Environmental exposure to the oxidant-producing herbicide paraquat has been implicated as a risk factor in Parkinson's disease. Although intraperitoneal paraquat injections in mice cause a selective loss of dopaminergic neurons in the substantia nigra pars compacta, the exact mechanism involved is still poorly understood. Our data show that paraquat induces the sequential phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun and the activation of caspase-3 and sequential neuronal death both in vitro and in vivo. These effects are diminished by the specific JNK inhibitor SP600125 and the antioxidant manganese(III) tetraakis (4-benzoic acid) porphyrin in vitro. Furthermore, JNK pathway inhibitor CEP-11004 effectively blocks paraquat-induced dopaminergic neuronal death in vivo. These results suggest that the JNK signaling cascade is a direct mediator of the paraquat-mediated nigral dopaminergic neuronal apoptotic machinery and provides a molecular linkage between oxidative stress and neuronal apoptosis.

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the midbrain. It affects over one million people in North America (1, 2). Epidemiological evidence has suggested that environmental agents in combination with genetic susceptibility may be responsible for the associated neurodegeneration in PD (3). Exposure to agricultural chemicals via living in a rural environment, drinking well water, or occupational exposure has been postulated to be a potential environmental risk factor for the disease (4–10). The widely used herbicide 1,1′-dimethyl-4,4′-bipyridium (paraquat) has been suggested as a prime risk factor for PD based on reports of parkinsonism correlated with exposure to the agent (4, 8) and on the similarity of the chemical structure of paraquat to the active metabolite of the parkinsonism-inducing agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridium ion (MPP⁺). Although recent studies have shown that paraquat, either alone or in combination with the diethercarbamate fungicide manganese ethylenebisdithiocarbamate, results in preferential degeneration of the nigrostriatal system (11, 12), the molecular mechanisms by which paraquat induces dopaminergic neuronal cell death are not yet fully understood. Understanding the mechanism that mediates paraquat-selective degeneration of nigral dopaminergic neurons may provide insights into more effective therapeutic approaches for the disease.

A variety of extracellular stimuli elicit cellular activities such as survival, proliferation, differentiation, and apoptosis through the activation of a family of mitogen-activated protein kinases (MAPKs) consisting of the extracellular signal-regulated kinases (ERKs), the p38 MAPK, and the c-Jun N-terminal kinases (JNKs) (13, 14). The JNKs, also known as stress-activated protein kinases (SAPKs), serve as phosphorylation substrates for MAP kinase kinases (MKKs) such as MKK4/7, which are activated in turn by phosphorylation via MAP kinase kinase kinases (MKKKs). JNK is activated in response to many different stress factors including heat shock, inflammatory cytokines, protein synthesis inhibitors, growth factor withdrawal, chemotherapeutic drugs, and ultraviolet irradiation (15, 16). However, the role of JNK signaling pathway in cell death is highly controversial. JNK activation has been implicated in both cell survival and cell death in response to stress depending on the cell type or the stimulus (13, 15).

Several studies have suggested that the JNK signal transduction pathway may be involved in PD and that blocking the activity of the JNK protein kinases may be effective in preventing disease-related neuropathology (for a review, see Ref. 17). For example, the activation of the JNK signaling pathway converging on the phosphorylation of c-Jun has been associated with the induction of apoptosis in MPTP-treated mice (18, 19). These and related findings prompted us to assess the changes in c-Jun and the JNK pathway activity in dopaminergic neurons during paraquat-induced cell death, a recently established model of the disease (12). We report here evidence that suggests a role for the JNK pathway in paraquat-mediated apoptosis of midbrain dopaminergic neurons.

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† To whom correspondence should be addressed: Buck Institute for Age Research, Novato, California 94945 and the Program in Molecular Biology, Department of Biological Sciences, University of Southern California, Los Angeles, California 90089.*
clonal antibodies were obtained from Chemicon (Temecula, CA). Media and sera were from Invitrogen. The ERK inhibitor PD98059, the p38 inhibitor SB202190, and the JNK inhibitor SP600125 were from Calbiochem. Benzoyloxycarbonyl-DEVD-fluoromethylketone (Z-DEVD-fmk) was purchased from BD Biosciences. Rabbit anti-4-hydroxyxenonalenol antibody was from Alpha Diagnostic International (San Antonio, TX). Manganese(III) tetrakis (4-benzoic acid) porphyrin (MnTBPAP) was purchased from OXIS International Inc. (Portland, Oregon). CEP-11004 was a gift from Cephalon (West Chester, PA).

Cell Culture—N27 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell viability was determined by MTT incorporation (20). To evaluate the effect of signaling pathways on cell death, the inhibitors listed above were added 1 h prior to exposure.

Caspase-3 Activity Assay—Caspase-3 activity was examined using a commercially available kit according to the manufacturer’s directions (Bio-Rad) (20). Briefly, 50 μg of protein from each sample was incubated with the synthetic substrate benzoyloxy-Asp-Glu-Val-Asp-7-aminofluoromethyl coumarin (Z-DEVD-APC) for 2 h at 37 °C. Fluorescence was measured using a microplate spectrofluorometer (excitation at 490 nm, emission at 530 nm). Serial dilutions of AQC were used as standards. A negative control in which caspase-3 inhibitor (Ac-DEVD-CMK) was added and a positive control containing apoptosis were used to test the efficacy of the assay.

Paraquat Administration—Eight-week-old male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were intraperitoneally injected with either saline or 7 mg/kg paraquat dichloride hydrate (dissolved in saline) at 2-day intervals for a total of 10 doses. Animals were killed at day 7 or 8 after the last administration. The JNK pathway inhibitor CEP-11004 (1 mg/kg) was administered to mice by subcutaneous injection 1 day prior to paraquat administration at 2-day intervals for a total of 10 doses and for 7 days following the last paraquat injection on alternative days. Experimental protocols were in accordance with the National Institutes of Health Guidelines for Use of Live Animals and were approved by the Animal Care and Use Committee at the Buck Institute.

Primary Mesencephalic Cultures—Primary mesencephalic cell cultures were obtained from embryonic gestation day 14 mouse embryos. Ventral mesencephalic tissue was mechanically dissociated by mild trituration in ice-cold calcium and magnesium-free Hank’s balanced saline solution (Invitrogen) and incubated with 0.05% trypsin/EDTA at 37 °C for 15 min. Dissociated cells were transferred to neurobasal medium (Invitrogen) containing 2% B27 supplement, 2 mM glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded onto poly-D-lysine-coated 24-well culture plates at a density of 7 × 10^4 cells/well. Cultures were maintained at 37 °C for 15 min. Dissociated cells were transferred to neurobasal medium (Invitrogen) containing 2% B27 supplement, 2 mM glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded onto poly-D-lysine-coated 24-well culture plates at a density of 7 × 10^4 cells/well. Cultures were maintained at 37 °C in a 5% CO2 incubator.

Cultures were fixed with 4% paraformaldehyde in PBS for 1 h, washed in PBS, and then incubated in 0.3% Triton X-100 in PBS for 1 h. Cultures were permeabilized with 0.3% Triton X-100 in PBS for 1 h. Cultures were permeabilized with 0.3% Triton X-100 in PBS for 1 h.

Immunohistochemistry—Following perfusion with saline and 4% paraformaldehyde in phosphate-buffered saline, brains were removed, immersion-fixed in the same fixative overnight, and cryoprotected in 20% sucrose. Serial coronal sections (40 μm) were cut on a cryostat.

For double immunolabeling studies, sections were incubated with blocking solution (2% horse serum, 1% bovine serum albumin, and 0.1% Triton X-100) in phosphate-buffered saline, pH 7.5) and then with primary antibodies at 4 °C overnight followed by secondary antibodies in blocking solution at room temperature for 2 h. The primary antibodies used were sheep anti-TH polyclonal antibody (1:200), rabbit polyclonal anti-JNK (1:100), rabbit polyclonal anti-phospho-c-Jun (1:100), and rabbit polyclonal anti-cleaved caspase-3 (1:100). The secondary antibodies were rhodamine-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch; 1:200) and fluorescein isothiocyanate-conjugated pig anti-rabbit IgG (Vector Laboratories; 1:200). Sections were mounted with Vectashield (Vector Laboratories), and fluorescence signals were detected using a Zeiss Axioplan2 photomicroscope equipped with a Neuro lucida stereo investigator (MicroBrightField, Williston, VT).

RESULTS

JNK Pathway Involved in Paraquat-induced Apoptosis

N27 is a dopaminergic cell line derived by SV40 immortalization of rat midbrain neurons from isolated mesencephalic cultures (22). The N27 cell line produces dopamine and expresses the dopamine-synthesizing enzyme tyrosine hydroxylase and the dopamine transport. This cell line has been used for comparing the effects of various neurotoxins in dopaminergic neurons and is therefore a suitable model system to study the role that paraquat-mediated oxidative stress may have on JNK activation as it relates to Parkinson’s disease. Incubation of N27 cells with various concentrations of paraquat for 24 h significantly affected cell viability. As shown in Fig. 1A, paraquat reduced cell viability in a dose-dependent manner. Cell death was accompanied by an increase in caspase activation; Western blot analysis of the caspase-3 cleavage product p17 protein in cells 18 h after paraquat treatment showed a marked increase in its levels as compared with the control protein (Fig. 1B). This suggests that paraquat-induced cell death is at least in part apoptotic.

Paraquat Activates JNK but Not ERK and p38 in Vitro—Paraquat is known to generate superoxide (23). The specific signal transduction pathways involved in cellular responses to
superoxide vary with cell type. For example, Chinese hamster lung fibroblast (V79) cells respond with a rapid but transient activation of both p38 and JNK (24), whereas ERK and p38 are selectively activated in primary rat microglia (25). To determine whether specific MAPK signal transduction pathways are involved in cellular responses to paraquat, we measured changes in the expression of phosphoactivated forms of JNK, p38, and ERK. Using phosphospecific antibodies against JNK, p38, and ERK, we found that exposure to paraquat for 12–18 h resulted in a progressive increase in immunoreactivity for phospho-JNK, whereas antibody against total JNK showed no paraquat-induced change in expression (Fig. 2). In contrast, paraquat did not induce the phosphoactivation of either p38 or ERK. Because JNK activation has been suggested to be involved in the induction of apoptosis by different insults (13, 15), we examined whether paraquat was able to activate JNK in cells through phosphorylation of its specific substrate c-Jun transcription factor. As shown in Fig. 2, the level of phospho-c-Jun increased as the duration of paraquat exposure increased up to 18 h. This sustained phosphorylation did not result from the increased expression of c-Jun as protein levels were unaltered relative to untreated samples.

**Fig. 2. Activation of the JNK signal transduction pathway in response to paraquat in N27 cells.** Cells were treated with 400 μM paraquat for 12 and 18 h and then harvested. Protein samples from whole cell extracts were loaded on 12% SDS-PAGE gels and transferred to PVDF membranes for Western blot analysis. Phospho-JNK (p-JNK), phospho-c-Jun (p-c-Jun), phospho-ERK1/2 (p-ERK), phospho-p38 (p-p38), total JNK, and total c-Jun from whole cell extracts were detected with the specific antibodies. Data shown are representative blots from three independent experiments per panel. β-Actin was used as a loading control. C, control.

**Inhibition of JNK Kinase Attenuates Paraquat-induced Cell Death in Vitro**—To investigate further the relationship between paraquat induction of MAPK signaling pathways and paraquat-induced cell death, we used a series of kinase inhibitors to inactivate the ERK, p38, and JNK receptors. We expected that whether a particular kinase were critical for the effects of paraquat on cell death, inhibiting its activity should reverse these effects. As shown in Fig. 3A, treatment with the JNK inhibitor SP600125 (7 μM) blocked paraquat up-regulation of caspase-3 activity, whereas the ERK inhibitor PD98059 (20 μM) and the p38 inhibitor SB203580 (10 μM) were ineffective, although SB203580 (10 μM) inhibited the phosphorylation of p38 and PD98059 (20 μM) inhibited the phosphorylation of ERK in N27 cells (Fig. 3C). Both the superoxide dismutase mimetic manganese(III) tetrakis (4-benzoic acid) porphyrin (200 μM) and a relatively specific caspase-3 inhibitor Z-DEVD-fmk (25 μM) also attenuated paraquat-mediated caspase-3 activity. Similarly, MnTBAP, SP600125, and Z-DEVD-fmk also protected N27 cells from paraquat-induced death, whereas the other inhibitors were ineffective in this regard (Fig. 3B). The above results collectively suggest that both superoxide production and JNK activation are required for paraquat-induced caspase-mediated cell death of N27 cells in vitro.

**Paraquat Induces JNK Activity in Primary Mesencephalic Cultures**—N27 is a transformed cell line that may have survival mechanisms that are absent in normal primary dopaminergic neurons. Therefore, to verify the role of the JNK signaling pathway in paraquat-induced cell death on a cellular level in primary dopamine neurons, we explored the effects of paraquat treatment in primary mesencephalic cultures via immunofluorescence with antibodies specific for both tyrosine hydroxylase and phospho-c-Jun, phospho-JNK, and activated caspase-3. In untreated control cultures, there was virtually no colocalization of phospho-c-Jun, phospho-JNK, and activated caspase-3 in TH-positive neurons (Fig. 4). In primary mesencephalic cultures treated with 40 μM paraquat for 18 h, however, colocalization of phospho-c-Jun, phospho-JNK, and activated caspase-3 was apparent in TH-positive neurons (Fig. 4).

To investigate further the relationship between paraquat induction of the JNK signaling pathway and paraquat-induced TH-positive neuronal death, we first treated neurons with MnTBAP (100 μM) or the JNK inhibitor SP600125 (5 μM), beginning 1 h prior to paraquat treatment. Fig. 5A shows that...
both MnTBAP and SP600125 reduced the colocalization of phospho-c-Jun and phospho-JNK and activated caspase-3 with TH-positive neurons after 18 h of paraquat treatment. Cells were stained for TH at 24 h after paraquat treatment, and TH-positive cells were counted. As shown in Fig. 5B, treatment with the JNK inhibitor SP600125 (5 \( \mu \)M) protected TH-positive neurons from paraquat-induced death, whereas the ERK inhibitor PD98059 (10 \( \mu \)M) and the p38 inhibitor SB203580 (8 \( \mu \)M) were ineffective. Both MnTBAP (100 \( \mu \)M) and Z-DEVD-fmk (25 \( \mu \)M) also attenuated paraquat-mediated TH-positive neuron death (Fig. 5B). These results suggest that superoxide production via paraquat results in the activation of the JNK signaling pathway and subsequent apoptotic neuronal death of dopaminergic midbrain neurons.

Paraquat Treatment Results in JNK Activation in Nigral Dopaminergic Neurons in Vivo—To determine whether JNK is activated in SNpc neurons after paraquat administration in vivo, we performed immunofluorescent double-labeling experiments using TH as a dopaminergic marker. In midbrain tissues from untreated mouse brains, phospho-JNK and phospho-c-Jun immunostaining were weak and diffusely cytoplasmic in nigral dopaminergic neurons (Fig. 6A). However, in tissues from mice treated with paraquat, this cytoplasmic immunolocalization within dopamine neurons exhibited a bright speckled appearance that became progressively more intense by 7 days (Fig. 6A) after the last paraquat administration. At the same time point after paraquat treatment, we detected the expression of activated caspase-3 in the cytosol of a substantial number of dopaminergic neurons using an antibody specifically recognizing cleaved caspase-3 (Fig. 6A). Similar results were found with regard to the differences in duration of JNK activation, c-Jun phosphorylation, and caspase-3 activation via Western blot analysis (Fig. 6B).

Paraquat Decreases the Number of Nigral Dopaminergic Neurons—To examine the neurodegenerative effects of systemic paraquat administration on SNpc dopaminergic neurons in mice, we also assessed midbrain sections immunostained with TH antibody. As shown previously (12), TH immunohistochemistry revealed a significant depletion of TH-positive neurons in the SNpc at day 8 after the last injection (Fig. 7A). In contrast, no marked reduction in TH-positive neurons was
seen in the ventral tegmental area as compared with that in controls (Fig. 7A). By using stereological techniques, we counted the number of nigral TH-positive neurons in saline- or paraquat-injected mice at day 8 after the last administration. As shown in Fig. 7B, paraquat administration results in a 29% reduction in nigral TH-positive neurons in mice as compared with saline-injected controls. Conversely, administration of CEP-11004 immediately prior to paraquat completely protected against these toxic effects, and indeed, the counts of TH-positive neurons were not different in the substantia nigra of mice injected with paraquat plus CEP-11004 as compared with saline-treated controls (Fig. 7B), suggesting the critical role of the JNK signal pathway in paraquat-mediated nigral dopaminergic neuronal death.

**DISCUSSION**

The major finding of this study is the critical role for JNK/c-Jun in mediating signals contributing to the selective dopaminergic neuronal death induced by paraquat. Our data demonstrate that neurons undergoing paraquat-induced apoptosis activated the activator protein-1 (AP-1) transcription factor c-Jun. This activation was regulated by post-translational mechanisms through the sustained activation of the JNK pathway. Induction of c-Jun activity in degenerating dopaminergic neurons preceded caspase-3 activation and neuronal death. These events appear to have functional importance in regulating dopaminergic neuronal survival and death from neurotoxins and environmental stresses.

The mechanism of paraquat neurotoxicity is most likely mediated via oxidative stress. Paraquat generates superoxide by both electron transfer reactions with NADH-dependent oxireductases and redox cycling via reaction with molecular oxygen (23, 26, 27). Previous studies indicate that neuronal-derived superoxide plays an important role in paraquat neurotoxicity...
Several neurotoxicants, including MPP⁺, rotenone, diethrin, manganese, and salsolinol, induce increases in superoxide and related reactive oxygen species and subsequent activation of the JNK pathway, which precedes apoptosis (29). In agreement with these data, our data show that MnTBAP, a unique category of superoxide dismutase mimetic, prevents paraquat-induced JNK activation and subsequent apoptosis. Other antioxidants including N-acetylcysteine and catalase have also been shown to inhibit dopamine-induced apoptotic death via an oxidative stress-involved JNK signaling pathway (30).

Following paraquat administration, the following sequence of events presumably takes place: superoxide production triggers JNK activation and phosphorylation of c-Jun. These effects preceded and triggered up-regulation of caspase-3 activation, which in turn contributes to the apoptotic response. The fact that inhibition of activated ERK by PD98059 and p38 by SB203580 did not suppress apoptosis suggests that ERK and p38 are not significantly involved in the paraquat-induced apoptotic pathway of dopaminergic neurons. On the other hand, inhibition of JNK activation by the novel JNK inhibitor SP600125 (31) significantly attenuated activation of caspase-3 and subsequent apoptosis, suggesting that JNK activation occurs upstream of caspase-3.

The results described in this study map the early signaling events by which the activation of JNK after paraquat treatment can lead to apoptosis in dopaminergic neurons. JNK is involved in many cellular responses such as proliferation, differentiation, and apoptosis (13, 15). It is thought that the duration of its activation may be crucial in the signaling decision; sustained JNK activation participates in the dopaminergic neuronal apoptosis induced by several neurotoxicants (18, 19, 29, 30). Increased oxidative stress via antisense inactivation of superoxide dismutase in PC12 cells in vitro was found to activate JNK (32). We have shown that prolonged phosphorylation of JNK, accompanied by phosphorylation of c-Jun, is an important step in the apoptotic signaling cascade induced by paraquat. The activation of JNK results in c-Jun phosphorylation, suggesting that this protein kinase plays an important role in regulating c-Jun transcriptional activity. Activated c-Jun is implicated in many neuronal cell death paradigms (33, 34). Phosphorylation of c-Jun has been shown to be essential for dopaminergic neuronal apoptosis induced by MPTP (19). Inhibition of c-Jun activity by dominant-negative overexpression, neutralizing antibody injection, and genetic disruption blocks sympathetic neuronal apoptosis induced by nerve growth factor withdrawal (35, 36). Moreover, sympathetic neurons carrying a mutant c-Jun gene (JunA) that lacks the two critical N-terminal phosphorylation site serines 63 and 73 are resistant to apoptosis induced by the excitatory amino acid kainate (37).

In addition, we investigated the protective effects of a selective caspase-3 inhibitor as well as the JNK inhibitor SP600125 in preventing paraquat-mediated cell death (Figs. 3 and 5). Cytochrome c and Smac (second mitochondria-derived activator of caspase) or DIABLO are two key modulators of the intrinsic cell death pathway. Numerous recent studies have shown that activated JNK is involved in cytochrome c or Smac/ DIABLO release into the cytoplasm, where it can complex with apoptosis-activating factor 1 (Apaf-1) and caspase-9, causing the activation of this initiator caspase (38, 39). Caspase-9, in turn, can cleave and activate the downstream executioner caspase-3. This results in the cleavage of additional cellular substrates, leading to the morphological changes associated with apoptosis, including chromatin condensation and DNA fragmentation (40).

In support of the idea that the JNK signaling cascade is a direct activator of the paraquat-mediated SNpc dopaminergic neuronal death, we found that paraquat-induced death is suppressed by CEP-11004 (Fig. 7). Screening of upstream kinases in the JNK pathway has demonstrated that CEP-11004 acts by inhibiting upstream mixed-lineage kinases (MLKs) (41). CEP-11004 has been found to reduce apoptosis in several neuronal apoptosis paradigms, including nerve growth factor deprivation, Aβ42, and MPP⁺ (41). Furthermore, CEP-11004 has been shown to be an effective neuroprotectant in the MPTP model of PD in vivo (41, 42).

In summary, our data support the conclusion that the activation of JNK is a major component of the mechanism of sensitization of dopaminergic neurons to paraquat-mediated apoptosis. MnTBAP can block both paraquat-induced JNK activation and apoptosis, indicating that paraquat-induced oxidative stress is involved. Understanding the mechanisms leading to paraquat-induced neuronal death may lead to novel therapeutics in the treatment of neurodegenerative disorders such as PD. Indeed the JNK inhibitor CEP-1347, an analogue of CEP-11004, is currently in clinical trials as a treatment for PD. Our data, demonstrating the involvement of the JNK/c-Jun pathway in yet another model of the disease, strengthen the argument that JNK/c-Jun inhibitor may be an effective therapy for the disease.

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The Herbicide Paraquat Induces Dopaminergic Nigral Apoptosis through Sustained Activation of the JNK Pathway
Jun Peng, Xiao Ou Mao, Fang Feng Stevenson, Michael Hsu and Julie K. Andersen

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