INDUCTION OF THE PROTECTIVE ANTIOXIDANT RESPONSE ELEMENT PATHWAY BY 6-HYDROXYDOPAMINE IN VIVO AND IN VITRO

Rebekah J. Jakel*,†,‡, Jonathan T. Kern*, Delinda A. Johnson*, Jeffrey A. Johnson*†,§,¶

*Department of Pharmaceutical Sciences, School of Pharmacy. † Medical Scientist Training Program, Medical School. ‡Neuroscience Training Program. §Center for Neuroscience, Molecular and Environmental Toxicology, and Waisman Center. University of Wisconsin-Madison USA.

RUNNING TITLE: 6-OHDA activates the ARE

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¶Correspondence directed to: Jeffrey A. Johnson
School of Pharmacy, University of Wisconsin
777 Highland Avenue
Madison WI 53705
Telephone: (608) 262-2893 Fax: (608) 262-5345
e-mail: jajohnson@pharmacy.wisc.edu

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ABSTRACT

Parkinson’s disease, a progressive neurodegenerative disorder, is characterized by loss of midbrain dopaminergic neurons. The etiology of sporadic Parkinson’s disease is unknown; however, oxidative stress is thought to play a major role in disease pathogenesis. Little is known regarding the transcriptional changes that occur in Parkinson’s disease. The antioxidant response element is a cis-acting enhancer sequence that is upstream of many phase II detoxification and antioxidant genes. Here we show that 6-hydroxydopamine, a mitochondrial inhibitor used to model Parkinson’s disease, activates the antioxidant response element both in cultured neurons and in the striatum and brainstem of 6-OHDA-lesioned mice. Pretreatment with antioxidants or NMDA receptor antagonists reduced but did not abolish activation. Further induction of this pathway with tert-butylhydroquinone was able to significantly reduce cell death due to 6-OHDA in vitro. These observations indicate that 6-OHDA activates the antioxidant response element through components of oxidative stress, excitotoxicity, and potential structural factors. Further induction of this endogenous defense mechanism may suggest a novel therapeutic venue in Parkinson’s disease.

Keywords: 6-hydroxydopamine, Parkinson’s disease, oxidative stress, antioxidant response element, tert-butylhydroquinone
INTRODUCTION

Parkinson's disease (PD), the most common adult-onset neurodegenerative movement disorder, is characterized by loss of the pigmented dopaminergic neurons in the substantia nigra pars compacta leading to a loss of striatal dopamine. The hallmark features of PD include akinesia, tremor, rigidity, and postural instability. Most cases of PD are sporadic, with a minority caused by known mutations. Although the etiology of sporadic PD is unclear, oxidative stress, mitochondrial dysfunction, and excitotoxicity likely play a role in pathogenesis (Jenner and Olanow 1998). Indirect evidence of reactive oxygen species (ROS) in PD has come from observations of increased oxidized end-products such as 8-hydroxy-2-deoxyguanosine, 4-hydroxy-2-nonenol, and protein carbonyls in post mortem brain tissue from patients with Parkinson’s disease (Alam et al. 1997a; Alam et al. 1997b; Castellani et al. 2002; Dexter et al. 1986; Dexter et al. 1989a; Dexter et al. 1994; Jenner et al. 1992; Saggu et al. 1989; Schapira et al. 1990; Sian et al. 1994a; Sian et al. 1994b).

There are several potential sources of ROS in PD. Impairment of the respiratory chain can cause oxidative stress through superoxide production. There is evidence for complex I dysfunction in post mortem human brain from PD patients (Schapira et al. 1989; Schapira et al. 1990). Indeed, PD is modeled in vitro and in vivo using complex I inhibitors such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and 6-hydroxydopamine (6-OHDA) (Betarbet et al. 2002). Mitochondrial inhibition can also generate free radicals via an excitotoxic mechanism (Albin and Greenamyre 1992;...
Brouillet and Beal 1993; Srivastava et al. 1993). Additionally, oxidative stress may be a consequence of high iron levels naturally present in the nigra, or due to changes in iron regulation observed in PD brains (Dexter et al. 1990; Dexter et al. 1987; Dexter et al. 1989b).

Another source of free radicals in PD may be intrinsic to the nigrostriatal dopaminergic system. Dopamine (DA), a catecholaminergic neurotransmitter, is essential to normal basal ganglia function; however, it can be oxidized to generate prooxidant species through autooxidation and enzymatic catabolism via monoamine oxidase, prostaglandin H, or tyrosinase (Graham 1978; Graham et al. 1978; Hastings 1995; Maker et al. 1981; Nappi and Vass 2001; Tse et al. 1976). DA toxicity is most likely mediated by an oxidative stress mechanism (Hastings et al. 1996; Maker et al. 1981; Stokes et al. 2000). 6-OHDA, a hydroxylated analog of the DA used to model PD, is a catecholaminergic neurotoxin via mitochondrial complex I inhibition and oxidative stress (Adams et al. 1972; Soto-Otero et al. 2000), and may be formed via DA oxidation (Jellinger et al. 1995).

One cellular defense mechanism to cope with oxidative stress is the antioxidant response element (ARE), a cis-acting enhancer element that is upstream of many phase II detoxification and antioxidant enzymes such as heme oxygenase-I and glutathione-S-transferases (Friling et al. 1990; Rushmore et al. 1990; Rushmore et al. 1991; Rushmore and Pickett 1990, 1991). The ARE is induced by xenobiotics, changes in the redox status,
as well as catechol and quinone structures (Nguyen et al. 2004). NF-E2 related factor (Nrf2), a basic leucine zipper transcription factor, is known to drive ARE-mediated gene expression (Nguyen et al. 2004). Following exposure to activators, Nrf2 translocates to the nucleus where it binds the ARE and activates transcription [reviewed by (Jaiswal 2004)]. Nrf2-knockout mice demonstrate decreased basal activity of some ARE regulated genes and normal expression of others; however, these animals do not display inducible ARE activity (Lee et al. 2003a). Because of the prominent role of oxidative stress in PD, we hypothesized that the ARE may be induced in response to the cellular dysfunction specific to this disease.

Previous research from our lab has shown that cultured neurons from Nrf2 knockout mice are more vulnerable to 1-methyl-4-phenylpyridinium (MPP+) and rotenone (Lee et al. 2003b). This suggests that the ARE system is critical in mediating PD pathogenesis. ARE-inducers have been able to protect against death due to DA and 6-OHDA in vitro (Duffy et al. 1998; Hara et al. 2003). Analysis of post mortem PD brains has revealed increased ARE-regulated enzymes such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase-1 (NQO1) also suggesting the potential for common transcriptional regulation (Schipper et al. 1998; van Muiswinkel et al. 2004; Yoo et al. 2003).

The current work tests the hypothesis that 6-OHDA induces the ARE. Specifically, we evaluated (1) whether 6-OHDA activates the ARE in vivo and in vitro, (2) the roles of
oxidative stress and excitotoxicity on ARE activation in vitro, and (3) whether further induction of the ARE with tert-butylhydroquinone (tBHQ) would protect against 6-OHDA-mediated cytotoxicity in vitro.

MATERIALS & METHODS

Animals

All animals were housed at the University of WI School of Pharmacy Vivarium and treated in accordance with all IACUC regulations. All mice were maintained under standard laboratory conditions with food and water available ad libitum in a 12-hour light/dark cycle. The transgenic ARE-human Placental Alkaline Phosphatase (hPAP) animals were generated as described previously (Johnson et al. 2002). The presence of the transgene was confirmed by PCR amplification of a portion of the inserted gene. ARE-hPAP-negative littermates were used as background controls for endogenous alkaline phosphatase assays.

Chemicals and Reagents

All chemicals were dissolved in neurobasal media (as described below) and from Sigma unless specifically noted. 6-hydroxydopamine (RBI) was dissolved in 0.5% ascorbate in 0.9% sterile saline. Apomorphine hydrochloride was dissolved in 0.15% ascorbate in saline. Dizocilpine (MK801) was dissolved in 0.5% dimethylsulfoxide (DMSO). Tert-butylhydroquinone and di-tert-butylhydroquinone (tBHQ and dtBHQ, Acros) were dissolved in 0.1% DMSO, with appropriate DMSO vehicle controls.
Primary Cortical Culture

Primary cortical neuronal cultures were derived from E16-18 embryos pooled from litters resulting from crossing ARE-hPAP+/− males with ARE-hPAP−/− female mice as previously described (Lee et al. 2003b). Briefly, following trypsin dissociation, cells were plated on poly-D-lysine coated 96-well plates or on CC2-treated chamber slides (LabTek) in media containing modified eagle media (MEM), fetal bovine serum, horse serum, L-glutamine, and penicillin/streptamicin/fungicide (PSF) for 24 hours. Cells were then transferred to media containing neurobasal (Gibco BRL), B27, PSF, and L-glutamine for the duration of the experiment. All toxin exposures lasted 24 hours. MK801 and antioxidant pretreatments (N-acetylcysteine 0.5mM, catalase 100 units/ml, and reduced glutathione 0.5mM) commenced one hour prior to toxin exposure. All treatments were started on 3-7DIV with exception of the cultures pretreated with tBHQ for 48 hours starting on 2DIV prior to toxin exposure.

Stereotaxic injections

16-25 week old male and female mice were anesthetized with isoflurane and received intrastriatal stereotactic injections of 6-OHDA (6μg, 1μl) with contralateral vehicle control administration into the following coordinates: 0.5mm anterior to bregma, ± 2.0mm lateral to midline, and 3.1mm ventral to dura. A Hamilton syringe was inserted and allowed to equilibrate for 2 minutes followed by injection over 3 minutes. The
syringe was allowed to equilibrate again for 2 minutes, and then the syringe was withdrawn over 3 minutes.

Behavioral Assessment
Mice in the 7-day time-point group for tissue assays were administered 1mg/kg apomorphine HCl s.c. (0.15% ascorbate in 0.9% sterile saline). Mice were observed for turning behavior for 20 minutes during the initial pretest 24-48 hours prior to surgery. One week following surgery, animals were again administered apomorphine and observed for 40 minutes for turning. Animals not exhibiting contralateral turning stereotypy were excluded from analysis (1 animal).

Tissue collection and Histology
All animals were euthanized with CO₂ and transcardially perfused with PBS. Tissues collected for hPAP tissue enzyme assay were first hemisected then dissected to remove cortex, brainstem, and striatum, which were snap frozen and stored at -80°C until assayed. Tissues collected for histology were post-fixed overnight with 4% paraformaldehyde and cryoprotected with 30% sucrose. Brains were sectioned on a cryostat (Leica, Deerfield IL). Serial sections were taken as free-floating in PBS + azide (40 μm) or directly onto slides (20 μm). Free-floating and mounted sections were stored at 4°C and -20°C, respectively until analysis.

Immunochemical Staining
Free-floating sections were incubated in 100% methanol containing 1% \( \text{H}_2\text{O}_2 \) to abolish endogenous peroxidase activity. Sections were blocked with PBS + 0.3% Triton-X 100 (PBST) with 10% normal goat serum. Sections were incubated in anti-tyrosine hydroxylase (Chemicon, 1:800). Sections were then exposed to biotinylated goat anti-rabbit IgG followed by the ABC and DAB reaction kits (Vector). All washes were completed with PBST. Sections were mounted on glass slides, dried, and cleared with xylenes before coverslipping.

Primary cultures were blocked with PBS containing 1% BSA, 10% NGS and/or NHS, and 0.2% Triton-X 100. Slides were exposed to anti-beta-III-tubulin (Promega, 1:200), anti-heme oxygenase-1 (Stressgen, 1:200) or anti-Glial Fibrillary Acidic Protein [(GFAP), Dako, 1:1000 and Chemicon, 1:200] overnight. Secondary antibodies used include rabbit anti-goat conjugated to Texas Red, goat anti-rabbit conjugated to Texas Red or fluorescein and horse anti-mouse conjugated to Texas Red or fluorescein depending on whether the samples were co-labeled with Vector Red or TUNEL as described. All secondary antibodies came from Vector Labs. Cells were counterstained with Hoescht 33258 to visualize nuclei. A Zeiss photomicroscope was used to acquire all images, which were analyzed using Axiovision software.

**Alkaline Phosphatase Assays**

For alkaline phosphatase tissue activity, tissues were homogenized in TMNC buffer (50mM Tris, 5mM MgCl2, 100mM NaCl, 4% CHAPS) and refrozen. Samples were
heat-inactivated at 65°C (to destroy endogenous phosphatase activity). HPAP activity was assayed in a 96-well format using the chemiluminescent CSPD substrate (Tropix) with Emerald™ (Tropix) enhancement in diethanolamine. Activity was measured in a luminometer and calculated relative to protein concentration as was determined by BCA kit (Pierce). Primary cortical cultures were also assayed for activity using this method using known cell numbers.

Alkaline phosphatase tissue histochemistry was assayed as follows. 20μm frozen sections were stored at -20°C until thawed and rehydrated in TMN (50mM Tris, 5mM MgCl2, 100mM NaCl). Sections were heat-inactivated in TMN (65°C) and treated with BCIP/NBT (Promega) at 37°C until color reaction product developed. Vector red alkaline phosphatase substrate (Vector Labs) was used on fixed primary cells as follows. Cells cultured on CC2-treated Lab-Tek chamber slides were incubated in TMN and heat inactivated as above, followed by incubation with Vector Red kit as per manufacturers instructions.

Cytotoxicity Measurements

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Roche) staining for primary cortical cells was completed as per manufacturer’s instructions. Cells were further counterstained with Hoescht. Five fields from each condition were quantified for number of either TUNEL+ or Hoescht+ cells by a non-biased observer who was blinded to the conditions of the experiment. The MTS assay [3-
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(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; Promega] was also used as per instructions.

Statistical Analysis
All data reported as averages ± SEM, using p<0.05 as the cutoff for significance. For primary culture data, all data points were collected in triplicate and analyzed with unpaired, two-tailed student t-tests. For tissue assays, paired, two-tailed student t-tests were used to analyze the data. Actual p values are reported in figure legends.

RESULTS
6-OHDA activates the ARE in vitro
Primary cortical neurons containing an ARE-driven reporter transgene were exposed to 6-OHDA (1, 25, or 75μM) for 24 hours at three time-points in vitro and harvested for hPAP activity (Johnson et al. 2002). 6-OHDA induced ARE activation in a dose-dependent fashion at all 3 time-points. At 7DIV, 75μM 6-OHDA was sufficient to induce an over 50-fold increase in ARE-hPAP activity over vehicle control (Figure 1A).
As time in vitro progressed, the degree of ARE activation increased (Figure 1A). Pretreatment with antioxidants (N-acetylcysteine, catalase, and reduced glutathione) significantly reduced ARE activation due to 75μM 6-OHDA by approximately 50% (Figure 1B).

ARE induction by 6-OHDA is not contingent upon ability to cause neurotoxicity
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6-OHDA and diethyl maleate (DEM), a known ARE activator through an oxidative stress mechanism, activate the ARE as compared to vehicle control. As shown in Figure 1C, pretreatment with antioxidants was sufficient to significantly reduce ARE activation by 6-OHDA and DEM. In contrast, 75μM MPP⁺ and 75mM glutamate, known oxidative stressors, fail to activate the ARE at relevant doses as compared to vehicle control, with or without antioxidants (Figure 1C).

6-OHDA-induced ARE activation is reduced by NMDA receptor antagonism

At 3 and 7 DIV, primary hPAP+ neurons were exposed to 6-OHDA (75μM) with or without pretreatment with MK801 (10μM) and/or antioxidants. As shown in Figure 1D, at both time points, 6-OHDA exposure led to significantly increased ARE activation (fold change over vehicle control) which was reduced by pretreatment with antioxidants. At 3DIV, pretreatment with MK801 did not have any significant effect on 6-OHDA-induced ARE-activation in the absence of antioxidants; however, in the presence of antioxidants, 6-OHDA-induced ARE activation was significantly reduced, but not to the level of ARE activity in the presence of MK801 alone (Figure 1D).

At 7DIV, when primary cortical cells are vulnerable to excitotoxicity (Frandsen and Schousboe 1990), pretreatment with MK801 significantly reduced ARE activation by approximately 50% (Figure 1D). Pretreatment with MK801, however, did not fully abolish ARE activity due to 6-OHDA. Pretreatment with antioxidants in addition to MK801 did not further attenuate ARE activation (Figure 1D). There was no significant
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difference between 6-OHDA + antioxidants and MK801 + 6-OHDA + antioxidants, suggesting that MK801 is blocking ROS due to excitotoxicity.

**ARE activation due to 6-OHDA is primarily in astrocytes**

Cultured neurons exposed to various conditions were assayed for hPAP histochemistry using the fluorescing substrate Vector Red followed by immunostaining for either GFAP or beta-III-tubulin to discern astrocytes versus neurons, respectively (Figure 2). Vehicle treated cells showed very little ARE-hPAP histochemistry (Figure 2A & B). Treatment with 6-OHDA generated ARE-hPAP histochemistry primarily in astrocytes (Figure 2C) as opposed to neurons (Figure 2D). Treatment with MPP⁺ did not reveal hPAP histochemistry in either astrocytes (Figure 2E) or neurons (Figure 2F) confirming hPAP activity measures in Figure 1C.

To confirm that increased hPAP activity correlates with protein expression, we examined heme oxygenase-1 (HO-1). HO-1 expression is known be regulated in part by the ARE and has been shown previously to correspond to striatal injury due to 6-OHDA (Munoz et al. 2005; Prestera et al. 1995). Increased HO-1 is seen in 6-OHDA-treated cultures (Figure 3). tBHQ treatment is a positive control for heme oxygenase-1 induction (Figure 3C).

In order to determine if ARE activation was a component of a more general neurotoxic response to complex I inhibitors, we assayed for cell death using the TUNEL-labeling.
Both 6-OHDA (75μM) and MPP⁺ (75μM) caused significantly increased apoptotic cell death as revealed by TUNEL staining and observable pyknotic nuclei in Hoescht-stained images (Figure 4). However, as demonstrated in figures 1 and 2, MPP⁺ failed to activate the ARE. This suggests that the structural properties and/or the mechanism of cell death due to 6-OHDA may account for its induction of the ARE.

6-OHDA activates the ARE in vivo in the striatum and brainstem

Thirty-two adult ARE-hPAP transgenic reporter mice received midstriatal 6-OHDA lesions with contralateral vehicle control injections. For tissue hPAP activity, animals were sacrificed either at 24 hours or 7 days post-injection. To test for the efficacy of the lesions, animals were administered apomorphine (1mg/kg) and observed for turning behavior 7 days post-injection (Ungerstedt and Arbuthnott 1970). All but one 7-day animal demonstrated a significant increase in turning contralateral to the hemisphere of the lesion (data not shown). The animal that did not show a contralateral turning preference was omitted from the study. 24-hour animals were not tested for rotational turning as this behavior is not present at early time points.

Tissue hPAP activity assays did not demonstrate induction due to 6-OHDA in tissues collected at 24 hours post-injection (Figure 5A). However, by 7 days post-injection, hPAP activity was significantly activated in the brainstem and striatum as compared to contralateral vehicle control hemisphere. The greatest fold change activation due to 6-OHDA lesions was found in the striatum, which demonstrated over 6-fold activation as
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compared to paired vehicle-treated contralateral hemisphere (Figure 5B). There was no change in the cortex, a negative control region, due to 6-OHDA at 7 days (data not shown).

Increased ARE activity correlates with loss of tyrosine hydroxylase immunoreactivity (THir) as seen in sections from identically treated animals in a parallel study (Figure 5C). At 24 hours, there is no loss of THir; however, by one week, the 6-OHDA lesion was nearly complete (Figure 5C).

Sections were taken from 6-OHDA-injected brains at 24 hours, 96 hours, and one week post-lesion for hPAP histochemistry and counterstained with nuclear fast red. At 24 hours, there were no hPAP+ cells present (data not shown). This agrees with data from tissue hPAP assays which did not reveal changes in ARE activity at 24 hours (Figure 5A). hPAP-negative tissue did not demonstrate any staining at any time point assayed (Figures 6A and E). At 96 hours post-injection, half of the animals assayed demonstrated hPAP+ cells at the penumbra of the lesion (Figures 6C and D), but not in the vehicle control-treated hemisphere (Figure 6B). At one week, all animals assayed demonstrated hPAP+ cells encroaching into the core of the lesion (Figure 6G and H), but not in the vehicle treated hemisphere (Figure 6F). No visible increase in hPAP+ cells was seen in the brainstem (data not shown). The issue of specific cell type expressing hPAP in and around the lesion is discussed subsequently.
Induction of ARE can reduce apoptosis due to 6-OHDA in vitro

tBHQ (10μM), a known ARE activator, can cause an over 30-fold induction in ARE activity, significantly more potent than 6-OHDA (75μM; Figure 7A). dtBHQ, a structural analog of tBHQ, does not activate the ARE and was used as a negative control (Figure 7B). Treatment with both tBHQ, and 6-OHDA does not significantly increase ARE induction over tBHQ alone (Figure 7A). This suggests tBHQ (10μM) saturates the Nrf2-ARE induction cascade.

Primary cortical cells were exposed to 6-OHDA for 24 hours following 48 hours of pretreatment with tBHQ or vehicle. 6-OHDA led to loss of cellular viability in a dose-dependent fashion (Figure 7B). Pretreatment with tBHQ significantly increased viability as compared to vehicle pretreated cells (Figure 7B).

Cells from the same culture were plated in chamber slides and exposed to 6-OHDA (75μM) following pretreatment with vehicle or tBHQ. After 24 hours, cells were fixed and assessed for apoptotic nuclei using the TUNEL assay and counterstained with Hoescht to indicate total cells in the field (Figure 7C). 6-OHDA caused significantly increased TUNEL+ cells (Figure 7C, middle panel and D) as compared to vehicle control (Figure 7C, top panel). Pretreatment with tBHQ decreased the amount of TUNEL+ cells by approximately 35% indicating a reduction in apoptosis (Figure 7C, bottom panel and D).
DISCUSSION

In the current study, we have shown that 6-OHDA, a catecholaminergic neurotoxin used to model PD, activates the ARE both in vivo and in vitro. Oxidative stress is a critical factor in PD pathogenesis and consequently, we hypothesized that the cellular injury in PD may lead to activation of the ARE. Although known ARE-regulated genes such as HO-1 and NQO1 are increased in the PD brain (Schipper et al. 1998; van Muiswinkel et al. 2004), the nature of the regulation of these changes on a transcriptional level has not been elucidated. The ARE is an enhancer sequence found in the promoter of many cytoprotective genes. Oxidative stress and xenobiotic exposures can lead to Nrf2 translocation to the nucleus and subsequent ARE-regulated transcription. In this way, the ARE can coordinate the upregulation of a multitude of protective genes with a single insult.

In primary neuronal cultures from reporter mice, 6-OHDA activated the ARE in a dose-dependent fashion over a 7-day period (Figure 1A). ARE-driven hPAP activity was observed primarily in astrocytes rather than in neurons (Figure 2C and D). This agrees with previous work that ARE-mediated activity is primarily induced in astrocytes in vitro (Eftekharpour et al. 2000; Kraft et al. 2004; Shih et al. 2003). ARE activation due to 6-OHDA (75μM) was reduced but not eliminated in the presence of antioxidants (Figures 1B-D). At 7DIV, pretreatment with MK801, an NMDA antagonist, also reduced, but did not eliminate ARE activity (Figure 1D). Antioxidants in combination with MK801 did not further reduce the ARE activation. Therefore, 6-OHDA activates the ARE by a
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combination of factors including oxidative stress generated in part through an excitotoxic mechanism. In addition, 6-OHDA may activate the ARE due to its catecholamine structure that is independent of ROS formation. The latter mechanism of activation is probably the same used by tBHQ.

DA and its metabolites share structural similarities to tBHQ and hydroquinone. tBHQ activates the ARE without producing ROS, suggesting that its mode of induction is purely structural. MPP⁺, another chemical used to model of PD, does not induce the ARE (Figures 1B, 2E and F) in cell culture and lacks structural similarities to known ARE activators. Experiments designed to determine the effect of MPTP administration in vivo are currently underway. The pro-oxidant nature of the quinones and catecholamines suggests that DA breakdown may be a contributing factor to PD pathogenesis. However, these chemicals, by virtue of their structure, may induce the ARE. If 6-OHDA and DA behave like tBHQ in the ARE induction cascade, it is possible that they alter the redox status of Keap1 and stabilize Nrf2 protein, allowing for enhanced binding to the ARE (Dinkova-Kostova et al. 2002; Nguyen et al. 2003). Further studies are needed to confirm the mechanism of direct activation of the ARE by catecholamines like 6-OHDA.

Direct intrastriatal administration of 6-OHDA in vivo lesions the nigrostriatal dopaminergic pathway modeling PD pathology in the live animal. 6-OHDA induces ARE activation in ARE reporter mice at one week, but not 24 hours post-injection (Figure 5). The loss of THir, indicating loss of nigrostriatal terminals, is observable at 96
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hours and nearly complete by one week. This suggests that ARE induction follows a time
course similar to retrograde degeneration. ARE induction, as measured by a tissue assay,
occurs primarily in the brainstem and striatum. In the striatum, ARE activation appears at
the penumbra of the lesion at 96 hours (Figure 6C and D). Previous work in a
Huntington’s disease model suggests that these cells may be reactive astrocytes (Calkins
et al. 2005). It is possible that a small number of surviving nigral neurons of the
lesioned hemisphere may also be differentially active, as there is observable basal hPAP
expression in this region of the brain (data not shown). This could explain the
mechanism underlying the expression of NQO1 observed in nigral neurons of human PD
brains (van Muiswinkel et al. 2004).

The importance of ARE induction in PD pathogenesis is currently being explored.
Previously we have shown that Nrf2 is important for determining the sensitivity of
primary neurons to complex I inhibitors (Lee et al. 2003b). Although the ARE is induced
by 6-OHDA, it is clear that this host response is insufficient to quell pathogenesis (Figure
5C). However, further induction of the ARE may protect against cell death. Preliminary
in vitro data shown herein imply that pre-activation with tBHQ can protect against 6-
OHDA-induced cell death. We have also shown that Nrf2-mediated protection is
efficacious in the malonate model of Huntington’s disease (Calkins et al. 2005). We are
currently exploring the potential for using ARE inducers in vivo in the Parkinson’s
disease animal models. Successful translation of this work into animal models of PD
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could lead to new approaches for the treatment of PD via activation of the Nrf2-ARE pathway.

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FIGURE LEGENDS:

Figure 1: 6-OHDA activates the antioxidant response element in vitro.

6-OHDA activates the ARE in a dose-dependent fashion at three time-points [3DIV (vehicle vs 1μM, p=0.03; 25μM, p= 0.04; 75μM, p= 0.0003); 5DIV (vehicle vs 1μM, p > 0.05; 25μM, p=0.01; 75μM, p=0.0006); 7DIV (vehicle vs 1μM, p=0.008; 25μM, p=0.008; 75μM, p=0.001); A]. 6-OHDA-induced ARE activation can be partially reduced by pretreatment with antioxidants (p=0.02; B). 6-OHDA and DEM activate the ARE in the absence of antioxidants (p=0.00001 and p=0.002, respectively), and in the presence of antioxidants (6-OHDA, p=0.001; DEM, p=0.03; C). However, MPP⁺ and glutamate fail to activate the ARE in vitro (C). Like B, pretreatment with antioxidants was sufficient to reduce ARE activation by 6-OHDA (p=0.002) and DEM (p=0.003) but had no effect on MPP⁺ (p>0.05). 6-OHDA activated the ARE compared to vehicle control in the absence of antioxidants (3DIV, p=0.01; 7DIV, p=0.005) and the presence of antioxidants (3DIV, p=0.002; 7DIV, p=0.001). ARE activation was reduced by antioxidants (3DIV, p=0.02; 7DIV, p=0.01). At 3DIV, in the presence of antioxidants, 6-OHDA-induced ARE activation was significantly reduced by MK801 (p=0.05), but not back to level of ARE activity due to the presence of MK801 alone (p=0.04; D). At 7DIV, MK801 significantly reduced ARE activation (p=0.01; D). Pretreatment with MK801 did not fully abolish ARE activity due to 6-OHDA (p=0.003). Unless noted, data shown as relative light units (RLU).

* significantly different from corresponding vehicle-treated control sample (p<0.05).
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† significantly different from corresponding sample in the absence of antioxidants (p<0.05)

# significantly different from corresponding sample in the absence of MK801 (p<0.05).

‡ significantly different from corresponding sample in the absence of 6-OHDA (p<0.05).

Figure 2: ARE-hPAP activation in vitro.

Primary cultures derived from ARE-hPAP mice were stained for hPAP histochemistry (red), Hoescht (blue), and either anti-GFAP or anti-beta III tubulin (green) in order to assess the identity of ARE-active cells (40x). Vehicle treated cells demonstrate little ARE-activity (A and B). 6-OHDA-treated cultures demonstrate induction primarily in astrocytes (C) as compared to neurons (D). Cells treated with MPP⁺ demonstrate little ARE activation (E and F).

Figure 3: Increased HO-1 expression in 6-OHDA- and tBHQ-treated cultures (40x).

HO-1, an ARE-regulated enzyme is upregulated in cells treated with either 6-OHDA (B) or tBHQ (C) as compared to vehicle controls (A). * indicates HO-1 immunoreactive cells.

Figure 4: Apoptotic cell death as demonstrated by TUNEL-labeling (20x).

As compared to vehicle control cells (A), 6-OHDA (B) and MPP⁺ (C) both lead to apoptotic cell death, with differential activation of the ARE. A, B, and C shown as merged pseudo-colored overlay images (Hoescht, blue; TUNEL, green). Corresponding
Hoescht photos displayed below demonstrate increased pyknotic nuclei, indicative of apoptosis. Figure D shows quantification of TUNEL counts. Approximately 10% of vehicle cells demonstrated apoptotic nuclei, whereas 6-OHDA and MPP+ treatment caused approximately 41% and 31% TUNEL+ cells, respectively. 6-OHDA and MPP+ induced significantly more apoptotic cells than vehicle control cultures (p=0.004; p=0.001), but were not significantly different from each other the specific dose shown (p>0.05).

**Figure 5: 6-OHDA activates the ARE in vivo.**

At twenty-four hours and one week post-lesion, individual brains were hemisected and dissected for regions of interest. 6-OHDA injections did not lead to increased ARE activation at 24 hours post-lesion (N=5; A). By one week, tissue ARE assays demonstrated significantly increased activity in the striatum (p=0.02) and brainstem (p=0.02) as compared to vehicle control hemisphere (N=7; B). Data shown as relative light units (RLU) per tissue weight. Sections taken from unilateral (right) 6-OHDA-lesioned animals with contralateral vehicle controls (left) were immunostained for TH. At 24 hours, there was little loss of TH+ cells in the striatum (1x); however, by 1 week, there is a nearly complete lesion (C).

**Figure 6: In vivo ARE-hPAP staining at 96 hours (A-D) and 1 week (E-H).**

Sections were taken at 24 hours, 96 hours, and 1 week post-injection for hPAP histochemistry (10x, scale bar = 100μm). At 24 hours, there were no hPAP+ cells (data
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not shown). Figures 6A and E show sections from an ARE-hPAP (-) mouse, processed for hPAP histochemistry, and counterstained with nuclear fast red at 96 hours and one week, respectively. The contralateral striatum receiving vehicle did not demonstrate hPAP+ cells (B and F). However, by 96 hours, there was increased ARE activity circumscribing the lesion in half of the animals assayed (C), which was present at one week in all animals (G). Inlays D and H show same sections as C and G prior to nuclear fast red processing.

Figure 7: tBHQ protects against 6-OHDA-induced cell death.

tBHQ (10µM) is a known ARE activator (p=0.00001) that is significantly more potent than 6-OHDA (75µM; p=0.01; A). A structural analog of tBHQ, dtBHQ, fails to activate the ARE (A). Data shown as relative light units (RLU). Pretreatment with tBHQ for 48 hours lead to increased viability following 6-OHDA treatment as demonstrated by the MTS assay at 2 different doses (75µM 6-OHDA, p=0.04; 125µM 6-OHDA, p=0.0006; B). C shows condensed TUNEL+ nuclei (right) and Hoescht-stained nuclei (left). As compared to vehicle control (C, top panel), 75µM 6-OHDA generated significantly more TUNEL+ cells (C, middle panel). Pretreatment with tBHQ reduced the amount of TUNEL+ cells (C, bottom panel). Images taken at 20X. TUNEL+ cells were quantified (D). Treatment with 6-OHDA significantly increases the number of TUNEL+ cells (p=0.000001). tBHQ pretreatment significantly reduced TUNEL+ cells by 37% (p=0.01).
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* significantly different from corresponding vehicle-treated control sample (p<0.05).

# significantly different from corresponding sample in the absence of tBHQ (p<0.05).
Figure 1: 6-OHDA activates the antioxidant response element in vitro.
Figure 2: ARE-hPAP activation *in vitro.*
Figure 3: Increased HO-1 expression in 6-OHDA- and tBHQ-treated cultures (40x).
Figure 4: Apoptotic cell death as demonstrated by TUNEL-labeling (20x).

A. Vehicle
B. 75μM 6-OHDA
C. 75μM MPP+

D. % TUNEL+ cells

- Vehicle
- 6-OHDA
- MPP+
Figure 5: 6-OHDA activates the ARE in vivo.
Figure 6: *In vivo* ARE-hPAP staining at 96 hours (A-D) and 1 week (E-H).
Figure 7: tBHQ protects against 6OHDA-induced cell death.