** Effect of pharmacological inhibitors of p38 and JNK signaling on paraquat- or rotenone-induced loss of TH⁺ neurons in primary cultures. Primary ventral mesencephalic cultures were prepared from E14 rats and pretreated with 10µM of the p38 inhibitor SB202190 or the JNK inhibitor SP60025 for 1 h, followed by paraquat or rotenone treatment for an additional 24 h. (A) Inhibition of JNK but not p38 attenuates paraquat-induced dopaminergic neuron loss. (B) Both JNK and p38 are critical for rotenone-induced dopaminergic neuron death. All TH⁺ cells on each coverslip were counted. Vehicle-treated cells represent 100% survival.
to determine what contribution glial cells make in rotenone- or paraquat-induced dopaminergic neuron death.

The JNK and p38 MAP kinases are activated in response to a variety of cellular stresses and toxicants (Davis, 2000). Prolonged activation of these signaling pathways has been implicated in several forms of neuronal apoptosis (Caughlan et al., 2004; Figueroa-Masot et al., 2001; Namgung and Xia, 2000; Newhouse et al., 2004; Xia et al., 1995). Our data indicate that rotenone-induced apoptosis in SH-SY5Y cells (Newhouse et al., 2004) and primary cultured dopaminergic neurons requires both JNK and p38 activity. Thus, activation of JNK and p38 may be a general requirement for rotenone-induced dopaminergic neuron toxicity. We also discovered that both JNK and p38 contribute to paraquat-induced apoptosis in PC12 cells. However, paraquat-induced loss of dopaminergic neurons in primary cultures requires JNK but not p38, consistent with a recent report (Peng et al., 2004). Thus, p38 activation may increase the susceptibility to paraquat in some cells, but not others.

Although paraquat and rotenone toxicity may have distinct cellular targets and different requirements for p38, our data identify JNK MAP kinase as a common downstream effector mediating paraquat and rotenone toxicity for dopaminergic cell lines and primary cultured dopaminergic neurons. Together with existing evidence in the literature concerning MPTP and 6-hydroxydopamine (Peng and Andersen, 2003), our data suggest the interesting possibility that JNK activation may play a major role in dopaminergic neuron death associated with several PD models.

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