Paraquat, N-methyl-4-phenyl-1,2,3,6 tetrahydropyridine, and rotenone have been shown to reproduce several features of Parkinson’s disease in animal and cell culture models. Although these chemicals are known to perturb dopamine homeostasis and induce dopaminergic cell death, their molecular mechanisms of action are not well defined. We have previously shown that paraquat does not require functional dopamine transporter and does not inhibit mitochondrial complex I in order to mediate its toxic action (Richardson et al., 2005). In this study, we show that paraquat specifically oxidized the cytosolic form of thioredoxin and activated Jun N-terminal kinase (JNK), followed by caspase-3 activation. Conversely, 1-methyl-4-phenylpyridinium (MPP+) and rotenone oxidized the mitochondrial form of thioredoxin but did not activate JNK-mitogen–activated protein kinase and caspase-3. Loading cells with exogenous dopamine did not exacerbate the toxicity of any of these compounds. These data suggest that oxidative modification of cytosolic proteins is critical to paraquat toxicity, while oxidation of mitochondrial proteins is important for MPP+ and rotenone toxicity. In addition, intracellular dopamine does not seem to exacerbate the toxicity of these dopaminergic neurotoxicants in this model.

**Key Words:** Paraquat; Parkinson’s disease; MPTP; rotenone; thioredoxin; MAPK.

Parkinson’s disease (PD) is a chronic neurodegenerative disorder that is characterized by bradykinesia, rigidity, resting tremor, postural instability, and gait abnormalities and affects more than one million people in the United States. The presence of intracytoplasmic inclusions called Lewy bodies and the loss of dopamine containing neurons in the substantia nigra pars compacta are the pathological hallmarks of PD (Braak et al., 2003). Environmental factors along with gene-environment interactions are considered to be major contributing factors for the development of sporadic PD, which represents more than 90% of PD cases (Tanner et al., 1999). Although progress is being made in understanding the pathogenesis of PD, the molecular mechanisms leading to the demise of dopaminergic neurons are not clear (Boye et al., 2005).

The discovery of N-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), the synthetic heroin contaminant (Langston et al., 1983) paved the way for understanding the molecular mechanisms of dopaminergic cell death in various animal and cell culture models (Speciale, 2002). MPTP is oxidized to the active metabolite 1-methyl-4-phenylpyridinium (MPP+) in the brain and is selectively toxic to dopaminergic neurons expressing dopamine transporter (DAT) (Chiba et al., 1985; Gainetdinov et al., 1997; Javitch et al., 1985). The mechanism of toxicity of MPP+ has been proposed to be mediated through inhibition of mitochondrial complex I (Niklas et al., 1985). The rotenone model of PD (Betarbet et al., 2000) has reinforced that complex I inhibition may be a key factor involved in the death of dopaminergic neurons and development of parkinsonism. MPTP has also been shown to release massive amounts of striatal dopamine (Rollema et al., 1986), which in turn may generate more reactive oxygen species (ROS) contributing to dopamine neuron death. This supports the hypothesis that intracellular dopamine-mediated oxidative stress is a contributing factor in the death of dopamine neurons. The herbicide paraquat has been shown to be a risk factor for the development of PD (Semchuk et al., 1992). Since paraquat is structurally similar to MPP+, some have speculated that it is transported through DAT (Shimizu et al., 2003) and inhibits mitochondrial complex I (Dawson and Dawson, 2003; Fukushima et al., 1994; Tawara et al., 1996). However, recent work from our laboratory has shown that paraquat is neither transported by DAT nor inhibits complex I (Richardson et al., 2005). The finding that the route of entry and early site of action is different for paraquat compared to MPTP and rotenone warranted further study to understand the downstream mechanisms of toxicity in this system.
Several lines of evidence from toxicant-induced animal models have shown the involvement of ROS-mediated oxidative stress in the pathophysiology of PD (Bove et al., 2005). Redox (reduction-oxidation) balance plays a key role in living cells and influences several major cellular pathways including cell death. Many redox-active proteins that exist in both reduced and oxidized forms (redox-active couples) protect eukaryotic cells from ROS-induced insults (Jones et al., 2004). Various redox couples have been shown to operate independently in several systems (Hansen et al., 2004; Harper et al., 2001; Watson et al., 2003). Thioredoxin (Trx), a ubiquitous thiol protein, is one of the most important regulators of redox balance in the cell and has been implicated to play a determinative role in cell survival in many disease conditions including cancer and neurodegenerative diseases (Burke-Gaffney et al., 2005). Trx is functional in the nucleus, cytosol, and mitochondria and exists as two distinct systems. The cytosolic form of Trx (Trx1) is the cytoplasmic/nucleus form, whereas mitochondrial form of Trx (Trx2) is a unique gene product and specific to mitochondria (Hansen et al., 2006b). Over expression of Trx1 has been shown to be cytoprotective against paraquat and MPTP-induced oxidative stress (Bai et al., 2002; Byun et al., 2005), whereas over expression of Trx2 exacerbates rotenone toxicity in HEK-293 cells (Damdimopoulos et al., 2002). The development of methods to simultaneously measure redox states of Trx1 and Trx2 provides the possibility to discriminate between chemicals that cause mitochondrial oxidative stress from those that cause oxidative stress in other subcellular compartments. Since paraquat is not a complex I inhibitor (Richardson et al., 2005), unlike MPTP or rotenone, the goal of this study was to characterize and compartmentalize the toxic mechanisms of actions of these parkinsonian chemicals in a neuroblastoma cell line stably expressing functional DAT.

MATERIALS AND METHODS

Cell culture and treatments. SK-N-MC cells (The American Type Culture Collection, Manassas, VA) stably expressing DAT (SK-DAT; Stephens et al., 2002) were cultured at 37°C (5% CO2 and 95% air) in minimum essential media supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 50 U/ml penicillin, 50 μg/ml streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, and 1% nonessential amino acids. The neurotoxants, MPP+ , rotenone, and paraquat were handled according to procedures approved by the Emory University Institutional Health and Biosafety Committee. Cells were plated in culture dishes and allowed to grow for 24 h before treatment of toxicants. Prior to treatment, the media was changed with fresh media containing all supplements. Cell permeable caspase-3 inhibitor was obtained form Calbiochem (EMD Biosciences, La Jolla, CA).

Cell viability assay. Cell viability was measured using WST-1 assay (modified MTT assay-Roche, Mannheim, Germany). The tetrazolium salt WST-1 is cleaved by mitochondrial dehydrogenase in viable cells into a dark red formazan that is spectrophotometrically quantified at 450 nm. The concentration of formazan formed is directly proportional to the number of viable cells. 0.5 × 105 cells were plated in 24-well plates. After 24 h, the media was changed with fresh media, and cells were treated for 48 h with rotenone (5, 10, 20, 50, 100, or 200μM) or paraquat (10, 20, 40, 100, 200, or 400μM). For dopamine or D1 receptor antagonist SCH23390 experiments, cells were pretreated either with dopamine alone or SCH23390 for 1 h followed by dopamine for 1 h and washed twice with cold phosphate-buffered saline (PBS) before treatment with MPP+, rotenone, or paraquat. For mechanistic studies, we used 50μM MPP+, 100nM rotenone, or 200μM paraquat that induce 70–80% cell death. After treatment, the media was again changed before addition of WST-1 reagent. Four hours after the addition of WST-1, the plates were read at 450 nm using a plate reader. The cell viability was calculated keeping the viability of untreated cells as 100%. Data were normalized to total protein using DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Dopamine content assay. SK-DAT cells were grown in 6-well plates (3 × 104 cells) for 24 h and treated with 20, 50, 100, or 200μM of dopamine in complete media with serum. After 1 h of treatment, the cells were washed twice with cold PBS and were sonicated in 100 μl of 0.1M perchloric acid containing 347μM sodium bisulfite and 134μM EDTA. Homogenates were centrifuged at 15,000 × g for 20 min at 4°C, the supernatant removed, and filtered through a 0.22-μm filter by centrifugation at 15,000 × g for 20 min. The supernatants were then analyzed for levels of dopamine, using HPLC with an eight-channel coulometric electrode array (ESA Coularray, Chelmsford, MA) as described previously (Richardson and Miller, 2004). Quantification was made by reference to calibration curves made with individual monoamine standards.

Trx redox Western analysis. Trx1 redox state analysis was performed as previously described (Watson et al., 2003). Separation of the oxidized and reduced forms of Trx1 was performed via Western blotting by native nondenaturing polyacrylamide gel electrophoresis (15% acrylamide). Proteins were electroblotted onto a nitrocellulose membrane prior to the immunodetection by a goat primary antibody for Trx1 (American Diagnostics, Greenwich, CT) and a donkey anti-goat Alexafluor 680 secondary antibody (Invitrogen, Carlsbad, CA). Membranes were scanned using the Odyssey Scanning system (Li-Cor, Lincoln, NE). Densometric analysis of membranes was performed with the Odyssey Scanning software. Redox state of Trx2 was determined by redox Western analysis with 4-acetamido-4′-maleimidylstilbene-2, -2′-disulfonic acid (AMS) as described by Halvey (Halvey et al., 2005) based upon the original method of Damdimopoulos et al. (2002). Briefly, proteins are precipitated with ice-cold 10% trichloroacetic acid for 30 min on ice, washed briefly with acetone, and then labeled with AMS for 30 min. Proteins were separated by nonreducing SDS-PAGE (15% acrylamide), electroblotted onto nitrocellulose membrane and then probed with a rabbit primary antibody for human Trx2. Secondary detection was performed with an anti-rabbit Alexafluor 680 secondary antibody (Invitrogen). Densometric analysis of membranes was performed with the Odyssey Scanning software. Band densitometric values were used with the Nernst equation to estimate steady-state redox potential of Trx1 and Trx2 (Hansen et al., 2006a).

Western blot analysis. 12.5 × 105 cells were plated in 100-mm dishes and grown for 24 h. After changing the media, the cells were treated with toxicants for various period of time (2–36 h). After treatment, cells were washed twice with ice-cold PBS, and total protein was extracted by sonication the cells in lysis buffer containing 20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium ortho vanadate, and 1 μg/ml leupeptin (Cell Signaling Technology, Beverly, MA), supplemented with 1mM PMSF, protease inhibitor cocktail (Roche), phosphatase inhibitor cocktails I, II (Sigma-Aldrich, St. Louis, MO), and 0.5 % SDS. The extract was kept on ice for 5 min and centrifuged at 10,000 × g for 5 min. The supernatant was used for Western blots. Protein was estimated using DC protein assay kit (Bio-Rad Laboratories). Equal amounts of total protein (50 μg) were separated on SDS-PAGE and transferred to PVDF membranes. After blocking and incubating with appropriate primary and secondary antibodies (Cell Signaling Technology), chemiluminescence was developed using Super Signal (Pierce Biotechnology Inc., Rockford, IL) and imaged on an Alpha Innotech Fluorochrom system and stored as digital image.

Statistical analysis. All cell culture experiments represent minimum of three independent experiments performed in triplicate. Statistical analyses were
performed on raw data by one-way ANOVA. Post hoc analysis was performed using Student-Newman Keuls test. Significance is reported at $p < 0.05$.

RESULTS

Paraquat, MPP$^+$, and Rotenone Induce Cell Death in SK-DAT Cells

The toxic mechanisms of parkinsonism-inducing compounds MPP$^+$, rotenone, and paraquat have been studied independently. In order to compare the molecular mechanisms of action of these compounds in a single system, we used neuroblastoma cells stably expressing DAT. Actively dividing SK-DAT cells were treated with various doses of MPP$^+$, rotenone, or paraquat in growth media containing serum. All three toxicants induced dose-dependent cell death after 48 h of treatment (Fig. 1). Rotenone is highly potent with IC50 of 22.5nM, whereas MPP$^+$ and paraquat are less potent with IC50 of 7.65µM and 121.8µM, respectively (Fig. 1). It is interesting to note that the dose-response curve for MPP$^+$ and rotenone are biphasic with initial steep decrease in viability followed by a gradual cell death (60% of rotenone- or MPP$^+$-treated cells die in a linear fashion, whereas the rest 30% cells die gradually). In contrast, paraquat toxicity is linear with steep decrease in viability with increasing dose (Fig. 1). The different kinetics of toxicity observed for MPP$^+$ and paraquat are in agreement with an earlier report in a nigral dopaminergic cell line, SN4741 (Chun et al., 2001).

Intracellular Dopamine Does Not Play a Role in MPP$^+$, Rotenone, or Paraquat-Induced Cell Death

The role of intracellular dopamine in the selective toxicity of dopaminergic neurons is uncertain, since reports show evidence for both presence and absence of a role for dopamine (Hasbani et al., 2005; Hastings et al., 1996; Rabinovic et al., 2000). In order to understand the role of intracellular dopamine in MPP$^+$-, rotenone-, or paraquat-induced cell death, we investigated whether exogenous dopamine transported into SK-DAT cells by DAT influences the toxicity. Although these cells do not contain endogenous dopamine (Table 1), when incubated with various concentrations of dopamine in the media (20–200µM), we found intracellular dopamine indicative of active uptake through DAT (Table 1). It is interesting to note that increasing concentrations of extracellular dopamine did not show a linear increase in intracellular dopamine indicating that cells reach saturation level for intracellular dopamine with the treatment of 50µM dopamine (Table 1). Extracellular dopamine is toxic to the cells in a time- and dose-dependent manner (Fig. 2A). Dopamine is not toxic up to 20µM, but induces cell death in approximately 90% cells when treated at 100µM (Fig. 2A). Pretreatment of cells for 1 h with 20µM dopamine, which is not toxic by itself, exacerbates the toxicity of paraquat (Fig. 2B). However, similar pretreatments did not exacerbate the toxicity of MPP$^+$ and rotenone (data not shown). Extracellular dopamine was completely washed before treatment of toxicants in these experiments. Furthermore, using radiolabeled dopamine pulse, we confirmed that intracellular dopamine taken up by DAT is not reverse transported, indicating, toxicokinetics of the compounds is not affected by dopamine pretreatment in SK-DAT cells (data not shown). Since, SK-N-MC cells are known to express D1 receptor (Sidhu et al., 1999), we pretreated the cells with D1 receptor antagonist SCH23390 before treatment with dopamine or dopamine plus paraquat in order to delineate the mechanism of dopamine-induced exacerbation of paraquat toxicity. The results indicate that exacerbation of paraquat toxicity by dopamine is mediated through the D1 receptor, since the effect is abolished by the pretreatment with a D1 receptor antagonist (Fig. 2B). These data indicate that, alteration in the toxicity of

![FIG. 1. MPP$^+$, rotenone, and paraquat induce dose-dependent cell death.](https://academic.oup.com/toxsci/article-abstract/95/1/163/1689664)

TABLE 1

<table>
<thead>
<tr>
<th>Treatment (µM)</th>
<th>Intracellular (ng/mg total protein) (± SD)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>22.5 ± 3.5</td>
</tr>
<tr>
<td>50</td>
<td>34.2 ± 3.8</td>
</tr>
<tr>
<td>100</td>
<td>35.8 ± 2.8</td>
</tr>
<tr>
<td>200</td>
<td>31.9 ± 2.9</td>
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Note. Cells were treated with indicated concentrations of dopamine in 6-well plates for 1 h in complete media with serum. After treatment, cells were washed twice in PBS, and intracellular dopamine was extracted in 100 µl of 0.1M perchloric acid and measured using HPLC with an eight-channel coulometric electrode array. The data are representative of three independent experiments.
Paraquat by dopamine pretreatment is not due to the intracellular dopamine-mediated toxicity.

**Paraquat Oxidizes Trx1, Whereas MPP⁺ and Rotenone Oxidize Trx2**

MPTP, rotenone, and paraquat are known to induce oxidative stress as part of their mechanisms of toxicity (Bove et al., 2005). Oxidative stress–mediated redox signaling is known to be compartmentalized within the cell (Hansen et al., 2006b). Since Trx is a major redox regulating protein in living cells, we investigated whether paraquat, MPTP, and rotenone target either Trx1 or Trx2. The redox status of Trx1 and Trx2 were independently studied by redox Western blot analysis, using isoform specific antibodies. SK-DAT cells were treated with paraquat, MPP⁺, or rotenone for 4 h, and the cellular proteins were extracted under nondenaturing condition. Levels and the redox state of Trx1 and Trx2 were analyzed by redox Western analysis. Trx1, which is the Trx1, is strongly oxidized by paraquat treatment in a dose-dependent manner, whereas MPP⁺ and rotenone show no significant oxidation of Trx1 (Fig. 3A). On the contrary, the Trx2 is oxidized by MPP⁺ and rotenone (Fig. 3B). Redox potentials of Trx1 and Trx2 calculated from the densitometric analyses of the bands are shown in Table 2. Interestingly, paraquat treatment does not oxidize Trx2 even at a concentration of 500µM (Fig. 3B).
Hydrogen peroxide was used as a positive control, which induces the oxidation of both Trx1 and Trx2. These results indicate that paraquat-induced oxidative stress is mainly cytosolic, whereas MPP\(^+\) and rotenone-induced oxidative stress occurs primarily in the mitochondria.

**Paraquat Toxicity Is Mediated by Jun N-Terminal Kinase and Caspase-3–Dependent Apoptosis**

Mitogen-activated protein kinases (MAPKs) have been shown to play a key role in a variety of cellular signaling including dopaminergic cell death in cell culture and animal model systems (Hunot et al., 2004, Peng et al., 2004). Hence, we investigated the role of MAPKs in MPP\(^+\), rotenone-, or paraquat-induced SK-DAT cell death. Among the three major subfamilies of MAPKs, Jun N-terminal kinase (JNK) was activated by paraquat treatment in SK-DAT cells. JNK was activated as early as 2 h after treatment and levels peaked at 12 h of treatment, indicated by Western blot showing the level of active phosphorylated JNK (Fig. 4). Although there was a mild activation of JNK in the rotenone-treated cells after 2 h of treatment, this was not as robust as observed with paraquat treatment and was not observed in the later time points (Fig. 4). There was a mild induction of p38 MAPK, only in the case of paraquat treatment, which was not observed in the case of MPP\(^+\) or rotenone treatment (Fig. 4). None of the treatments including 20\(\mu\)M dopamine-induced extracellular signal-regulated kinase (ERK) activation in SK-DAT cells (data not shown). In order to investigate potential downstream effectors of cell death, we studied the activation of caspase-3 in SK-DAT cells. Paraquat treatment induced caspase-3 activation after 12 h and reached maximum activation after 36 h of treatment (Fig. 5A). Caspase-3 activation was not observed in MPP\(^+\) or rotenone treatment, suggesting that these compounds do not induce caspase-3–dependent apoptosis in this model system (Fig. 5A).

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>(E_0) Trx1 (mV) ± SD</th>
<th>(E_0) Trx2 (mV) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP(^+)</td>
<td>0</td>
<td>−281 (± 6.1)</td>
<td>−352 (± 6.8)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>−282 (± 5.4)</td>
<td>−321 (± 7.1)*</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>−279 (± 8.3)</td>
<td>−319 (± 8.8)*</td>
</tr>
<tr>
<td>Rotenone</td>
<td>0</td>
<td>−285 (± 4.1)</td>
<td>−361 (± 9.8)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>−280 (± 6.6)</td>
<td>−324 (± 7.7)*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>−278 (± 6.0)</td>
<td>−319 (± 4.1)*</td>
</tr>
<tr>
<td>Paraquat</td>
<td>0</td>
<td>−281 (± 4.4)</td>
<td>−354 (± 9.1)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>−268 (± 7.4)*</td>
<td>−350 (± 11.1)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>−257 (± 4.8)*</td>
<td>−344 (± 12.0)</td>
</tr>
</tbody>
</table>

*Note. Oxidized and reduced forms of Trx1 and Trx2 were separated by redox Western analyses, and the band densitometric values were used with Nernst equation to estimate steady-state redox potentials. Asterisks denote a statistically significant difference from untreated controls (\(p < 0.05\)).

Poly ADP ribose polymerase (PARP), the key enzyme involved in DNA repair is the major substrate for activated caspase-3. During the induction of apoptosis, activated caspase-3 cleaves active PARP (116 kDa) into inactive fragment of 89 kDa. The

**FIG. 4.** Paraquat activates robust JNK and mild p38 MAPK. Western blot analyses of total protein extracts of cells treated with 200\(\mu\)M of paraquat, 50\(\mu\)M MPP\(^+\), or 100nM rotenone in complete media with serum for various time periods. JNK is activated as early as 2 h after paraquat treatment and peaked at 8–12 h. p38 MAPK is induced moderately at 4 h and continued to be active until 30 h after paraquat treatment. Data are representative of three separate experiments. Tubulin was used as protein loading control.

**FIG. 5.** Paraquat activates caspase-3 in SK-DAT cells. (A) Caspase-3 activation. Cells were plated in 100-mm plates and treated with 200\(\mu\)M of paraquat, 50\(\mu\)M of MPP\(^+\), or 100nM of rotenone in complete media with serum for various time periods. Active caspase-3 (generated by the proteolytic cleavage of procaspase-3) was analyzed by Western blot of total protein extracts. (B) Western blot analyses of PARP using total protein extracts isolated from cells treated with 200\(\mu\)M of paraquat, 50\(\mu\)M of MPP\(^+\), or 100nM of rotenone. PARP was analyzed using an antibody that recognizes both the intact (116 kDa) and the cleaved inactive PARP (89 kDa).
formation of the inactive fragment (89 kDa) of PARP is one of the hallmarks of caspase-mediated apoptosis. Treatment of SK-DAT cells with 200\(\mu\)M paraquat-induced PARP degradation as early as 12 h after treatment indicative of caspase–3–mediated apoptosis, which was not observed in the case of MPP\(^+\) or rotenone treatment (Fig. 5B). Pretreatment with 1\(\mu\)M of cell permeable caspase-3 inhibitor (cell permeable DEVD-CHO-EMD Biosciences, La Jolla, CA) 1 h before the treatment of paraquat protects the cells from paraquat-mediated toxicity (Fig. 6). It is noteworthy that caspase-3 inhibitor pretreatment offers almost complete protection from paraquat toxicity, indicating that paraquat toxicity is primarily caspase-3 mediated in SK-DAT cells. Pretreatment of caspase-3 inhibitor did not alter the toxicity of MPP\(^+\) and rotenone, confirming the absence of caspase-3–mediated apoptosis in the case of MPP\(^+\) and rotenone-induced cell death (Fig. 6).

**DISCUSSION**

Paraquat, MPP\(^+\), and rotenone have been used in several *in vitro* and *in vivo* model systems to understand the molecular mechanisms of neurodegeneration. However, the toxic mechanisms of these compounds are not conclusive, since several studies report evidence for both apoptotic and nonapoptotic forms of cell death (Ahmadi *et al.*, 2003; Blum *et al.*, 2001; Choi *et al.*, 1999; Hartmann *et al.*, 2001; Isenberg and Klaunig, 2000; Newhouse *et al.*, 2004; Vila *et al.*, 2001). MPP\(^+\) and rotenone are known to inhibit complex I of the mitochondrial electron transport chain, which in turn reduces the ATP-producing capacity of cells and increases the generation of ROS (Eckert *et al.*, 2003; Glinka *et al.*, 1997; Li *et al.*, 2003; Przedborski and Jackson-Lewis, 1998). We have shown the involvement of mitochondrial impairment–induced oxidative damage in rotenone toxicity in SK-N-MC cells (Sherer *et al.*, 2003). A recent study in human neuronal stem cells shows that rotenone induces caspase-independent cell death in undifferentiated cells and caspase-dependent cell death in differentiated cells (Li *et al.*, 2005), documenting the importance of the nature of the cell in determining the mode of cell death. Our study shows no evidence of caspase-dependent apoptosis in SK-DAT cells during MPP\(^+\) or rotenone-induced toxicity. This is in agreement with the earlier findings that complex I inhibitors including MPP\(^+\) do not induce apoptotic form of cell death (Jackson-Lewis *et al.*, 1995; Shimizu *et al.*, 1996).

Several reports have shown that dopamine can redox cycle, and the products including several quinones may be toxic to cells (Blum *et al.*, 2001; Dryhurst, 2001; Stokes *et al.*, 1999). Since dopamine containing neurons die in PD, it was hypothesized that dopamine plays a key role in the demise of nigral neurons. There are several lines of evidence *in vivo* as well as *in vitro*, supporting this hypothesis (Hastings *et al.*, 1996; Rabinovic *et al.*, 2000). However, dopamine depletion either by genetic ablation of tyrosine hydroxylase, the biosynthetic enzyme which makes dopamine, or by pharmacological alpha methyl-p-tyrosine treatment does not protect against acute MPTP toxicity in mice, indicating that dopamine may not be the major culprit at least at these models of neurotoxicity (Hasbani *et al.*, 2005). Our data are in agreement with these recent findings. Our study also indicates that, in SK-DAT cells, intracellular dopamine does not seem to play a significant role in the toxicity of MPP\(^+\), rotenone, or paraquat. It should be noted that we observed exacerbation of paraquat toxicity by dopamine, which is mediated by the activation of the endogenously expressing D1 receptor, rather than intracellular dopamine-induced oxidative stress. The additive effect of a nontoxic dose of 20\(\mu\)M dopamine in paraquat-induced cell death suggests that paraquat-mediated cell death signaling may cross talk with D1 receptor signal transduction pathway culminating in cell death (Chen *et al.*, 2004). Although dopamine treatment was shown to activate ERK, JNK and p38 MAPKs in SK-N-MC cells, only ERK activation was shown to be involved in D1 receptor activation and cell death. It is noteworthy that, although significant MAPK activation and cell death occurred at concentrations higher than 50\(\mu\)M, these authors observed moderate MAPK activation by as low as 10\(\mu\)M dopamine (Chen *et al.*, 2004). We have shown a robust JNK and moderate p38 MAPK activation during paraquat-induced cell death, which is exacerbated by the pretreatment of 20\(\mu\)M dopamine. We did not observe ERK induction in SK-DAT cells following 20\(\mu\)M dopamine treatment, indicating that, in this system, D1 receptor–mediated downstream signaling may cross talk with paraquat-induced toxic mechanisms and exacerbate its toxicity without the involvement of ERK.

Trx performs multiple functions including the regulation of redox-sensitive transcription factors and, through peroxiredoxins, detoxification of ROS. We have recently shown in HeLa cells that, tumor necrosis factor-\(\alpha\)–induced oxidative stress is specifically localized to mitochondria, which distinctly oxidizes
mitochondrial Trx2 (Hansen et al., 2006b). In this study, we found that paraquat oxidizes only the cytosol-specific isoform of Trx, whereas MPP⁺ and rotenone preferentially oxidize the mitochondrial form, suggesting the subcellular localization of oxidative stress is distinct for the different toxicants. Although the glutathione system is also present in the mitochondria, flux occurs between the mitochondria and cytosol. The Trx2 system is the only antioxidant system that is specific to mitochondria. Although we have not studied other redox-active couples like glutathione, data presented in the present study indicate that, oxidative stress generated by paraquat is compartmentalized to cytosol whereas, MPP⁺- and rotenone-induced oxidative stress is mainly mitochondrial. Disruption of redox balance and, hence, the redox signaling has been linked to cell death in many cases. Recently, reduced Trx was shown to inhibit rotenone-induced activation of p38 MAPK pathway by binding to apoptosis signal–regulating kinase1 (ASK1), whereas oxidation of Trx releases ASK1 and activates the MAPK pathway (Hsieh and Papaconstantinou, 2006). Our data, demonstrating that reduced Trx2 system is mainly mitochondrial. Disruption of redox balance and, hence, the redox signaling has been linked to cell death in many cases. Recently, reduced Trx was shown to inhibit rotenone-induced activation of p38 MAPK pathway by binding to apoptosis signal–regulating kinase1 (ASK1), whereas oxidation of Trx releases ASK1 and activates the MAPK pathway (Hsieh and Papaconstantinou, 2006). Our data, demonstrating that paraquat oxidizes Trx1 prior to activating MAPK, indicates the significance of the compartmentalization of redox signaling machinery.

Since paraquat is structurally similar to MPP⁺, it has been hypothesized that the mechanism of paraquat-mediated neurotoxicity is also similar (Shimizu et al., 2001). However, recently, we have shown that paraquat is neither a complex I inhibitor nor a substrate for DAT (Richardson et al., 2005), indicating that the molecular mechanism of neuronal cell death induced by paraquat may be different from MPP⁺. JNK, which is also known as stress-activated protein kinase has been shown to be involved in both survival and cell death depending on the cell type and stimulus (Davis, 2000; Kyriakis and Avruch, 2001). JNK activation was shown to be closely associated with the dopaminergic cell death observed in PD (Hunot et al., 2004; Peng and Anderson, 2003; Peng et al., 2004). Data presented in this study shows that paraquat induces Jun N-terminal MAPK-mediated caspase-3–dependent cell death, unlike MPP⁺- or rotenone-induced cell death supporting our hypothesis. Paraquat has been shown to induce oxidative stress followed by Jun N-terminal MAPK-mediated caspase-3 dependent cell death in several model systems (McCarthy et al., 2004; McCormack et al., 2002; Peng et al., 2004). However, these studies did not compare the mechanisms of action of MPP⁺- or rotenone with that of paraquat. We tested MPP⁺-, rotenone, and paraquat in the same system and found that their mechanisms of action are different. Indeed, a caspase-3 inhibitor completely protected cells from paraquat toxicity but not from MPP⁺- or rotenone-mediated toxicity. Our data also show a moderate induction of p38 MAPK during paraquat-induced cell death, which is not observed with MPP⁺ or rotenone. Activation of p38 was known to induce proapoptotic response in several systems (Davis, 2000). However, in this case, since the p38 induction was not robust, it may act as an additive factor in the JNK-mediated toxic response. Moreover, in some systems, p38 was activated as a compensatory or pro-survival response to the toxic insult mechanism (Caughan et al., 2004; Mao et al., 1999).

Cell death or survival is controlled at several stages. Although several death mechanisms involve oxidative stress as initiator of death process, the downstream molecular pathways are dependent on the cell type, toxicant, and the kinetics of treatment. In addition, the distinct cellular compartments in which these processes occur may influence the downstream signal transduction pathways in a diverse manner, ultimately culminating in cell death. Understanding these different signal transduction pathways initiated by various toxicants, which likely cross talk to induce cell death, may help understand the mechanism of cell death observed in neurodegeneration. In this study, we show that the mechanism of action of the herbicide paraquat is fundamentally different from that of MPP⁺ and rotenone. MPP⁺ and rotenone being mitochondrial complex I inhibitors primarily target mitochondria and induce oxidative stress, whereas paraquat induced cytosolic oxidative stress followed by caspase-3–mediated cell death. The distinct molecular mechanism of toxicity of these parkinsonism-inducing compounds should be taken into consideration when designing experiments aimed at understanding the pathogenesis of PD.

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REFERENCES


