Paraquat (PQ) causes selective degeneration of dopaminergic neurons in the substantia nigra pars compacta, reproducing an important pathological feature of Parkinson disease. Oxidative stress, c-Jun N-terminal kinase activation, and α-synuclein aggregation are each induced by PQ, but details of the cell death mechanisms involved remain unclear. We have identified a Bak-dependent cell death mechanism that is required for PQ-induced neurotoxicity. PQ induced morphological and biochemical features that were consistent with apoptosis, including dose-dependent cytochrome c release, with subsequent caspase-3 and poly(ADP-ribose) polymerase cleavage. Changes in nuclear morphology and loss of viability were blocked by cycloheximide, caspase inhibitor, and Bcl-2 overexpression. Evaluation of Bcl-2 family members showed that PQ induced high levels of Bak, Bid, BNip3, and Noxa. Small interfering RNA-mediated knockdown of BNip3, Noxa, and Bak each protected cells from PQ, but Bax knockdown did not. Finally, we tested the sensitivity of Bax-deficient mice and found them to be resistant to PQ treatments that depleted tyrosine hydroxylase immuno-positive neurons in the substantia nigra pars compacta of wild-type mice.

Early cell loss in specific neuron populations is common to several neurodegenerative disorders, including Parkinson disease (PD), Alzheimer disease, and spinocerebellar atrophy (1). In PD, dopaminergic neurons in the substantia nigra pars compacta (SNpc) are depleted, with clinical signs of bradykinesia and rigidity appearing once the loss of those cells surpasses 70%. Familial mutations, metabolic disorders and exposure to environmental agents are implicated as potential causative factors in many neurodegenerative disorders (2). Sporadic PD is thought to have an environmental component, which is supported by epidemiological studies showing strong correlations between exposure to agricultural pesticides and a higher risk for late onset PD (3–9). A prime candidate is the widely used herbicide 1,1′-dimethyl-4,4′-bipyridium (paraquat/PQ) because it can cause selective degeneration of tyrosine hydroxylase immuno-positive (TH⁺) neurons in the SNpc, and long term exposure was shown to increase PD risk 6-fold in a Taiwan population that sprays it on rice fields (10–12).

The depletion of dopaminergic neurons in PD is thought to result from programmed cell death (13), a process that is critical for eliminating cells in development and homeostasis. The most common form of programmed cell death is Type I/apoptosis, which can be triggered through extrinsic and intrinsic cell death pathways (14). Activation of the intrinsic pathway centers on the release of apoptogenic factors from mitochondria, including cytochrome c (15). Cytochrome c normally shuttles electrons between complexes III and IV in intermembrane space of mitochondria, but once released into the cytoplasm it complexes with Apaf-1 and procaspase-9 to form an active apoptosome (16). Because apoptosome formation quickly leads to executioner caspase activation and apoptosis, the release of cytochrome c is tightly regulated. Pro-apoptotic members of the Bcl-2 family facilitate cytochrome c release by causing transient membrane disruptions, referred to as mitochondrial outer membrane permeabilization (MOMP; 17). Once unrestrained, Bcl-2 family members Bak and Bak trigger MOMP. In healthy cells Bak and Bax are blocked by anti-apoptotic members such as Bcl-2 and Bcl-xL (18). Apoptotic stimuli can disrupt these interactions to disinhibit Bak and/or Bax and cause MOMP. Pro-apoptotic BH3-only members of the family bind to anti-apoptotic members, such as Bcl-2 and Bcl-xL, thereby freeing Bak and Bax to cause MOMP and release cytochrome c.

The cytotoxic actions of PQ clearly involve reactive oxygen species (ROS), but its specificity for dopaminergic neurons and possible involvement of the intrinsic cell death pathway are unclear (9). Although positively charged, PQ is actively transported across the blood-brain barrier by the same neutral amino acid transporter used by L-valine and L-dopa (12, 20). Inside neurons, PQ induces oxidative stress by producing superoxide anions through redox cycling (11). Dopaminergic neurons may be particularly sensitive to high ROS because of dopamine metabolism that creates hydrogen peroxide and superoxide radicals, which in turn react with nitric oxide to form highly toxic peroxynitrite (21). High ROS production stretches metabolic resources as the cell removes misfolded proteins, replaces oxidized lipids, and repairs damaged DNA (22). The cells that cannot compensate for this oxidative stress undergo default apoptosis or necrosis under severe conditions,
but PQ-treated cells can be protected by superoxide dismutase/catalase mimetics (23–25). Although it is known that PQ-induced ROS activates JNK (24, 26) and leads to caspase-3 cleavage, mechanisms acting in between have not been identified.

In the present study, we investigated PQ-induced cell death, which shows morphological and biochemical features that are consistent with apoptosis. PQ induced high levels of pro-apoptotic Bcl-2 family members. Loss-of-function approaches demonstrated that Bak knockdown protected cells from PQ, and Bak-deficient mice were resistant to PQ neurotoxicity. We found that PQ neurotoxicity is mediated by a Bak-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

Reagents—Unless otherwise indicated all reagents were purchased from Sigma. Dulbecco’s modified Eagle’s medium: Ham’s F-12 medium was from BioWhittaker and fetal calf serum was from Hyclone (Logan, UT). Parafilm was from Chem Service (West Chester, PA), and z-VAD-fmk was from Enzyme Systems (Aurora, OH). Hoechst 33342, propidium iodide, CalceinAM, and TMRE were from Molecular Probes-Invitrogen (Eugene, OR).

**SK-N-SH Cell Lines**—Parental lines of SK-N-SH were obtained from ATCC maintained in Dulbecco’s modified Eagle’s medium: Ham’s F-12 medium (1:1) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were grown and treated in a humidified incubator with 5% CO2 and 10% O2 at 37 °C. Thawed vials of SK-N-SH cells were used for no more than 15 passages.

Treatment Conditions—Typically, the cells were plated at 3.4 × 10^5/cm² (3.0 × 10^5/well) in each well of a 6-well plate and grown for 24 h prior to experimental procedures. Cell death was induced by incubation with 200 or 400 μM paraquat/PQ (unless otherwise indicated) for 18 h. When cycloheximide (1 μM) or z-VAD-fmk (50 μM) were used, they were added to media at time 0. Staurosporine was used at 250 nM.

Assessment of Cell Viability and Apoptosis—To assay nuclei for apoptotic morphology, the cells were incubated in medium containing 25 μM Hoechst 33342 for 5 min at room temperature, and the images were captured using a Nikon TE2000 inverted fluorescent microscope with a 20× FLUOR objective and a Hamamatsu ORCA-AG 12-bit CCD camera using Image-Pro Software (3–6 images/well). For CalceinAM/propidium iodide viability assay, the cells were incubated in medium containing 5 μM CalceinAM and 100 ng/ml propidium iodide for 15 min at 37 °C, and then the images were captured with a fluorescent video microscope (3–6 images/well). For Δψ cells were incubated in medium containing 50 nM TMRE for 20 min at 37 °C and kept on ice until analysis with a BD FACScan.

**Cytochrome c Release from Mitochondria**—The cells were fractionated into cytosolic and heavy membrane fractions, as previously described (27). Briefly, the cells were lysed in cold homogenization buffer (20 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol) containing 250 mM sucrose and homogenized on ice with a Dounce homogenizer. The homogenates were centrifuged at 700 × g for 10 min at 4 °C, and the supernatants were centrifuged at 22,400 × g for 15 min at 4 °C. The resulting supernatants were the cytosolic fraction, and the pellets were mitochondria-rich. The pellets were resuspended in mitochondria buffer (50 mM HEPES-KOH, pH 7.4, 1 mM EDTA, 0.2 mM dithiothreitol, 1% Nonidet P-40) containing 10% glycerol and further centrifuged at 22,400 × g for 15 min at 4 °C. The resulting supernatants were heavy membrane/mitochondrial fractions. The fractions were separated and immunoblotted with cytochrome c-specific antibody (no. 556433; BD Biosciences).

**Stable SK/Bcl-2 Lines**—Stable Bcl-2-expressing SK-N-SH lines were made by transfecting SK-N-SH cells with His₆-tagged human Bcl-2/pDNA3 (gift of Dr. Doug Green) using Lipofectamine 2000, followed by selection for G418 resistance. Expression of the transgene was confirmed by immunoblotting whole cell extracts with anti-His antibody (sc-8036; Santa Cruz).

**Immunoblotting**—Whole cells lysates were prepared from treated cells in Laemmli buffer with 2-mercaptoethanol. Samples were sonicated to disrupt genomic DNA and then boiled in sealed tubes for 5 min. Equal volumes of samples were loaded onto SDS-PAGE gels (4–15% gradient gels; Bio-Rad), separated, and blotted onto polyvinylidene difluoride (Amersham Biosciences) in Tris-glycine-MeOH buffer. Nonspecific binding was blocked by incubation of the membrane with 1% cold fish gelatin in PBS for 1 h at room temperature. The blots were probed with primary antibodies specific for actin (A-2066; Sigma) caspase-3 (no. 622701; Biolegend), Bak (no. 556382; BD Biosciences), Bax (no. 556467; BD Biosciences), Bid (no. 550365; BD Biosciences), Bik (no. 557040; BD Biosciences), Bnip3 (B7931; Sigma), Mcl-1 (M-863-P0; Lab Vision), and Noxa (OP-180; Calbiochem). Primary antibodies were incubated in 1% milk in PBST (PBS with 0.05% Tween 20) for 1 h at room temperature or overnight at 4 °C, with rocking. The blots were rinsed three times for 15 min with PBST and then incubated with Alexa-conjugated secondary antibodies (anti-mouse and total RNA was isolated with TRIzol (Invitrogen). One-voluminated with equal volumes of isopropanol at room temperature for 30 min. Precipitated RNA was concentrated by centrifugation at 14,000 g (g 4 °C) for 30 min and then rinsed with RNase-free 90% EtOH, followed by a second centrifugation. After removal of trace EtOH, the RNA pellet was allowed to air dry for 5–10 min and resuspended in RNase free 10 mM Tris. RNA was quantified by spectrophotometry (A₂₆₀), and 1 μg of total RNA was used for each reverse transcriptase reaction with Superscript III (Invitrogen) and oligo(dT) primers, at 65 °C for 5 min, on ice for 1 min, at 50 °C for 60 min, and at 70 °C for 15 min. Each qPCR contained 1/50th of a single reverse transcription reaction (20 ng of initial template), primers, and qPCR master mix containing SYBR green (Abgene, UK). Primer sets that cross an intron-
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exon boundary were preferentially used to prevent background amplification of genomic DNA. Triplicate reactions for each oligonucleotide primer set were prepared and run simultaneously in a 96-well plate using an ABI PRISM 7700 sequence detection system with the following conditions: 15 s at 95 °C and 1 min at 60 °C for 40 cycles. Glyceraldehyde-3-phosphate dehydrogenase and actin primers served as internal controls.

siRNA—Small interfering RNA (siRNA) for human Bak, Bax, and BNip3 were designed with the Whitehead Institute siRNA selection program (28). The sequences for siRNA were: human BNip3 sense, 5′-GAA GAU GGG CAG AUC AUG UUU; human BNip3 antisense, 5′-ACA UGA UCU GCC CAU CUU; human Bak sense, 5′-CCG ACC UGA UCA CUC AGA CUU; human Bak antisense, 5′-CUC UGA GUC AUA GCG UCG GUU; human Bax sense, 5′-CCG CUC CAC UCA CCA UCU GGU; and human Bax antisense, 5′-UUG GCG GAG UGA GUG GUA GAC. Those siRNA were synthesized at the DNA/RNA Peptide Synthesis Facility in the Beckman Research Institute at the City of Hope Medical Center (Duarte, CA). Noxa siRNA were purchased (sc-37305; Santa Cruz Biotechnology). Duplexed siRNA was transfected into SK-N-SH cells using HiPerFect (Qiagen) as per the manufacturer’s instructions. Knockdown was confirmed with immunoblots, using whole cell lysates.

Immunostaining—Wild-type C57BL/6 mice were obtained from Charles River (Hollister, CA). They received two intraperitoneal injections of either saline or 10 mg/kg PQ separated by a 1-week interval and were sacrificed 2 days after the second injection. The time point was chosen because it coincides with the onset of cell loss in the SNpc (46). The mice were deeply anesthetized and intracardially perfused with heparinized saline, followed by 4% paraformaldehyde. All of the animal studies were done according to National Institutes of Health and Institutional Animal Care and Use Committee guidelines. The brains were excised and cryoprotected by immersion in sucrose solution prior to sectioning on a cryostat. Coronal sections through the entire midbrain were made at 40 μM and 10% saline. Midbrain sections in 80 °C 10 mM citrate (pH 8.5) for 20 min. Endogenous 3% H2O2 for 10 min and rinsing three times in PBS. The sections were then depleted by immersing sections in whole cell lysates.

Bak Knock-out Mice—Eight-week old mice that were deficient in Bak (strain B6-129-Bak1fng1Thor) and the appropriate control strain (C57BL/6) were obtained from Jackson Laboratories (Bar Harbor, ME). The animals received three intraperitoneal injections of either PQ or saline spaced 1 week apart. The mice were sacrificed 1 week after the last injection. A block containing the midbrain was immersion-fixed in 4% paraformaldehyde overnight at 4 °C prior to cryoprotection in graded sucrose solutions. The tissue block was snap frozen in cold isopentane and sectioned into 40 μM sections on a cryostat for stereological cell counting.

RESULTS

PQ Induces Apoptosis—Apoptotic cells usually display a condensed brightly stained nucleus (Fig. 1A). PQ significantly increased the percentage of apoptotic nuclei in cultures of SK-N-SH neuroblastoma cells treated for 18 h (Fig. 1B). This cell death could be blocked by the ribosome inhibitor cycloheximide or the caspase inhibitor z-VAD-fmk. Another feature of apoptotic cells is the activation of caspase 3 and the cleavage of caspase substrates such as poly(ADP-ribose) polymerase, and both occurred in PQ-treated cells (Fig. 1C). These findings were consistent over three separate experiments and in studies involving longer time points (not shown). A key step in the intrinsic cell death pathway is the release of cytochrome c from the intermembrane space of mitochondria. Subcellular fractionation showed dose-dependent increases of cytochrome c in cytosolic extracts, indicative of release (Fig. 1D). Cytochrome c release in response to PQ could be blocked by cycloheximide, but not z-VAD-fmk, indicating caspase-independent release. Together, these findings demonstrate that PQ neurotoxicity induces typical apoptosis, which requires new protein synthesis and caspase activity.

To determine whether cycloheximide and z-VAD-fmk blocked cell death and not simply the appearance of apoptotic nuclei, we also evaluated cells for viability using calceinAM (Fig. 2A and supplemental Fig. S1) and YOPRO3 (supplemental Fig. S1) assays. Cultures treated with PQ showed a significantly lower percentage of viable cells compared with controls. Cycloheximide and z-VAD-fmk provided significant protection
Paraquat Neurotoxicity through Bak

FIGURE 1. PQ induces apoptosis in SK-N-SH neuroblastoma cells. A, photomicrograph of Hoechst 33342-stained nuclei after PQ treatment shows a mixture of apoptotic (arrows) and nonapoptotic nuclei. B, PQ significantly increases the percentage of apoptotic nuclei, but cycloheximide and z-VAD-fmk protect. The cells were treated with the indicated combinations for 18 h; the error bars represent S.E. *** p < 0.005. C, immunoblot showing dose-dependent caspase-3 cleavage in response to PQ at 200 and 400 μM for 18 h. Densitometry values indicate full-length (32 kDa) and cleaved caspase-3 normalized to actin and the control lane. D, poly(ADP-ribose) polymerase cleavage is increased by PQ. Normalized densitometry values are shown for cleaved poly(ADP-ribose) polymerase. E, cytosolic extracts from PQ-treated SK-N-SH cells (18 h) show dose-dependent cytochrome c (cyto C) release. The densitometry values are normalized to actin and the control lane.

FIGURE 2. Cycloheximide, caspase inhibitor, and Bcl-2 maintain cell viability after PQ treatment. A, calceinAM viability assays showed that PQ reduces viability, but z-VAD and cycloheximide (chx) maintain viability for at least 18 h. The cells overexpressing Bcl-2 (SK/Bcl-2) were almost completely protected from PQ neurotoxicity. B, PQ reduces the percentage of cells with functioning mitochondria, as indicated by Δψ, but cycloheximide, z-VAD, and Bcl-2 maintain more cells with intact mitochondria. Staurosporine (sts) effects on wild-type and Bcl-2 overexpressing SK-N-SH cells are shown for comparison. The results were representative of three separate experiments. The error bars represent S.E. *** p < 0.005.

To determine whether MOMP may be relevant for PQ-induced apoptosis, we generated SK-N-SH cell lines that stably express anti-apoptotic Bcl-2 (supplemental Fig. S1). SK/Bcl-2 clones were highly resistant to PQ neurotoxicity (Fig. 2A). The resistance of SK/Bcl-2 clones to PQ was consistent over three experiments. These findings establish that PQ-induced apoptosis can be blocked by at least one anti-apoptotic member of the Bcl-2 family, suggesting that the mechanism may involve MOMP.

Cycloheximide-mediated protection established that PQ neurotoxicity requires new protein synthesis that may include pro-apoptotic members of the Bcl-2 family. We evaluated expression patterns of key Bcl-2 family members using qPCR and immunoblots. Bak and Bax are key players in MOMP as bak−/−/bax−/− cells are resistant to DNA damage, endoplasmic reticulum stress, and many other apoptotic stimuli (32, 33). PQ induced bak mRNA (3x) (Fig. 3A) and Bak protein (1.5x) (Fig. 3B). Interestingly, PQ did not induce bax mRNA (Fig. 3A) or Bax protein (Fig. 3B). Bak is constitutively localized to the outer mitochondrial membrane where it is inhibited by Bcl-xL and Mcl-1 (34, 35). PQ induced bcl-xL and bfl-1/A1 expression (Fig. 3A) but not mcl-1. Protein levels of Bcl-xL and Bfl-1 were also higher (Fig. 3B).

Anti-apoptotic members use direct binding to prevent Bak and Bax from causing MOMP and permitting cytochrome c release. This inhibition can be disrupted by BH3-only members that bind to anti-apoptotic members, freeing Bak and Bax to cause MOMP. PQ induced the BH3-only members bnip3, noxa, and bid (Fig. 3C). Interestingly, BNip3 and Noxa bind to Bcl-xL and...
Mcl-1, respectively, two important inhibitors of Bak. BNip3 (1.6x) and Noxa (2.5x) protein levels were also higher after PQ treatment. Full-length Bid is cytoplasmic until cleaved by caspases-8, -10, or -2 to form tBID, which then translocates to mitochondria and facilitates both Bak and Bax activation. Although PQ potently induced Bid expression in SK-N-SH cells, it is noteworthy that SH-SY5Y cells, the parental line of SK-N-SH cells, do not express caspase-8 because of DNA methylation (36, 37). Taken together, these findings demonstrate that PQ induces the pro-apoptotic Bcl-2 family member Bak and two of its activators, BNip3 and Noxa.

**Bak, BNip3, and Noxa Knockdown Protect against PQ Neurotoxicity**—To determine the physiological significance of BNip3, Noxa, and Bak in PQ neurotoxicity, we knocked down the expression of each one using siRNA. Bak siRNA protected SK-N-SH cells completely at 200 μM and partially at 400 μM of PQ.

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**FIGURE 3.** PQ effects on mRNA and protein levels of key Bcl-2 family members. A, real-time PCR analysis of anti-apoptotic and BH1–3 members of the Bcl-2 family in SK-N-SH cells treated with PQ (200 μM) or control (Ctrl) media for 18 h. B, immunoblots of whole cell lysates from SK-N-SH cells treated with PQ (200 μM) or control media for 18 h. The samples were probed with primary antibodies for Bak, BNip3, Noxa, Bax, Bid, Bfl-1, and Bcl-xL. Densitometry values below each lane were normalized to actin levels and the control lane in each blot. C, qPCR analysis of BH3-only members in cells treated with PQ or control medium. Each PCR was done in triplicate; the error bars represent S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.005.

**FIGURE 4.** Bak knockdown protects cells from PQ, but Bax knockdown does not. A, viability of SK-N-SH cells treated with Bak siRNA (gray bars) or scrambled siRNA (white bars) and then treated with 0, 200, or 400 μM PQ for 18 h. The bars represent percentage of cells (50,000 total) with functional mitochondria as indicated by ΔΨm. *, p < 0.05. B, immunoblotting confirmed knockdown of Bak in cultures assessed for viability in A. C, Bak knockdown did not provide any protection against PQ at 200 or 400 μM, as indicated by ΔΨm. D, immunoblotting confirmed knockdown of Bax protein using the experimental procedures in C. E, BNip3 knockdown protected SK-N-SH cells from PQ at 200 and 400 μM, as indicated by ΔΨm. F, immunoblotting confirmed BNip3 knockdown using the experimental conditions in E. G, Noxa knockdown protected SK-N-SH cells from PQ at 400 μM, as indicated by ΔΨm. H, Immunoblotting confirmed BNip3 knockdown using the experimental conditions in G. The error bars represent S.E. *, p < 0.05; **, p < 0.01.
Paraquat Neurotoxicity through Bak

**TABLE 1**

|          | TH | Bak | BNip3 | Noxa |
|----------|----|-----|-------|------|
| Saline   | 58 ± 8.0 | 67.3 ± 7.9 | 92.3 ± 5.9 | 225.4 ± 24 |
| PQ       | 46.8 ± 4.1 | 89.5 ± 5.1 | 112 ± 11.5 | 190.8 ± 32.7 |

*p < 0.05.
*p < 0.01.

PQ (Fig. 4A). Significant knockdown of Bak protein under those conditions was confirmed by immunoblotting (Fig. 4B). Bak is critical from MPTP-induced nigrostriatal degeneration (38) but was not strongly induced by PQ, so we used siRNA to knock down Bak as a control. Knocking down Bak protein levels did not protect SK-N-SH cells from PQ at 200 or 400 μM (Fig. 4C). Immunoblotting confirmed knockdown of Bak under these conditions (Fig. 4D). These findings suggested that PQ does not have a generalized effect on Bcl-2 members but depends specifically on Bak for MOMP. BNip3 knockdown also protected SK-N-SH cells from PQ at 200 and 400 μM (Fig. 4E). Immunoblotting revealed significant knockdown of BNip3 protein in cells treated with siRNA, but PQ induction of BNip3 was still noticeable even in extracts from knockdown cultures (Fig. 4F). SiRNA-mediated knockdown of Noxa protected SK-N-SH cells from the toxic effects of PQ at 200 and 400 μM (Fig. 4G). Protection from 400 μM PQ was higher with Noxa siRNA than with Bak or BNip3 knockdown. Immunoblots confirmed Noxa knockdown under the conditions tested (Fig. 4H). Taken together, lowering protein levels of Bak, BNip3, or Noxa each provided protection against PQ neurotoxicity.

**Bak Knock-out Mice Are Resistant to PQ Neurotoxicity**—We immunostained midbrain slices from PQ-treated mice and found significantly more Bak and BNip3 immuno-positive neurons in the SNpc of PQ-treated mice compared to control mice (Table 1 and supplemental Figs. S3 and S4). To conclusively demonstrate the significance of Bak in PQ neurotoxicity in vivo, we tested Bak-deficient (bak−/−) and control C57BL/6J mice for their sensitivity to PQ-induced depletion of TH+ neurons in the SNpc. Bak-deficient mice have no overt phenotype. Wild-type mice injected with saline had 5925 ± 54 TH+ neurons/side in cross-sections at the level of the third cranial nerve. However, PQ-treated mice had only 4110 ± 116 TH+ neurons (Fig. 5A), which was a statistically significant decrease (p < 0.005). In contrast, bak−/− mice injected with saline had 5671 ± 111 TH+ cells/side, and PQ-treated mice had 5435 ± 411, a difference that was not statistically significant. Nissl-stained neuron counts in the SNpc of these mice showed similar results. Wild-type mice injected with saline had 7122 ± 197 Nissl-stained neurons/side, but PQ-treated wild-type mice had 5136 ± 151 (Fig. 5B), a statistically significant decrease (p < 0.005). However, bak−/− mice injected with saline had 6688 ± 215 neurons in the SNpc/side, whereas PQ-treated knock-outs had 6508 ± 432, which was not significantly different. Together, these data indicate that PQ induces the production of new proteins that activate Bak, resulting in biochemical and morphological changes that are consistent with apoptotic cell death.

**DISCUSSION**

Since the discovery that MPTP can induce selective nigrostriatal neurodegeneration, there has been a concerted effort to identify environmental factors that contribute to sporadic PD. The discovery of several PD-linked loci has provided important insights into mechanisms that can kill nigrostriatal neurons, yet a growing body of work continues to show that late onset PD has a significant environmental component. A more thorough understanding of gene-toxicant interactions is required to determine how environmental triggers exacerbate genetic predisposition in some individuals but not others. The present study adds to this knowledge by establishing the significance of a key cell death pathway in the neurotoxicity of a widely used herbicide that has been implicated in sporadic PD.

Overproduction of ROS has been implicated in the pathophysiology of PD, Alzheimer disease, polyglutamine diseases, amyotrophic lateral sclerosis, and other neurodegenerative disorders (1). ROS modify proteins causing misfolding and aggregation, as well as the activation of cell death pathways, which are common features in neurodegeneration. ROS are incontestably involved in the mechanisms by which PQ kills dopaminergic neurons (26, 39); however, molecular links between PQ-induced oxidative stress and caspase activation remain unresolved. In the present study, we found that PQ triggers apoptosis through the intrinsic cell death pathway, which includes Bak-dependent MOMP, cytochrome c release, caspase-3 activation, and ultimately apoptotic cell death.

Although PQ shares some structural similarities with MPP+ (40, 41), early suggestions that they share a common mecha-
Paraquat Neurotoxicity through Bak

**FIGURE 6. Proposed mechanism for PQ neurotoxicity at the level of MOMP.** PQ induces the BH3-only members Noxa and BNip3, as well as Bak. Noxa specifically binds to Mcl-1 and BNip3 binds to Bcl-xL (34). Thus, PQ induces the expression of two BH3-only members that block two major inhibitors of Bak, Mcl-1 and Bcl-xL. Binding of Noxa to Mcl-1 and BNip3 to Bcl-xL causes disinhibition of Bak, making it available to create MOMP. MOMP releases cytochrome c from the intermembrane space into the cytoplasm where it can interact with Apaf-1 and procaspase-9 to create an apoptosome. The fully active apoptosome processes and activates executioner caspase-3 triggering apoptosis.

nism have not been supported. Unlike MPP⁺, PQ entry into neurons does not rely on DAT (42), and it has a lower binding affinity for complex I (43). The unresolved ability of PQ to inhibit mitochondrial complex I (44) has led to speculation that it may kill neurons through ATP depletion leading to necrotic death. We found that PQ neurotoxicity does not lead to the direct loss of Δψ through mitochondrial dysfunction, because caspase activity was required to compromise the inner mitochondrial membrane. PQ-induced apoptosis requires new protein synthesis to initiate apoptosis, and our findings suggest that BNip3 and Noxa are among the newly made proteins that may drive this process. Further divergence between MPTP/MPP⁺ and PQ effects on the oxidation status of thioredoxin have also been shown to impact caspase-3 cleavage (39). MPP⁺ activates cell death through JNK, p53, and ultimately Bax-mediated release of cytochrome c. Although PQ also activates JNK and its downstream target c-Jun (26), in knockdown studies we found that Bax is not required for PQ-induced cell death.

Links between ROS generation/JNK activation and caspase-3 activation remain a black box that may involve components, such as transcription factors, mitochondrial dysfunction, chaperones, the proteasome, and others. Our findings suggest that these upstream influences are integrated by Bcl-2 family member expression profiles. PQ neurotoxicity acts through a Bak-dependent mechanism that may rely on BNip3 and Noxa (Fig. 6). Our finding that Bak-deficient mice are resistant to PQ provides conclusive evidence that Bak is necessary for PQ neurotoxicity in vivo. Bak is constitutively present on the surface of mitochondria where it is inhibited by Mcl-1 and Bcl-xL but not Bcl-2 (34, 45). Huang and colleagues (34, 35) have reported that Bak-mediated cytochrome c release requires a double hit to neutralize both anti-apoptotic Bcl-xL and Mcl-1. Although PQ raised protein levels of Bcl-xL slightly, it strongly induced the expression of BH3-only members that interfere with Bcl-xL and Mcl-1 function. BNip3 specifically interacts with Bcl-xL and blocks its binding to Bak, whereas Noxa binds strongly to Mcl-1 and blocks its inhibition of Bak. PQ increased the number of Bak-positive cells in the SNpc and provided the double-hit necessary to neutralize the anti-apoptotic effects of Bcl-xL and Mcl-1. We found that PQ induced higher BNip3 and Noxa expression in vitro and that knocking down these proteins, or Bak, made cells more resistant to PQ-induced apoptosis. Basal expression of Noxa was detected as immuno-positive neurons in the SNpc of untreated B6 mice, and the decrease of Noxa-positive cells in PQ-treated mice may simply reflect depletion of neurons in this nucleus. Our findings show that Bak is a necessary component of PQ-induced cell death in vitro and in vivo and provides a novel apoptotic stimulus that works through a Bak-specific mechanism, perhaps independently of Bak.

The involvement of BNip3 and Noxa raises the possibility that another transcription factor, hypoxia-induced factor-1α, may also be an important upstream component because both genes are transcriptional targets for hypoxia-induced factor-1α (40, 46, 47). Because hypoxia-induced factor-1α expression increases under ischemic conditions in the brain and heart (48), it is possible that hypoxia or transient ischemia may further elevate BNip3 and Noxa and thereby enhance PQ-dependent neurotoxicity. Interestingly, head trauma often causes ischemic injuries, and head injury has been linked with an increased risk for PD (19, 49).

In summary, our in vitro and in vivo findings indicate that PQ-induced apoptosis results from Bak-mediated MOMP, with cytochrome c release and caspase-3 activation. This report reduces the number of unknown steps between ROS/JNK and caspase-3 activation and demonstrates that these steps act on a relatively small proportion of Bcl-2 family members that regulate Bak. Findings in this report have potential implications for human neurodegenerative processes and may help identify mechanisms by which environmental toxicants and other risk factors contribute to PD pathogenesis.

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