Parkinsonian Mimetics Induce Aspects of Unfolded Protein Response in Death of Dopaminergic Neurons*

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Genes associated with Parkinson’s disease (PD) have suggested a role for ubiquitin-proteasome dysfunction and aberrant protein degradation in this disorder. Inasmuch as oxidative stress has also been implicated in PD, the present study examined transcriptional changes mediated by the Parkinsonism-inducing neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP+™) in a dopaminergic cell line. Microarray analysis of RNA isolated from toxin treated samples revealed that the stress-induced transcription factor CHOP/Gadd153 was dramatically up-regulated by both 6-OHDA and MPP+™. Treatment with 6-OHDA also induced a large number of genes involved in endoplasmic reticulum stress and unfolded protein response (UPR) such as ER chaperones and elements of the ubiquitin-proteasome system. Reverse transcription-PCR, Western blotting, and immunocytochemical approaches were used to quantify and temporally order the UPR pathways involved in neurotoxin-induced cell death. 6-OHDA, but not MPP+™, significantly increased hallmarks of UPR such as BiP, c-Jun, and processed Xbp1 mRNA. Both toxins increased the phosphorylation of UPR proteins, PERK and eIF2α, but only 6-OHDA increased phosphorylation of c-Jun. Thus, 6-OHDA is capable of triggering multiple pathways associated with UPR, whereas MPP+™ exhibits a more restricted response. The involvement of UPR in these widely used neurotoxin models supports the role of ubiquitin-proteasome pathway dysfunction in PD.

Parkinson’s disease (PD)† involves an irreversible degeneration of the dopaminergic nigrostriatal pathway, resulting in marked impairments of motor control. Although the etiology of PD remains unknown, both genetic and environmental factors appear to play a role. For example, three genes and several putative loci have been identified (1), including two autosomal dominant mutations of the α-synuclein gene, that were linked to familial early-onset PD (2, 3). α-Synuclein has subsequently shown to be the major component of Lewy bodies, the hallmark inclusion of PD (4). Parkin, a second gene with mutations associated with PD (5), has been shown to be an ubiquitin-protein isopeptide ligase (6). Loss of Parkin activity is linked to endoplasmic reticulum (ER) stress and unfolded protein response (UPR; Refs. 7 and 8). Finally, a missense mutation in the gene encoding ubiquitin C-terminal hydrolase L1 is also associated with rare cases of PD (9). Thus, aggregation of α-synuclein together with defects in the ubiquitin pathway support the notion that a dysfunctional ubiquitin-proteasome system in which aberrant proteins are not cleared may play a major role in PD. The role of proteasomal impairment has been further emphasized by recent reports that pharmacological inhibition of proteasome function leads to selective degeneration of dopaminergic neurons in culture (10) as well as in vivo (11). In particular, cell death was associated with increased cytoplasmic levels of α-synuclein and ubiquitin, as well as the formation of inclusion bodies (10, 11). Taken together, accumulating genetic and molecular evidence suggests that defects in ER and ubiquitin-proteasomal processing contribute to the pathogenesis of PD.

Because PD is largely restricted to dopaminergic neurons and because dopamine is easily oxidized in vitro and in vivo to a variety of neurotoxic metabolites, dopamine itself is considered a major factor in this disorder. For example, dopamine is readily oxidized to highly cytotoxic quinone molecules via at least three different enzymatic pathways (for review see Ref. 12). Moreover, in the presence of transition metals and hydrogen peroxide, dopamine can be converted to 6-OHDA (for review see Ref. 13), a highly potent endogenous neurotoxin widely used to create animal models of PD (13). Both 6-OHDA and other dopamine quinine derivatives have been found in post-mortem Parkinsonian brains (14–16), a finding that, together with the extensive studies documenting 6-OHDA-induced nigral degeneration, underscores the role of dopamine plays in its own demise.

Similarly, another PD mimetic, N-methyl-4-phenyl-1,2,3,6-tetrahydroxyridine (MPTP) or its active derivative, MPP+™, is also thought to induce oxidative stress and impair energy metabolism (for review see Ref. 17). The original finding that human exposure to MPTP results in PD (18) has been replicated in various animal models including non-human primates (for review see Ref. 17). Thus, both 6-OHDA and MPP+™ have been shown to produce reactive oxygen species and to inhibit mitochondrial complex I, as well as to mimic many behavioral, pharmacological, and pathological symptoms of this disorder (for review see Refs. 13, 17, and 19). Despite these parallels, the molecular mechanisms by which these neurotoxins kill cells remain unclear. Further, their relevance to emerging genetic and pharmacological models investigating ubiquitin-proteasome pathway dysfunction and protein aggregation has yet to be studied.

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The abbreviations used are: PD, Parkinson’s disease; 6-OHDA, 6-hydroxydopamine; MPP+™, 1-methyl-4-phenylpyridinium; UPR, unfolded protein response; RT, reverse transcription; ER, endoplasmic reticulum; MPTP, N-methyl-4-phenyl-1,2,3,6-tetrahydroxyridine; PBS, phosphate-buffered saline; PERK, PKR-like ER kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; ANOVA, analysis of variance; TH, tyrosine hydroxylase; eIF, eukaryotic initiation factor.
Role of UPR in 6-OHDA and MPP⁺ Toxicity

Previous results from this laboratory and others have demonstrated that 6-OHDA and MPP⁺ trigger morphologically distinct forms of cell death in the dopaminergic cell line MN9D and mouse primary mesencephalic cultures (13, 20, 21). Markers of apoptosis such as chromatin condensation and caspase-3 cleavage are widespread in cells treated with 6-OHDA, but not with MPP⁺. Despite the different forms of cell death induced by either toxin, both types of cell death seem to be dependent on de novo protein synthesis (22, 23). However, few studies of gene expression in 6-OHDA or MPP⁺-induced dopaminergic cell death models have been done. Presumably, this is as a result of the scarcity and heterogeneity of the tissue involved as well as the technical limitation in analyzing a few genes at a time. Thus, at present, there is no information about the coordinated patterns of gene expression involved in 6-OHDA or MPP⁺ toxicity.

To unravel biological processes occurring in response to 6-OHDA and MPP⁺, we used microarray analysis of RNA isolated from the dopaminergic cell line MN9D (24) as a starting point to identify possible pathways induced by these Parkinsonian mimetics. These cells have been shown to mimic many aspects of the dopaminergic cell type from which they were immortalized (20–25). Capitalizing on the homogeneity and similarity in response of MN9D cells, the present study used microarray results, in addition to RT-PCR, Western blotting, and immunocytochemical approaches, to reveal that 6-OHDA triggers three separate signaling pathways associated with ER stress and UPR, whereas MPP⁺ seems to only involve one such signaling pathway. The unexpected identification of UPR induction in these models of dopaminergic cell death increases our understanding of how they may function to mimic the disease state and supports the theory that aberrations in the ubiquitin-proteasome pathway play an important role in PD.

MATERIALS AND METHODS

Cell Cultures—For primary cultures, the ventral mesencephalon was removed from embryonic day 14 CF1 murine embryos (Charles River Laboratories, Wilmington, MA) as described previously (21). Briefly, tissues were mechanically dissociated, incubated with 0.25% trypsin and 0.05% DNase in PBS for 20 min at 37 °C, and further triturated using a constricted Pasteur pipette. All plates were pre-coated overnight at room temperature with 0.5 mg/ml poly-D-lysine (Sigma) followed by 2.5 µg/ml laminin (BD Biosciences, San Jose, CA) for 2 h at 37 °C. Cells were maintained in serum-free Neurobasal medium (Invitrogen) supplemented with 1× B27 supplement (Invitrogen), 0.5 mM l-glutamine (Sigma), and 0.01 µg/ml streptomycin plus 100 units of penicillin. Half of the culture medium was replaced with fresh Neurobasal medium on the third and fifth day following plating. All experiments were conducted after 6 days in vitro.

MN9D cells were plated on dishes coated with 0.5 µg/ml poly-L-lysine for 1 h at 37 °C and then rinsed with sterile H₂O. Cells were maintained in Iscove’s Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in an incubator with 10% CO₂ at 37 °C. Cells were switched to serum-free Iscove’s Dulbecco’s modified Eagle’s medium/F-12 supplemented with 1× B27 prior to addition of experimental agents.

Cycloheximide Treatment and Determination of Cell Viability—MN9D cells were plated at a density of 40,000 cells/well in 24-well plates and treated after 3 days. One µg/ml cycloheximide (Calbiochem, La Jolla, CA) was added either immediately prior to, or at times following addition of 50 µM 6-OHDA or 100 µM MPP⁺ (all in 0.1% bovine serum albumin, PBS) to plated MN9D cells. After 48 h, cell survival was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay as previously described (22).

Microarray Analysis—MN9D cells were plated at a density of 200,000 cells/well in six-well plates. After 3 days, cells were treated with 50 µM 6-OHDA or 75 µM MPP⁺, or left untreated for control conditions. Total RNA was isolated after 9 h of neurotoxin treatment using an RNAeasy kit (Qiagen, Valencia, CA) according to the protocol from the manufacturer. Equal amounts of total RNA from three independent neurotoxin treatments were pooled together for each GeneChip hybridization experiment. Two separate GeneChip hybridizations of pooled, treated, and control RNA were performed, representing six independent experiments. A minimum of 20 µg of purified total RNA was sent to the Alvin J. Siteman Cancer Center GeneChip Core Facility (Washington University, St. Louis, MO) for generation of labeled cRNA target and hybridization against Affymetrix Murine Genome U74Av2 GeneChip arrays (Santa Clara, CA) using standard protocols (path- box.wustl.edu/~mgacore). Data were analyzed by Affymetrix Microarray Suite version 5.0, as well as Spotfire Decision Site for Functional Genomics (Somerville, MA). For those transcripts designated both “present” and “increasing” in each replicate by the software, a threshold of an average signal log ratio greater than 0.5 (~1.5-fold change) was set. Transcripts for which signal was less than 3% of the maximum signal were filtered out.

Reverse Transcription-PCR—MN9D cells were plated and treated exactly as described for microarray experiments. Total RNA was extracted after 1, 3, 6, 9, and 12 h. Primers to 18 S ribosomal RNA (26) were used to standardize amounts of RNA in each sample. RNA was reverse transcribed using gene-specific reverse primers, and resulting cDNAs were PCR-amplified. PCR primer sequences were used for: CHOP (++) and CHOP (−) described in Ref. 27, BIP/Fd (TGACTGGAATTCCCTCTGCT) and BiPRev (AGTCTTCAGTGTGGCGACTCC), c-junFwd (GCTGTAACGTGATACCGAAG) and c-junRev (CTTGGATCCGATCTGAGA), and Xbp1Fwd (TGGAGAAAGAGCCGGCTGA) and Xbp1Rev (CTCTGGGAGAGACATTGGA). PCR products were resolved on a 4% PAGE gel and analyzed with Vistra Green (Amersham Biosciences) detection and quantitative fluorescence imaging.

Western Blot Analysis—For MN9D Western blots, cells were plated and treated exactly as described for microarray experiments. For primary culture Western blots, 600,000 cells/well were plated in six-well plates and treated on the 6th day in vitro with 40 µM 6-OHDA or 1 µM MPP⁺ (21). MN9D lysates were taken at 1, 3, 6, 9, and 12 h, and primary lysates were taken at 6 and 12 h. Cells were washed once with PBS and harvested in ice-cold radioligand precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% NaoAc, 0.1% SDS, 50 mM Tris, pH 8.0) with protease inhibitor mixture (Roche, Mannheim, Germany) and placed on ice for 30 min. Insoluble cell debris was removed by centrifugation, and the protein concentration of cell lysates was determined using the Bio-Rad protein assay. Equal amounts of protein were run on SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Bio-Rad). Mouse monoclonal antibody against CHOP/Gadd153 (1:100) and goat polyclonal antibodies against Hsp60 (1:500) and BiP/Gp78 (1:125) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit polyclonal antibodies against cleaved caspase-3, phospho-c-Jun, phospho-eIF2α, and phospho-PERK (all 1:1,000) were purchased from Cell Signaling Technologies (Beverly, MA). After incubation with appropriate primary and horseradish peroxidase-conjugated secondary antibodies (anti-mouse 1:5000, Sigma; anti-goat 1:5000, Jackson Immunoresearch, West Grove, PA; or anti-rabbit 1:2000, Cell Signaling Technologies), specific protein bands were detected and analyzed by enhanced chemiluminescence substrate detection (ECL Plus; Amersham Biosciences) and quantitative fluorography.

Immunocytochemistry—MN9D cells were plated at a density of 300,000 cells/well on a four-well chamber slide. Twelve hours after plating, cells were treated with 75 µM 6-OHDA or 75 µM MPP⁺ and fixed 12 h later with 4% paraformaldehyde in PBS. Primary culture cells were plated at a density of 100,000 cells/35-mm microwell plate (1.25 × 10⁵ cells/mm², MatTek Corp., Ashland, MA). On day 6 in vitro, cells were treated with 40 µM 6-OHDA or 1 µM MPP⁺, and fixed after 12, 18, or 24 h with 4% paraformaldehyde in PBS. Cultures were double-stained with either mouse monoclonal anti-CHOP (1:300) or rabbit polyclonal anti-phospho-eIF2α (1:500), together with mouse monoclonal (1:500), Pei-Freeze, Rogers, AR) or mouse monoclonal (1:2,500), Immunostain, HUD, WI) antibodies against the dopaminergic neuron marker TH, respectively. Secondary antibodies conjugated with Cy3 (anti-mouse and anti-rabbit 1:300) and Alexa488 (anti-mouse 1:500; anti-rabbit 1:2000) were used. Cells were imaged using an Olympus FV500 confocal microscope.

Statistics—GraphPad Prism software (San Diego, CA) was used for statistical analysis. The significance of effects between control and drug conditions was determined by one-way ANOVA as indicated and post hoc Dunnett’s multiple comparison tests (GraphPad Prism software).

RESULTS

Cell Death Induced by 6-OHDA and MPP⁺ Is Blocked by Inhibition of Macromolecular Synthesis—Previous studies have characterized 6-OHDA-induced cell death as a caspase-

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require; addition of 1.0;
treated with the protein synthesis inhibitor cycloheximide. Ad-
many transcripts as MPP
2). Notably, 6-OHDA treatment affected almost three times as
expression following 6-OHDA and MPP
expression associated with the cell death process. Therefore, it may be possible to identify changes in gene

dependent, apoptotic process, whereas MPP+-induced cell
death can occur independent of caspase activation, and without
canonical markers of apoptosis (13, 20, 21, 28). Some forms of
apoptotic and non-apoptotic cell death require de novo syn-
thesis of cell death proteins (29, 30), whereas others do not (31, 32).
To determine whether 6-OHDA- or MPP+-induced cell
death require de novo macromolecular synthesis, cultures were
treated with the protein synthesis inhibitor cycloheximide. Ad-
dition of 1.0 μg/ml cycloheximide together with 100 μM
6-OHDA or 75 μM MPP+ provided significant protection. In
contrast, delaying addition of cycloheximide following neuro-
toxin treatment resulted in increasing cell death in a time-de-
pendent manner (Fig. 1). These data indicate that, although
6-OHDA induces an apoptotic form of cell death and MPP+
does not, both types of cell death require de novo protein syn-
thesis. Therefore, it may be possible to identify changes in gene
expression associated with the cell death process.

Microarray Analysis Identifies Distinct Changes in Gene Ex-
pression following 6-OHDA and MPP+ Treatment—Microarray
analysis was used to examine the expression profile of a large
number of transcripts. Out of the ~12,000 genes and expressed
sequence tags represented on the MG-U74Av2 GeneChip,
4,304 (~35% of total) were defined as “present” by the micro-
array analysis software for MPP+-treated samples. Similarly,
4,580 (~37% of total) were defined as present for 6-OHDA-
treated samples. Transcripts were subsequently grouped by
individual toxin treatment, or by both 6-OHDA and MPP+ (Fig.
2). Notably, 6-OHDA treatment affected almost three times as
many transcripts as MPP+. Specifically, 153 transcripts in-
creased in response to 6-OHDA, whereas only 55 transcripts
increased in response to MPP+. Results for decreasing tran-
scripts were similar (data not shown). Both neurotoxins in-
duced a number of the same transcripts, with 39 of the 55
transcripts induced by MPP+ also induced by 6-OHDA (Table
I). These included genes involved in cell cycle and/or differen-
tiation, signaling, stress, and transcription factors, indicating
possible common cell death mechanisms. The most highly
induced transcript in response to either treatment was that to
the stress protein CHOP/Gadd153. 6-OHDA also induced a
large number of transcripts that were unchanged by MPP+
treatment, including molecular chaperones and other genes
involved in protein folding, trafficking, and the ubiquitin-pro-
teasome pathway (Table II). These results support previous
findings showing that MPP+ and 6-OHDA promote distinct yet
overlapping programs of cell death.

CHOP Is Induced in Response to 6-OHDA and MPP+—To
confirm the microarray findings that CHOP mRNA was up-
regulated by 6-OHDA and MPP+ in MN9D cells, RT-PCR was
performed (Fig. 3A). 6-OHDA induced a large and rapid induc-
tion of CHOP mRNA that peaked between 6 and 9 h. MPP+
induction of CHOP mRNA lagged behind that of 6-OHDA, but
continued to increase for at least 12 h (Fig. 3, A and C). These
data are consistent with the GeneChip results from a 9-h time
point showing greater induction with 6-OHDA than with
MPP+ (Fig. 2 and Table I) Western blotting of MN9D total cell
lysates confirmed that levels of CHOP protein were also in-
creasing (Fig. 3, B and C). Again, 6-OHDA induced a larger and
more rapid increase in protein expression than did MPP+ (Fig
3C). To visualize CHOP induction in situ (Fig. 3D), treated cells
were fixed, stained, and imaged using confocal microscopy.
Control cultures had dim, diffuse staining, whereas both
6-OHDA and MPP+ treated cells showed intense nuclear stain-
ing. This localization is consistent with the role of CHOP as a
transcription factor. Together, these results confirm and ex-
tend the GeneChip findings that toxin treatment of dopamine-
ergic cells leads to an up-regulation of CHOP mRNA and pro-
tein levels.

RT-PCR Reveals Markers of Unfolded Protein Response Are
Up-regulated by 6-OHDA and MPP+ Treatment—CHOP is up-
regulated by a variety of cellular stresses including ER stress
(27, 33–35). Following confirmation of CHOP induction, further
analysis of GeneChip results revealed a pattern of induction of
other stress-induced genes including many involved in UPR
(Fig. 2, Tables I and II). These included molecular chaperones
such as BiP/Grp78 and UPR-induced transcription factors
other than CHOP (Atf4 and Xbp1). To examine the role that
UPR may play in 6-OHDA and MPP+ toxicity, induction of
these transcripts was verified by RT-PCR (Fig. 4, A and B). BiP
is an ER-resident chaperone protein central to UPR (36). Lev-
els of BiP mRNA were increased greater than 2-fold over con-
trol from 6 to 12 h following 6-OHDA exposure. BiP expression,
however, decreased slightly in response to MPP+ exposure over
12 h. These results were consistent with GeneChip results at
9 h for both 6-OHDA and MPP+ (Table II). Although not
specific to ER stress, activation of the c-Jun N-terminal kinase/
stress-activated protein kinase pathway (JNK/SAPK) occurs
during UPR (37, 38). Expression of c-Jun mRNA was increased
rapidly by 6-OHDA and then maintained at levels 5–6-fold that
of control at 3 to 12 h following exposure. MPP+ treatment
resulted in a rapid induction of c-Jun mRNA to 3-fold that of
control at 1 h, identical to exposure to 6-OHDA. However,
MPP+ induction of c-Jun mRNA was not sustained and re-
turned to control levels by 9 h.

Another feature of the UPR pathway is the non-conventional
removal of 26 base pairs of Xbp1 mRNA by the ER membrane
resident protein, Ire1α/β, under conditions of ER stress (39, 40). Moreover, levels of unprocessed Xbp1 mRNA are also increased by ER stress. In response to 6-OHDA but not MPP⁺, Xbp1 was induced almost 2-fold according to the GeneChip analysis (Fig. 2, Table II). To determine whether Xbp1 mRNA was processed, primers flanking the excised portion of Xbp1 mRNA were used to reveal a shift in size of the RT-PCR product (Fig. 4A). As indicated in Fig. 4B, 6-OHDA produced a large, transient induction of processed Xbp1 mRNA peaking at 3–6 h and returning to near control levels after 12 h. In contrast, MPP⁺ treatment resulted in a sustained inhibition of Xbp1 mRNA processing from 3 to 12 h.

**Western Blotting Reveals Markers of Unfolded Protein Response Are Up-regulated by 6-OHDA and MPP⁺ Treatment**—
Induction of the UPR pathway triggers not only transcriptional changes, but also involvement of protein kinase signaling pathways. One such pathway is that of JNK/SAPK, activation of which leads to phosphorylation of c-Jun (37, 38). In addition to changes in c-Jun mRNA expression (Fig. 4, A and B), Western blot analysis using antibodies against phospho-c-Jun indicated that 6-OHDA administration increased phosphorylation of c-Jun —6-fold over control levels at 9–12 h (Fig. 5, A and B). In contrast, treatment with MPP+ induced a transient increase of phosphorylated c-Jun at 3 h, returning to control levels by 6–9 h. These data are consistent with the RT-PCR results indicating a slight, early MPP+ mediated increase in c-Jun mRNA that was not sustained (Fig. 4, A and B). Taken together these results indicate that cellular responses to 6-OHDA led to the activation of the JNK/SAPK pathway.

Another consequence of UPR is translational attenuation caused by phosphorylation of eIF2α by the ER membrane resident kinase PERK. Western blotting using antibodies against phospho-eIF2α revealed that both 6-OHDA- and MPP+ -mediated toxicity resulted in eIF2α phosphorylation (Fig. 5, A and B). Specifically, MPP+ exposure induced a rapid, transient response, whereas 6-OHDA exposure resulted in sustained phosphorylation of eIF2α from 3 to 12 h. The eIF2α kinase PERK is itself activated by phosphorylation, and Western results indicated that MPP+ induced PERK phosphorylation in a profile almost identical to eIF2α phosphorylation. In contrast, PERK phosphorylation induced by 6-OHDA exhibited delayed kinetics, staying at baseline levels for 3 h following treatment, and then rising 3-fold over the next 9 h. BiP protein levels showed a slight increase over 12 h with 6-OHDA treatment, but not with MPP+ (Fig. 5A), again consistent with both GeneChip and RT-PCR data. In accordance with previous reports that 6-OHDA induced apoptosis (20, 21), but MPP+ does not, activated caspase-3 was detected only in 6-OHDA-treated cultures (Fig. 5A). Collectively, these data reveal that many components of UPR, including multiple signaling pathways, were up-regulated in response to 6-OHDA toxicity. In contrast, treatment with MPP+ led to the up-regulation of some, but not all, markers of UPR. Thus, MPP+ may ultimately lead to dopaminergic cell death by a pathway that is at least partially independent of UPR.

6-OHDA, but Not MPP+, Induces Components of the UPR Pathway in Primary Mesencephalic Cultures—To determine whether UPR induction could be observed in primary mesencephalic cultures following neurotoxin treatment, Western blot analysis and immunocytochemistry were performed. Similar to results from the dopaminergic MN9D cells, 6-OHDA increased levels of CHOP protein at 6 and 12 h (Fig. 6A). 6-OHDA also increased phosphorylation of eIF2α and c-Jun. In contrast, none of the markers seen in the dopaminergic cell line were up-regulated in mesencephalic cultures treated with MPP+.

Neither 6-OHDA nor MPP+ induced significant changes in levels of BiP protein over 12 h (data not shown).

Immunostaining of primary cultures with CHOP and phospho-c-Jun antibodies allowed individual dopaminergic neurons to be examined via co-staining with TH. 6-OHDA-treated cultures displayed intense nuclear staining of CHOP in both dopaminergic neurons as well as in many other cell types. Cultures treated with MPP+ did not appear different from controls in overall expression of CHOP, nor was CHOP induction detected in dopaminergic neurons over a 24-h period. Similarly, increased expression of phospho-c-Jun was widespread with 6-OHDA treatment in both dopaminergic and non-dopaminergic neurons, whereas there was no obvious change in phosphorylation of c-Jun following MPP+ administration. Taken together, these results suggest that MPP+ can induce a partial UPR response in the MN9D cell line but not in cultured dopaminergic neurons. In contrast, 6-OHDA induces a broad spectrum of UPR responses in both MN9D cells as well as in dissociated dopaminergic neurons. Thus, these cells will serve as a useful model in determining the temporal and molecular events associated with 6-OHDA neurotoxicity.

**DISCUSSION**

Accumulating evidence suggests that ER stress induced by aberrant protein degradation plays a role in PD. Beginning with a functional genomics approach to identify transcriptional alterations in a well characterized model of 6-OHDA and MPP+ toxicity, the present study identified numerous changes in genes associated with UPR. Notably, a major target of the UPR pathway, the transcription factor CHOP, was dramatically up-regulated at both the mRNA and protein levels by either 6-OHDA or MPP+. Moreover, 6-OHDA activated numerous other markers of UPR including BiP, splicing of Xbp1 mRNA, the JNK/SAPK pathway, as well as proteins involved in the
Fig. 3. **CHOP is up-regulated following 6-OHDA and MPP⁺ administration.** Treatment with 6-OHDA and MPP⁺ increased levels of CHOP mRNA isolated from MN9D cells as detected by RT-PCR (A) and levels of CHOP protein isolated from MN9D cells as detected by Western blot analysis (B). Equivalent loading was monitored by 18 S rRNA and Hsp60, respectively. C, quantification of CHOP mRNA and protein induced by 6-OHDA (squares) and MPP⁺ (triangles) was performed as described in text. Values represent mean ± S.E. of triplicate RT-PCRs and Western blots. *, p < 0.05; **, p < 0.01 compared with untreated control (one-way ANOVA with post-hoc Dunnett’s multiple comparison test). Error bars of less than 2% are buried in the symbol. D, MN9D cells were fixed after 12 h of neurotoxin treatment and stained with an antibody against CHOP. **Left panels** are phase bright images showing the morphology of MN9D cells. **Middle panels** show CHOP immunostaining. Nuclear localization of CHOP can be observed in the **merged right panels**.
attenuation of translation such as PERK and eIF2α. In contrast, MPP⁺ effects appeared restricted to events associated with PERK and eIF2α phosphorylation. In confirmation of these cell line results, 6-OHDA also triggered UPR responses in primary cultures of dopaminergic neurons. Collectively these data emphasize that 6-OHDA and MPP⁺ induce distinct cellular stress responses. Inasmuch as 6-OHDA is widely used to create animal models of PD, the present findings further support the notion that ER stress and ubiquitin-proteasome dysfunction is associated with this disorder.

**Biological Sequelae Associated with PD Mimetics—Oxidative stress and mitochondrial dysfunction have long been implicated in PD (41). Because of this, two neurotoxins exhibiting specificity toward dopaminergic neurons, 6-OHDA and MPP⁺, are commonly used to model nigral degeneration. 6-OHDA is a potent inducer of oxidative stress that can be endogenously converted from dopamine (13). Dopamine quinone derivatives including 6-OHDA have been found in post-mortem PD brains (14–16), implicating dopamine itself as a factor in this disorder. MPTP was originally identified because accidental human exposure led to PD (18, 42). MPTP, and its active metabolite MPP⁺, are also thought to induce oxidative stress in addition to inhibiting mitochondrial function (17). The discovery that mutations in α-synuclein (2, 3), parkin, and UCH-L1 (5, 9, 43, 44) are associated with PD led to the recognition that impaired protein degradation is also an important factor in this disorder. Mechanistically, however, it is still unclear what the common thread is among these seemingly disparate cellular responses.

The present study utilized gene expression profiling to assess thousands of genes to obtain a more detailed understanding of the molecular programs utilized by dopaminergic cells in response to 6-OHDA and MPP⁺. Two important outcomes from this study include the identification of a previously unsuspected link between these known oxidative stress inducers and aspects of ER stress/UPR, as well as the identification of at least a subset of common transcriptional changes associated with toxin-mediated events. The latter observation emphasizes the overlapping yet divergent nature of cell death in response to 6-OHDA versus MPP⁺.

Commonality in response to 6-OHDA and MPP⁺ is highlighted by the finding that the most highly induced transcript by either toxin was CHOP, a stress-induced transcription factor implicated in cell death (34, 45). The temporal and spatial up-regulation of CHOP was confirmed and extended by RT-PCR, Western blot analysis, and immunocytochemistry (Fig. 3). In support of the present findings, microarray analysis of MPP⁺-treated SH-SY5Y cells also resulted in an up-regulation of CHOP, albeit with a much later, more prolonged time course (46). Similarly, microarray analysis of the dopaminergic cell line, SN4741, revealed induction of stress indices following MPP⁺ treatment (47). To date, however, this is the first report that 6-OHDA up-regulates CHOP, and that it does so to a much greater extent than MPP⁺.

Additional transcripts identified via microarray analysis revealed that 6-OHDA induced a large number of genes that were not positively affected by MPP⁺, many of which were involved in protein folding, trafficking, or degradation (Table II). In contrast, the subset of genes induced by both drugs included amino acid transporters, tRNA-synthetases, ion channels, and stress-induced transcription factors (Table I). A small number of genes was induced by MPP⁺ but not 6-OHDA. These included Dnaja3, adaptor-related protein complex AP-3 β1 subunit, and myelin transcription factor 1. Currently, the significance of these changes is unclear. Overall, MPP⁺-induced transcripts appeared to primarily represent a subset of genes induced by 6-OHDA.
UPR Signaling Pathways—Three signaling pathways have been associated with UPR that are triggered by the ER proteins, Ire1α/β, ATF6, and PERK review (48). The Ire1α/β pathway is thought to activate caspase-12, the JNK/SAPK pathway, as well as Xbp1 mRNA splicing (37, 39, 40, 49). Translocation of ATF6 to the nucleus leads to the up-regulation of Xbp1 as well as various ER chaperones (48, 50). Finally, in addition to transcriptional changes, ER stress/UPR can down-regulate protein translation through phosphorylation of eIF2α via PERK kinase activity (48). Of interest, there is some redundancy in these cascades. For example, CHOP can be up-regulated by both the ATF6 and PERK pathways (50, 51). CHOP, as well as many chaperone proteins, contains a binding site called the ER stress element in its promoter region. In the nucleus, ATF6 binds to ER stress element sites activating CHOP transcription. In addition, CHOP contains a second site called the amino acid response element that is bound by the transcription factors ATF4 and C/EBPβ. ATF4 is activated when eIF2α is phosphorylated by PERK (48) or other eIF2α kinases (52, 53). Thus, signaling through PERK also leads to the up-regulation of CHOP.

GeneChip analysis indicated that many of the genes induced by either MPP⁺ or 6-OHDA were increased to a similar extent. A notable exception, however, was that 6-OHDA induced CHOP 26-fold compared with 9-fold with MPP⁺ (Fig. 2, Table I). Moreover, although both neurotoxins increased ATF4 and C/EBPβ, only 6-OHDA increased Xbp1 mRNA levels (Fig. 2). These data are consistent with the notion that 6-OHDA triggered both ATF6 and PERK pathways leading to the dual activation of the CHOP promoter. Moreover, processing of Xbp1 mRNA, indicating activation of the Ire1α/β pathway, was only observed with 6-OHDA. Although at present we have no clear evidence that caspase-12 is activated (data not shown), 6-OHDA but not MPP⁺ also dramatically up-regulated c-Jun mRNA and markedly increased phospho-c-Jun levels (Fig. 5). Taken together, it seems reasonable to propose that 6-OHDA is activating all three branches of the UPR signaling cascade, Ire1α/β, ATF6, and PERK, whereas MPP⁺ is only activating the PERK branch. One possible model summarizing these results is shown in Fig. 7.

Additional support for this hypothesis comes from studies showing that eIF2α can also be phosphorylated by other kinases such as GCN2 in response to amino acid starvation (52) or PKR in response to viral infection (53). Thus, phosphorylation of eIF2α does not require activation of the entire UPR and can lead to induction of genes downstream of ATF4, but not ATF6 (50, 51). The present findings are consistent with the model that MPP⁺ triggers eIF2α phosphorylation (Fig. 7) without involving ATF6 and Ire1α/β activation. These data are remarkably similar to a recent report showing that arsenite...
exposure of primary neuronal cells led to the up-regulation of CHOP expression without a concurrent activation of UPR (54). Thus, MPP⁺-mediated cell death parallels that described for amino acid starvation and/or toxin treatment. 6-OHDA- or MPP⁺-mediated Cell Death—Previously we and others have shown that, although 6-OHDA and MPP⁺ both generate oxidative stress, only 6-OHDA treatment resulted in activation of caspases and morphological changes associated with apoptosis (20, 21). Several lines of evidence from this laboratory suggest, however, that 6-OHDA does not mediate an intrinsic, mitochondrial dependent, apoptotic pathway. For example, overexpression of the anti-apoptotic protein, Bcl-2, did not attenuate 6-OHDA-induced cell death in either the MN9D cell line or in primary dopaminergic neurons (22, 25). Moreover, deletion of the pro-apoptotic Bcl-2 family member, Bax, did not rescue dopamine neurons from 6-OHDA toxicity (25), nor was Bax protein translocated to the mitochondria in response to this toxin. ³ Finally, microarray analysis failed to detect up-regulation of any BH3-only family proteins thought to act upstream of the intrinsic mitochondrial pathway, even though downstream caspases were activated (Fig. 5A). Thus, these data support a model in which 6-OHDA activates apoptosis without involving the intrinsic mitochondrial pathway.

Another possibility is that 6-OHDA activates the extrinsic, apoptotic pathway involving death receptors such as Fas and the induction of caspase-8. The extrinsic pathway can occur independent of de novo protein synthesis (32, 55, 56) as well as Bcl-2 family member expression (for review see Ref. 57). However, activation of the extrinsic pathway requires ligand-mediated death receptor multimerization, adaptor proteins such as FADD, as well as autoproteolysis of caspases-8 and -10 (for review see Ref. 58). In the case of 6-OHDA-induced apoptosis, utilization of the extrinsic pathway seems unlikely because it was dependent on new protein synthesis, known death-inducing ligands were not identified by microarray analysis, and so-called death receptors (Fas (APO-1, CD95), tumor necrosis factor receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand receptor I and II, etc.; Ref. 59) as well as Fas-associated death domain were not detected either. In contrast, a growing body of evidence indicates that ER stress can induce apoptosis independent of both extrinsic and intrinsic pathway factors requiring instead caspase-12 and caspase-9 (60, 61). Apoptosis mediated by 6-OHDA appears to have more characteristics in common with this alternative, non-mitochondrial, pathway, although the involvement of caspases-9 and -12 remains to be determined.

The present data as well as previous studies (20, 21) help to order and clarify the temporal events following neurotoxin treatment. Previous studies of primary dopaminergic neurons have shown that 6-OHDA induced an immediate increase (minutes) in reactive oxygen species (ROS) (21). The current findings suggest that following ROS generation 6-OHDA treatment quickly leads to the induction of c-Jun and processed Xbp1 mRNA (Fig. 4). These mRNAs are increased after 1 h and reach near maximal values by 3 h. Another early event is the phosphorylation of eIF2α, which is also increased significantly at 1 h, peaks at 3 h, and then stays elevated for the next 9 h (Fig. 5). Presumably triggered by the aforementioned primary events, a distinct second wave of transcriptional responses occurs, exemplified by CHOP and BiP. The latter are unchanged at 1 h and then rise rapidly (Fig. 3, 4). Phosphorylation of c-Jun also occurs during this time (Fig. 5). Reflecting an earlier increase in levels of CHOP mRNA, increased CHOP protein is detected after 6 h (Fig. 3). In addition, phosphorylation of PERK is not detected until 6 h following 6-OHDA exposure (Fig. 5). The last event to occur in this study was the activation of caspase-3, which was barely detectable at 9 h and only increased significantly after 12 h (Fig. 5A). Previous studies have shown that the pan-caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone blocks 6-OHDA toxicity in MN9D cells (20) and that the pan-caspase inhibitor boc-Val-Ile-Ala-Ome-fluoromethylketone is similarly effective in cultured dopaminergic neurons (21). Thus, a broad, multiphasic program of transcriptional, translational, and post-translational events precedes 6-OHDA-induced dopaminergic cell death.

Following transient increases, MPP⁺-induced phosphorylation of eIF2α, which is also increased significantly at 1 h, peaks at 3 h, and then stays elevated for the next 9 h (Fig. 5). In addition, phosphorylation of PERK is not detected until 6 h following 6-OHDA exposure (Fig. 5). The last event to occur in this study was the activation of caspase-3, which was barely detectable at 9 h and only increased significantly after 12 h (Fig. 5A). Previous studies have shown that the pan-caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone blocks 6-OHDA toxicity in MN9D cells (20) and that the pan-caspase inhibitor boc-

\[ ^3 \quad W. A. Holtz and K. L. O'Malley, unpublished observation. \]
6-OHDA. Why then are MPP \(H_1\) mediated changes transient? One possible explanation is that, although both toxins initially trigger the same response as a result of oxidative stress, this response diverges as MPP \(H_1\) more effectively depletes cellular energy. Conceivably, only 6-OHDA-treated cells retain sufficient energy to execute apoptosis. On the other hand, BiP and Xbp1 mRNA did not increase significantly at any time following MPP \(H_1\) treatment, but were induced by 6-OHDA. This might indicate that the two responses are distinct from the beginning, despite sharing common participants.

In primary cultures, the difference between 6-OHDA and MPP \(H_1\) appears to be even more distinct. Markers of UPR seen in 6-OHDA-treated MN9D cells were also seen in 6-OHDA-treated primary cultures (Fig. 6). In contrast, MPP \(H_1\) did not appear to up-regulate CHOP or to phosphorylate eIF2\(\alpha\) or c-Jun in dissociated dopaminergic neurons (Fig. 6). Further investigation will be needed to determine whether this is the result of differences between MN9D cells and primary cells, or of the manner or timing in which the cells were treated.

Unraveling the biological processes by which PD mimetics induce their neurotoxic effects is important to accurately model this disease. However, despite decades of use, the complex signaling pathways by which 6-OHDA and MPP\(H_1\) act remain unclear. The unsuspected finding that 6-OHDA and MPP\(H_1\) trigger components of the UPR pathway will lead to a better understanding of the application of these agents in models of nigral degeneration and improve the interpretation of the results. In addition, information obtained from 6-OHDA- or MPP\(H_1\)-mediated cell death may also contribute toward understanding other disorders such as excitotoxicity, amyotrophic lateral sclerosis, ataxias, etc. These findings support the emerging role of ubiquitin-proteasome system dysfunction in PD, and provide a connection between oxidative stress, mitochondrial dysfunction, and impaired protein degradation.

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Note Added in Proof—While this manuscript was under review, Ryu et al. (Ryu, E. J., Harding, H. P., Angelastro, J. M., Vitolo, O. V., Ron, D., and Greene, L. A. (2002) J. Neurosci. 22, 10690) demonstrated induction of the unfolded protein response in 6-OHDA-treated PC12 cells and sympathetic neurons. This supports our findings in MN9D cells and primary dopaminergic cultures that 6-OHDA is an inducer of ER stress.

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