Overexpression of superoxide dismutase or glutathione peroxidase protects against the paraquat+maneb-induced Parkinson’s disease phenotype*  

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Running Title: Model of Parkinson’s Disease and Antioxidant Enzymes

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Oxidative stress has been implicated in the pathogenesis of Parkinson’s disease based on its role in the cascade of biochemical changes that leads to dopaminergic neuronal death. This study analyzed the role of oxidative stress as a mechanism of the dopaminergic neurotoxicity produced by the combined paraquat and maneb model of the Parkinson’s disease phenotype. Transgenic mice overexpressing either Cu,Zn superoxide dismutase or intracellular glutathione peroxidase and non-transgenic mice were exposed to saline, paraquat, or the combination of paraquat + maneb twice a week for 9 weeks. Non-transgenic mice chronically exposed to paraquat+maneb exhibited significant reductions in locomotor activity, levels of striatal dopamine and metabolites and dopaminergic neurons in the substantia nigra pars compacta. In contrast, no corresponding effects were observed in either Cu,Zn superoxide dismutase or glutathione peroxidase transgenic mice. To begin to determine critical pathways of paraquat+maneb neurotoxicity, the functions of cell death-inducing and protective mechanisms were analyzed. Even a single injection of paraquat+maneb in the non-transgenic treated group modulated several key pro- and anti-apoptotic proteins, including Bax, Bad, Bcl-x(-) and upstream stress-induced cascade. Collectively these findings support the assertion that protective mechanisms against paraquat+maneb-induced neurodegeneration could involve modulation of the level of reactive oxygen species and alterations of the functions of specific signaling cascades.

Parkinson’s disease (PD) is a neurodegenerative disorder resulting, in part, from the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc). A lack of evidence for heritability of the idiopathic form of PD has pointed to environmental risk factors as potential contributors to the disease etiology. In accord with that possibility, exposures to environmental agents have repeatedly been linked with the development of PD (1-9). Animal models, epidemiological studies, and clinical case reports have repeatedly identified exposure to the herbicide paraquat (PQ) and the fungicide maneb (MB) as potential risk factors for the PD phenotype (1, 9-15). Our laboratories have extensively demonstrated that exposure to the combination of PQ+MB produces a potentiated loss of dopaminergic neurons in the SNpc of mice compared to either compound alone (16). This model exemplifies the “multiple-hit” hypothesis associated with PD. Furthermore, the neurotoxicity of PQ+MB seems to be highly selective to the nigrostriatal dopaminergic system.
Additionally, we have shown that exposure during critical periods of development and during advanced age as well as gender and genetic background all modify the susceptibility and response of dopaminergic neurons to PQ and MB (17-19).

While the exact mechanisms by which low chronic exposures to pesticides induce a Parkinson’s disease phenotype in experimental models are not known, several have been shown, even at relatively low levels to produce excessive generation of reactive oxygen species (ROS). Until recently, these chemically-induced ROS that lead to oxidative stress have been investigated for their ability to cause (often nonspecific) damage to intracellular molecules. However, current advances in understanding the importance of oxidative species in the regulation of signal transduction pathways suggest additional mechanisms of toxicity.

It is very likely that the specific mechanism(s) of neurotoxicity of systematic exposure to low levels of PQ+MB involves several targets. Studies to date have only examined the effects of each compound alone and mostly involved \textit{in vitro} assays, acute exposures or intracerebral administration of these xenobiotics. PQ is known as a potent redox cycler, which in the presence of molecular oxygen can generate superoxide anions leading to oxidative stress and consequently neuronal cell damage or death (20). There are also studies indicating the ability of PQ to cause oxidation of cellular NADPH and increased levels of lipid peroxidation (21). Intrahippocampal injection of PQ causes neuronal cell death in rats, mostly by apoptosis (22). Pre-treatment of these rats with the free radical scavenger, U74389G significantly reduced hippocampal injury. Intraneural injections of PQ in rats leads to the induction of oxidative stress, behavioral dysfunction, as well as physiological and neuropathological effects, all of which were prevented by pre-treatment with M40401, a non peptidyl superoxide dismutase mimetic (23). These observations have clearly demonstrated that paraquat causes neurotoxicity after intracerebroventricular or intracerebral injections in experimental animals. However, acute exposure using this route of administration does not exhibit selective neurotoxicity towards the nigrostriatal dopaminergic system. Although systemic administration of PQ has yielded varying outcomes in \textit{in vivo} models, more recent studies have consistently shown selective dopaminergic cell loss in the SNpc (17, 24, 25).

The mechanism(s) of neurotoxicity of MB is currently less well understood. Commercial Mb contains as a major active component, manganese-ethylenebis-dithiocarbamate (Mn-EBDC), but also includes other inert compounds. Direct delivery of Mn-EBDC to the lateral ventricles produces selective degeneration of dopaminergic neurons in rats (26). Most importantly, the ability of Mn-EBDC to preferentially inhibit mitochondrial complex III has been demonstrated. The Mn-EBDC complex has also been reported to produce oxidative stress by catalyzing catechol oxidation \textit{in vitro} (27), which may contribute to its selective neuronal vulnerability. A recent study (28) provided evidence that \textit{in vitro} exposure to Mn-EBDC is able to inhibit proteasomal function and induce \(\alpha\)-synuclein aggregation. It was suggested that the neurotoxic effects as well as proteasomal inhibition were associated with oxidative stress since they were prevented by pretreatment with antioxidants. Mn-EBDC is metabolized to the EBDC anion and manganese, both of which are neurotoxic. EBDC alone was capable of enhancement of MPTP-induced neurotoxicity (29).

The mechanisms of neurotoxicity associated with exposure to the combination of these two pesticides are not yet known, but is clearly of significance given its potential to induce a Parkinson’s disease phenotype. Although oxidative stress has been repeatedly implicated in the pathogenesis of Parkinson’s disease, the role that ROS plays in mediating oxidative stress and subsequently nigrostriatal dopamine system neurotoxicity following exposure to PQ+MB has not been determined. To further explore the mechanism(s) and the role of antioxidant enzymes in the neurotoxicity associated with PQ+MB, transgenic mice overexpressing the corresponding human proteins were used in these studies. The use of SOD and Gpx1 transgenes also allows a comparison of the effectiveness of intracellular endogenous antioxidants targeting different reactive oxygen species. PQ neurotoxicity, for example is thought to involve the generation of superoxide anions, which in the presence of excess Cu,Zn SOD might lead to increased levels of hydrogen peroxide and modulation of neurotoxicity. The current report demonstrates that overexpression of either Cu,Zn SOD or Gpx1 leads to significant neuroprotection against combined PQ+MB-induced DA neurotoxicity.
EXPERIMENTAL PROCEDURES

Transgenic Mice and treatment - The generation of transgenic mice with human GPx1 and SOD genes in a C57BL/6 X CBA/J background was previously reported (30-32). Mouse lines GPE23 and SOD42 containing 200 and 70 copies, respectively, of the corresponding human genes were used. Normal and transgenic animals for the experiments were obtained by breeding homozygous transgenic founders and nontransgenic mice with the same genetic background. SOD and GPx1 transgenic mice show increased activities of SOD and GPx1, respectively, in most tissues, as reported earlier. The total level of enzymatic activity in brain of corresponding enzyme was about 3.6 times higher in GPx1 animals and about 2.5 times higher in SOD transgenic mice. We also reported previously that neuronal, astrocytic and microglial cells overproduce corresponding antioxidant enzymes (31). Animals were maintained under a 12 h light/dark cycle at 25 °C and fed ad libitum. For PQ+MB treatment, nontransgenic and transgenic males matched for age were injected i.p. twice a week for 9 weeks (a total of 18 injections) with saline, 5 mg/kg PQ, or 5 mg/kg PQ + 15 mg/kg MB. Mice were sacrificed 1 week after the last injection and striatum, frontal cortex and midbrain dissected for various outcome measures. In a separate set of experiments, mice were treated with a single injection of saline or the doses of PQ+MB stated above, and sacrificed for analysis after 16 or 40 hrs. Tissues were harvested from striatum, midbrain and frontal cortex for biochemical analysis. Experimental protocols have been approved by the University of Medicine and Dentistry Animal Care and Use Committee.

Locomotor Activity - Automated locomotor activity chambers equipped with infrared photobeams (Opto-Varimex Minor, Columbus Instruments International Corporation, Columbus, OH) were used to quantify locomotor activity. Photobeam breaks were recorded each minute for 45 minutes for horizontal, vertical, and ambulatory movements. Mice were habituated to the locomotor activity chambers in three 45-minute sessions occurring on consecutive days, with all mice receiving i.p. saline injections prior to the session. After the third habituation session, PQ+MB treatment began, and effects on locomotor activity were assessed immediately and 24 hours after injections 1, 4, 8, 14, 16, and 18. Activity was also measured one week following the last PQ+MB exposure to determine the persistence of effects.

Dopamine and Metabolite Analyses - Neurotransmitter concentrations in the striatum and frontal cortex were measured one week following the last injection of SAL, PQ, or PQ+MB. Following cervical dislocation and rapid decapitation, frontal cortex and striatal blocks were dissected. HPLC-EC analysis was performed as previously described (33), and normalized to protein concentration as measured by the Bio-Rad DC protein assay (Bio-Rad, Richmond, CA). The concentrations of the neurotransmitters were expressed in units of ng/mg protein.

Immunohistochemistry (IHC) for Tyrosine Hydroxylase - Brains used for immunolabelling studies were post-fixed in 4% paraformaldehyde for 1 week and subsequently stored in 30% sucrose. Fixed brains were cut into 30 μm coronal sections and collected in cryoprotectant. Sections were prepared as previously described (18), incubating with a primary antibody to tyrosine hydroxylase (TH, Chemicon, Temecula, CA) for 48 hours at a dilution of 1:4000, and with secondary goat anti-rabbit antibody (Vector, Burlingame, CA) for 24 hours at a dilution of 1:500. Sections were incubated with avidin-biotin solution using the Vectastain Elite Kit (Vector) for 2 hours, and then developed in 3-3’-diaminobenzidine tetrachloride for 2-5 minutes. Sections were mounted on gelatin-coated slides, counterstained with cresyl violet for visualization of neuronal nuclei, and coverslipped for stereological analysis.

Stereological Analysis - After delineation of the SNpc at low magnification (4X objective), one side of every fourth section from the entire midbrain region was sampled at higher magnification (100X objective) using the stereology module of the Stereo Investigator imaging program (MicroBrightField, Inc., Williston, VT) with an Olympus Provis microscope. The optical fractionator method, an unbiased quantitative technique, was used for counting TH+ (TH-positive and cresyl violet positive neurons) and TH- (cresyl violet positive only) cells. Criteria for TH+ and TH- neurons were determined as previously described (19, 34). The mean thickness was determined by measuring two fields from five sections per sample, and the entire depth of field was sampled, ignoring the upper and lower 0.5 μm. All
samples were evaluated by one experimenter without knowledge of mouse status.

**Paraquat Toxicokinetic Study** - Male mice from all the 3 lines (nTg, Gpx1 and SOD) were treated with an acute dose of 5 mg/kg PQ + 15 mg/kg MB and sacrificed 1 hour after exposure. This time point was chosen based on our previous paraquat toxicokinetic studies that suggest the peak levels of paraquat in tissue occurs at this time-point. Mice were perfused with saline to remove all blood from brain to reduce variability in sampling. The frontal cortex, striatum, midbrain and cerebellum were dissected and weighed. The analysis of paraquat was performed by liquid chromatography coupled to ion trap mass spectrometry (LC/ITMS) using a Waters Alliance 2690 interfaced to a ThermoFinnigan LCQ Classic instrument. The tissues were first extracted, then separated on a 5 μm (15 cm x 4.6 mm) Zorbax RX C8 reverse phase column, with gradient elution from a highly aqueous to a highly organic mobile phase. Paraquat was extracted from the brain using a microwave assisted solvent extraction (MASE) method. Roughly 30 mg of brain tissue was extracted in acetic acid and water. The mixture was sonicated and then extracted using a moderate power microwave program. The analyte was isolated from larger mass molecules using a 10 kDa microcentrifuge membrane. The analyte was ionized by electrospray ionization (ESI) and extracted in positive mode. The spectrum was acquired in full scan (150-250 m/z and 150-1000 m/z) mode. The chromatograms for all brain extracts showed peaks corresponding to PQ (m/z 184-186). An m/z of 184 suggests other chemical species (possibly adducts) related to paraquat. A calibration curve using m/z of 186 was used and was linear over a concentration range of 0.4 ng to 25 ng. Paraquat concentration is expressed as ng/mg tissue.

**Lipid Peroxidation** - Levels of lipid peroxidation were measured in tissue homogenates using the Lipid Peroxidation Assay Kit II (Calbiochem). Tissues were homogenized in 10 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl₂ and 4 mM butylated hydroxytoluene (1:10 w/v ratio). Samples were pretreated with catalase for elimination of H₂O₂ interference. The assay is based on the oxidation of ferrous ions to ferric ions by hydroperoxides under acidic conditions. Stable complex of ferric ions with an indicator dye, xylene orange was measured at 560 nm according to the manufacturer’s protocol.

**Western Blotting** - Proteins were analyzed by Western blot analysis as reported previously (31). In brief, tissue samples were homogenized in RIPA buffer (50 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, aprotinin, pepstatin, and phosphatase inhibitors (10mM NaF, 1 mM NaVO₄, 1.5 mM Na₂MoO₄, 1 mM benzamidine, 20 mM glycerophosphate and 20 mM p-nitrophenyl phosphate). Samples were sonicated and SDS-PAGE (5x) sample buffer was added to the final concentrations of 50 mM Tris HCl, pH 6.8, 2%SDS, 100 mM DTT, 0.006% bromphenol blue, and 10% glycerol. Lysates were centrifuged at 12,000xg for 20 min, and supernatants were loaded on 10 or 15% PAGE. After electrophoresis, gels were electroblotted onto polyvinylidene difluoride membrane and analyzed using specific antibodies. Blocking and antibody incubation solutions contained 5% nonfat dry milk. Milk was replaced with bovine serum albumin for testing phosphorylated proteins. Proteins were detected using the Western Lighting™ Chemiluminescence reagent (PerkinElmer Life Sciences). Antibodies tested include: rabbit anti-Bax, rabbit anti-Bcl-xL, rabbit anti-Bad, mouse anti-Phospho-Bad (Ser112), rabbit anti-Phospho-p44/42 MAP kinase (Thr202/Tyr204), rabbit anti-Bak and rabbit anti-actin (all Cell Signaling Tech. Inc.). For quantitative analysis, bands on the films with linear exposure range were scanned on a model 300A densitometer using ImageQuant software (Amersham Biosciences).

**Statistical Analyses** - Body weights were analyzed using repeated measures ANOVAs (RMANOVAs) with time as a within group factor and treatment as a between group factor. Total locomotor activity counts for each session, levels of DA and serotonin and metabolites and turnover, levels of PQ in toxicokinetic studies, and levels of Bax, Bcl-xL, Bad and P-P44/42 MAPK were analyzed using two factor ANOVAs with transgene and treatment as between group factors. Post-hoc assessments were carried out based on main effects or interactions as appropriate. For all analyses, p values of ≤0.05 were considered statistically significant.
RESULTS

Gross effects of treatment - Body weights were monitored throughout the experimental duration. No treatment related changes in body weights were observed in any of the groups at any time point during the course of the treatment.

Locomotor Activity - Mice were habituated to the locomotor chambers during a three-day period and horizontal activity counts evaluated. All mice were also injected with saline on these days to habituate them to the injection procedure. Mice within each line were randomly assigned to treatment groups to ensure similar baseline activity levels. Exposure to PQ alone and PQ+MB significantly decreased locomotor activity immediately following each injection, but only in the non-transgenic line, with a greater decrease observed in the PQ+MB treated group as compared to PQ alone (data not shown). Both the GPx1 and SOD mice treated with either PQ alone or with PQ+MB displayed activity levels similar to the corresponding saline treated mice. Additionally, only the PQ+MB treated nTg mice showed residual reductions in locomotor activity when measured 24 hrs after injections, with all other groups returning to control levels at these time points. Locomotor activity was evaluated again one week after the last treatment where analysis of variance confirmed a significant effect of transgenic status ($F_{(2,66)} = 24.7, p < 0.0001$) and an interaction of transgenic status and treatment ($F_{(4,66)} = 2.58, p = 0.045$). As shown in Figure 1, the only group still demonstrating a significant decrease in locomotor activity one week post-termination of treatment was the nTg group treated with PQ+MB as compared to the saline and PQ alone treated groups ($p = 0.01$) (Fig. 1).

Dopamine and Metabolite Analysis - Striatal and frontal cortical levels of DA, DOPAC, HVA, DA turnover, HIAA and 5HT were evaluated in tissues obtained 1-week post-treatment to preclude measurement of any acute effects of treatment (Fig. 2). At this time, significant effects of transgenic status in levels of striatal DA were found ($F_{(2,62)} = 4.49, p = 0.01$), with post-hoc analysis confirming a significant decrease (30%) in striatal DA only in the nTg group treated with PQ+MB ($p < 0.05$), without corresponding changes in either the GPx1 and SOD groups. Similarly, striatal DOPAC levels were reduced by PQ alone and by PQ+MB and HVA by PQ+MB only in nTg mice (both $p < 0.05$). GPx1 and SOD mice treated with PQ alone or PQ+MB showed no change in DA, DOPAC or HVA levels as compared to their saline treated counterparts. Striatal levels of 5HT or its metabolite, HVA, and DA turnover, analyzed as (DOPAC+HVA)/DA or DOPAC/DA were unaffected by treatment in any of the groups (data not shown). Frontal cortical levels of DA, DOPAC, HVA, HIAA or 5HT were also unaffected by treatment in any of the groups (data not shown).

Stereological Analysis - Numbers of both dopaminergic (TH+) and non-dopaminergic (TH-) neurons in the SNpc were determined by unbiased stereology and corresponding counts are depicted in Figure 3. Non-transgenic mice treated with PQ alone showed an 18% decrease in TH+ neurons in the SNpc ($p = 0.02$). This is consistent with previous observations from our laboratory, where PQ alone failed to alter locomotor activity or decrease striatal DA levels, but still resulted in loss of TH+ neurons in the SNpc. Additionally, nTg mice treated with the combination of PQ+MB showed a further decrease in the number of TH+ neurons (37%, $p < 0.0001$). This decrease was also significantly greater than the nTg mice treated with PQ alone ($p = 0.02$), suggesting a potentiated loss of TH+ neurons with combined exposure. No observable difference in non-dopaminergic neurons was observed. In contrast, no reductions in TH+ neurons were seen in response to PQ or to PQ+MB in either the GPx1 or SOD-treated groups.

Paraquat Toxicokinetics - The concentration of PQ in different brain regions was quantified in the nTg, GPx1, and SOD mice to ascertain that the differences in toxicity were not due to differences in PQ toxicokinetics. PQ levels were determined in the striatum and midbrain, since these are the regions most affected by PQ and MB in the nTg mice. Analysis of variance showed that PQ levels in the nTg, GPx1, and SOD mice were significantly different in the striatum and midbrain ($F_{(3,13)} = 9.7, p = 0.0012$ & $F_{(3,13)} = 12.7, p = 0.0004$, respectively) (Fig. 4). Non-transgenic and GPx1 mice showed comparable PQ levels in both the striatum and midbrain, while SOD mice showed a significantly higher concentration of PQ in both regions than either nTg or GPx1 mice. While the mechanism associated with these transgenic differences in toxicokinetics is unknown at present, the observation demonstrates that the difference in dopamine neurotoxicity between the nTg and transgenic (GPx1
and SOD) mice cannot be a function of lower PQ levels in the transgenic groups.

*PQ+MB-induced lipid peroxidation* - Resistance of SOD and Gpx1 transgenic mice to PQ+MB induced dopaminergic neurodegeneration suggests an antioxidant mechanism of neuroprotection. To determine whether this effect correlated with the level of oxidative stress in vulnerable brain regions, levels of lipid peroxidation were determined in striatum, midbrain and frontal cortex from tissue obtained one week following termination of PQ or PQ+MB treatment. As shown in Figure 5A, statistically significant increases in the level of lipid peroxidation in the striatum and midbrain regions were found only in nTg mice exposed to PQ+MB (279% and 263% in the striatum and midbrain, respectively, as compared to saline treated controls). While a small increase in the level of oxidative stress was also observed in animals treated with PQ alone in these two regions, it did not achieve statistical significance. In contrast, neither of the transgenic lines exhibited increases in lipid peroxidation levels in these regions. No increase in lipid peroxidation levels was noted in frontal cortex of any of the groups.

To determine whether combined PQ+MB exposure was capable of increasing oxidative stress in these brain regions after acute treatment, levels of lipid peroxides were measured at 16 and 40 hrs after a single injection. A marked increase in lipid peroxides was observed in the midbrain and striatum of nTg mice in comparison to saline injected control animals (see Fig. 5B). Again, a regionally selective effect was noticed with no changes detected in frontal cortex of any of the experimental groups.

*Expression of Bax, Bcl-xL and Bad in Brain Regions After PQ+MB Administration* - To begin systematic analysis of the behavior of these molecules in this model system, measurement focused on early events, i.e., at 16 and 40 hrs after a single combined PQ+MB injection. Among Bcl-2 members, Bax appears to play a critical role as a pro-apoptotic mediator in several Parkinson’s disease animal models as well as in patients (35). Thus, the presence of Bax protein in tissue homogenates from midbrain and frontal cortex regions of normal and transgenic mice exposed to saline or PQ+MB was determined. No difference in the level of Bax or any other tested proteins in saline exposed normal or transgenic mice was found. As shown in Figure 6, increased expression of Bax was observed only in the midbrain of nTg mice at 16 hrs after an acute PQ+MB injection. The level of Bax increased 5.7 fold (Fig. 6) in comparison to animals injected with saline. Neither transgenic line revealed any changes in the level of Bax at either time point.

Western blotting analysis of the Bcl-xL protein in midbrain and cortex extracts indicated that at 40 hrs after a single PQ+MB injection, expression level of this protein had decreased 2 fold in nTg animals in comparison to saline injected controls (Fig. 7). No changes in Bcl-xL were observed at 16 hrs in these mice, or in either transgenic line at either time point. The effect was region specific, since no change in Bcl-xL expression in cortex after PQ+MB administration was observed. This analysis revealed that at 16 and 40 hrs after an acute exposure to PQ+MB, the ratio of Bax/Bcl-xL was shifted towards the induction of pro-apoptotic mechanisms.

Analysis of Bad expression in midbrain and cortex extracts of normal and transgenic mice exposed to PQ+MB indicated that at 16 and 40 hrs, total levels of this protein remain unchanged (Fig. 8). We also tested phosphorylation status of Bad at Ser-112, which is known to be controlled by the Ras-mitogen activated protein kinase pathway (36). Data show that in contrast to the total level of Bad, Ser-112 phosphorylation was significantly induced in the midbrain region of nTg animals at 16 and 40 hrs after PQ+MB administration (Fig. 8, bottom plot). This suggests that at least some cell-protective mechanisms are induced in animals exposed to PQ+MB and these mechanisms are not functional in the presence of increased levels of antioxidant enzymes. This observation supports an important role of ROS in inducing cell death as well as defense pathways targeting different members of the Bcl-2 family.

*Activation of P44/42 MAP kinase* - The P44/42 MAP kinase phosphorylation was analyzed in mice treated with a single dose of PQ+MB. Data from Western blot analysis with specific antibodies indicate that phosphorylation of both P42 and P44 proteins was induced at 16 hrs only in midbrain homogenates of nTg animals injected with PQ+MB (Fig. 9). These results are in agreement with early activation of Bad phosphorylation.

**DISCUSSION**

Behavioral, neurochemical, and pathological analyses in this study demonstrated that low level
exposure of wild type (nTg) mice to the pesticides PQ and MB, particularly in combination, resulted in selective adverse consequences to the nigrostriatal dopaminergic system. These observations are consistent with previous data from our laboratories, of selective loss of dopaminergic neurons in the SNpc and corresponding loss of striatal dopamine in C57BL/6 mice (16-18, 33). Similarly, behavioral deficits were evident only in the nTg mice treated with PQ+MB, present even one week after the last treatment. The adverse changes, however, were observed only in the nTg mice, while those overexpressing SOD and GPx1 were spared from virtually all adverse effects. These findings therefore implicate ROS as a mediator of neurotoxicity of PQ and MB. These results also indicate that both enzymes were equally capable of providing protection against oxidative stress induced by PQ+MB. The exact sources and types of ROS inducing cellular damage are not known at this point. Given the specific characteristics of both enzymes, it is apparent that effective prevention of superoxide-driven radical formation and detoxification of peroxides, especially lipid peroxides was important for the observed neuroprotection. Moreover, even though activities of catalase and glutathione peroxidase in the brain of SOD transgenic mice and wild type animals are the same under normal conditions (30) and may be enough to tolerate local increases in \( H_2O_2 \), the possibility still remains that a compensatory increase in the activities of these enzymes occurs with PQ+MB exposure.

Our previous studies suggest that the adverse effects of both PQ and of PQ+MB are attributable to PQ (17, 24), with co-administration of MB increasing the levels of PQ in brain and slowing its excretion (37). However, the toxicokinetic studies carried out here confirmed that the differences in susceptibility between the nTg and Tg mice is not due to a difference in PQ concentration in the midbrain and striatum, but must arise, instead, from the intrinsic neuroprotective effects of these enzymes against oxidative stress generated by these xenobiotics. Additionally, the dopamine cell loss and behavioral deficits observed in the nTg mice (C57BL/6 xCBA/J background) were similar to those observed in our previous experiments using C57BL/6 mice. Although PQ brain levels were actually higher in C57BL/6 mice (unpublished observation), the loss of dopamine neurons and behavioral changes were similar, suggesting strain differences arise at the cellular level via inherent differences in dopaminergic neurons. This is evident given that the nTg mice had significantly lower PQ levels but exhibited similar levels of dopaminergic neurotoxicity.

Our present results show that chronic PQ+MB exposure leads to sustained increases in lipid peroxidation. We were able to detect the presence of elevated oxidative stress levels one week after the last injection of the neurotoxicants. Importantly, even a single injection of PQ+MB caused a shift in ox/redox cellular balance in the striatum and midbrain of nTg animals, but not in the cortex. Both transgenic mouse lines were protected against injury, showing no changes in lipid peroxidation status. PQ is a well-known inducer of ROS following acute exposures, even though its entry across the blood-brain barrier is somewhat impeded. It is unclear whether the increase in ROS in susceptible brain regions after combined PQ+MB exposure is a result of direct ROS formation due to the redox cycling properties of PQ or it is a result of activation of other ROS-generating pathways through signaling mechanisms leading to the disruption of the electron transport chain or activation of enzymes such as NADPH oxidase. PQ is also capable of stimulation of glutamate efflux to initiate excitotoxicity. Depolarization of NMDA receptor channels and Ca\(^{2+}\) influx into the cells by activation of non-NMDA receptor channels might stimulate nitric oxide synthase (NOS) (38). NO released from these cells can interact with dopaminergic terminals leading to the generation of secondary ROS, mitochondrial dysfunction and long-lasting dopamine release. Another mechanism that should also be considered involves the PQ diaphorase activity of NOS (39). Due to this activity, PQ can use NOS to generate \( O_2^- \) at the expense of NO. It is also possible that several of these mechanisms may be functioning simultaneously.

Our data indicates that the combination of PQ with MB is significantly more potent than PQ alone following long-term exposure. While this is likely due to the fact that MB can increase the levels and duration of action of PQ in the brain, it is also possible that MB contributes directly to the toxicity. For example, recent reports demonstrate MB-induced dopamine efflux, proteasomal inhibition and elevated protein carbonyl content in cell culture; intraventricular injection models also suggest effects...
independent from PQ leading to the generation of oxidative stress (28). The ability of MB to alter the function of complex III in oxidative phosphorylation, which along with complex I is a major site for superoxide radical formation, is also noteworthy. NADPH oxidase from phagocytes as well as recently discovered non-phagocytic enzymes in neuronal cells might be considered as additional source of ROS inducing oxidative stress in neurons (40).

An important process associated with exposure to either PQ or MB is aggregation of α-synuclein. Although its exact role is not known, α-synuclein mutations in autosomal dominant familial cases and the presence of α-synuclein in Lewy bodies of Parkinson’s disease patients have suggested an important role of this protein in the pathophysiological processes underlying neurodegeneration (41). PQ triggers α-synuclein fibrillation both in vitro and in exposed mouse brains (42). MB is capable of inhibiting proteasomal activity and inducing α-synuclein aggregation in dopaminergic cell cultures (28). Oxidative stress detected with combined PQ+MB in vulnerable brain regions may contribute to malfunction of the proteasomal degradation system. Importantly, impairment of proteasomal degradation and accumulation of unfolded proteins in turn may cause oxidative stress and trigger cell death mechanisms, as implicated in the pathogenesis of Parkinson’s disease. In both cases, one would expect neuroprotection upon scavenging of ROS associated with these processes.

The mechanism leading to the selective death of nigral neurons in this model system is currently unknown. The challenge is characterizing the chronic process when at any given time a few cells are undergoing active degeneration. It also seems that the speed of cellular demise is very fast, making detection very difficult. Although there is much controversy about the role of apoptosis in the pathogenesis of Parkinson’s disease it seems very likely that this process contributes significantly to the dopaminergic neuron loss observed in this disorder (43). The exact dynamic of cell loss during chronic exposure to these pesticides has not yet been elaborated, but the gradual decrease in the ability of non-transgenic mice to regain normal locomotor function after successive injections of PQ+MB (44), the data from several earlier time points (45), and the fact that neither PQ nor MB accumulate in brain suggest that cell loss occurs gradually as well. Therefore, to begin a systematic analysis of the mechanism of cell death and possible ROS-sensitive pathways involved in this process, cellular response in different brain regions were investigated at 16 and 40 hrs after a single PQ+MB injection since early time points will be crucial for identifying the initial mechanisms and pathways. Indeed, combined PQ+MB induced oxidative stress in the midbrain and striatum at these time points. Our preliminary experiments demonstrated that levels of the expression of pro- and anti-apoptotic mediators were not affected in striatum (data not shown). This fact supports the notion of selective vulnerability of the dopaminergic neurons, with effects primarily on the cell bodies, which reside in the SNpc. According to the data of Zhang et al. (26), where they used direct delivery of purified Mn-EBDC, the major component of maneb, to the lateral ventricular system, selective degeneration of pre-synaptic dopaminergic nerve terminals were observed only in the striatum. Medium spiny neurons that represent more than 90% of striatal neurons and use GABA as their neurotransmitter and receive glutamatergic input from various brain regions were well preserved. Similar results were obtained in our studies with combined PQ+MB exposure in non-transgenic animals (44, 45). We focused in the current studies on death signaling pathways showed to be activated in patients with Parkinson’s disease as well as in experimental model systems. The p53-glyceraldehyde-3-phosphate dehydrogenase-Bax and FAS receptor-FADD-caspase 8-Bax pathways, probably interconnected, have recently gained attention as possibly being involved in the etiology of Parkinson’s disease (43). In both cases, Bax plays an important role, as a pro-apoptotic protein, inducing and augmenting the apoptotic cascade (46). Changes in the level of expression of pro- and anti-apoptotic Bcl-2 family members should have several important functions in the control of the cellular response to PQ+MB exposure. Bcl-xL is expressed in the nervous system and its complexation with Bax prevents it from clustering on mitochondria and interacting with mitochondrial proteins (47). Bcl-xL is capable of protecting neuronal cells from apoptosis induced by a variety of stimuli, including MPTP/MPP+ and 6-OHDA, while decreases in levels of this protein sensitizes cells to these neurotoxins (48, 49). The opposite effects have been
reported in regard to the mechanisms of action of Bax/Bad proteins (50).

An important connection lies between the Bcl-2 family proteins and regulation of the intracellular redox state. For example, Bcl-2/Bcl-xL can influence the cellular “redox pool” and alter cell behavior (48, 51). It is widely accepted that Bcl-2 expression leads to enhanced antioxidant capacities targeted at the suppression of oxidative stress signals, whereas Bax upregulation and translocation to the mitochondrial membrane induces ROS production (50). The exact source of the ROS is not clear, although mitochondria may be one of the major sources. It still remains to be determined whether changes in levels of Bcl-xL and Bax in the midbrain region of nTg mice administered PQ+MB impacted the level of ROS. It also has been reported that ROS can influence expression of Bcl-2 (52). Mechanism of activation/inhibition of pro- and anti-apoptotic members of Bcl-family is very complex and includes regulation at the level of transcription, translation as well as protein degradation. Therefore, level of Bcl-xL and Bax proteins observed in our experiments are the summation of all these processes; each of them may be affected by PQ+MB exposure. The early up-regulation of pro-apoptotic Bax and later down-regulation of pro-apoptotic Bcl-xL in our experiments suggested different mechanisms of their regulation. Selective effects on time course of expression of these proteins in response to oxidative stress as well as other inducers are widely reported (53, 54). The Bcl-2, Bcl-xL/Bax ratio was considered as an important determining factor for the cells fate in a variety of neurotoxic conditions (55-57). Importantly, even after a single injection of PQ+MB, the ratio of Bcl-xL/Bax in the midbrain regions of wild type animals was shifted to a proapoptotic state, which might lead, under conditions of chronic exposure, to behavioral abnormalities and dopaminergic neurodegeneration. This shift was completely eliminated by over-expression of antioxidant enzymes, suggesting that oxidative stress in this model system functions upstream of at least two critical proteins well known to be involved in the regulation of neuronal cell death. The redox-sensitive transduction pathways responsible for the upregulation of Bax levels and the downregulation of Bcl-xL expression in response to PQ+MB exposure remain to be characterized.

Another Bcl-xL and Bcl-2 death binding partner is Bad, the only BH3-subfamily member. Bad phosphorylation regulates apoptosis in several cell types, including neurons (58). Under normal conditions, phosphorylated Bad complexes with the molecular chaperone protein, 14-3-3. In response to an apoptotic stimulus, Bad will be dephosphorylated and released from a complex, after which it interacts with Bcl-xL, leading to the downstream Bax-dependent release of cytochrome c and consequently caspase activation. Phosphorylation states as well as intracellular localization of Bad are important features in cell-death pathways. We show here that Bad Ser-112 phosphorylation was induced by PQ+MB, whereas the total amount of protein was not affected. This effect seems to confer a protective effect, since it should result in dissociation from Bcl-2 and Bcl-xL and subsequent association with 14-3-3 protein. The released Bcl-xL is able to block the release of cytochrome c from mitochondria. It was previously reported that phosphorylation of Bad at Ser-112 is regulated by MAPK pathway (36). Neurons undergoing oxidative stress-related injuries typically display MAPK activation. A neuroprotective role as well as a detrimental role of MAPK kinase activation has been reported (59), the latter one usually associated with sustained activation of these kinases. Indeed, we observed activation of both P44 and P42 kinases in midbrain regions of nTg animals, but not in transgenic mice. Interestingly, both kinases had returned to normal levels at 40 hrs, whereas P-Bad was still induced. This result might indicate a significant stability of the modified protein, which was still present at a time, when kinase activation already ceased. Proapoptotic proteins are known for their long half-lives and in several cases their phosphorylation has been prevented by ubiquitination and degradation (60, 61). It is also possible that another kinase phosphorylates Bad at a later time point. Several other kinases besides MAPK have been shown to be capable of phosphorylating Bad at Ser-112, such as mitochondrion-associated protein kinase A and p21-activated kinase 5 (Pak5) (62). Phosphorylation of Bad at Ser-136 is mediated by the serine/threonine protein kinase Akt-1/PKB. We did not detect any activation of Akt in animals exposed to PQ+MB (data not shown). Interestingly enough, nicotine, which possesses neuroprotective properties in models of Parkinson's disease, induces phosphorylation of Bad at several sites including Ser-112 in cell culture (63).
It is likely that several other signaling pathways mediate PQ+MB neurotoxicity. To rationalize the search of these pathways for further investigation, it is logical to look at data already reported for in vitro and in vivo models after exposure to each of these compounds alone. Unfortunately, no data is available for MB, whereas several PQ-induced effects have been described. For example, it has been reported by several laboratories that PQ activates another MAPK cascade - JNK, leading to dopaminergic neuronal cell death in vitro and vivo (64, 65). This effect was diminished by application of the JNK inhibitor and antioxidant manganese (III) tetrakis (4-benzoic acid) in cell culture. Studies of these pathways as well as detailed analysis of the character of cell death are currently under the way.

REFERENCES


FOOTNOTES

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1The abbreviations used are: DA, dopamine; GPx1, intracellular glutathione peroxidase; MB, maneb; nTg, non-transgenic; Mn-EBDC, manganese-ethylenebis-dithiocarbamate; PD, Parkinson’s disease; PQ, paraquat; SNpc, substantia nigra pars compacta; SOD, Cu, Zn superoxide dismutase; TH, tyrosine hydroxylase;

FIGURE LEGENDS

Fig. 1. Changes in locomotor activity seven days after the last exposure in nTg, GPx1 and SOD transgenic mice. Data shown is total horizontal activity presented as percent of respective saline treated controls (mean ± SEM, n = 7 – 10 per treatment group). Only the non-transgenic mice treated with PQ+MB show a persistent decrease in activity levels seven days post-exposure, with all other groups evidencing full behavioral recovery. Post-hoc analysis revealed significant difference (p<0.05) from saline and paraquat alone treated nTg mice (*).

Fig. 2. Changes in striatal dopamine, metabolites and serotonin in nTg, GPx1 and SOD transgenic mice one week after the last exposure. Values were determined using HPLC analysis and are presented as ng/mg protein (mean ± SEM, n = 7 – 10 per treatment group). A significant decrease in dopamine and its metabolites were observed only in the non-transgenic mice treated with PQ+MB. The levels of 5HT were not altered in any of the treatment groups. Post-hoc analysis revealed significant difference (p<0.05) from saline treated nTg mice (*).

Fig. 3. Total number of TH+ neurons determined in the substantia nigra pars compacta of nTg, GPx1 and SOD transgenic mice using stereological techniques (mean ± SEM, n = 4 per treatment group). TH+/Nissl+ (TH+) neurons as well as TH/Nissl+ (TH) were counted. Only the non-transgenic mice treated with either PQ alone or PQ+MB showed significant loss of TH+ neurons in the SNpc, with a greater loss observed in the PQ+MB treated group. Both the SOD and GPx1 transgenic mice showed no significant neuronal loss with either PQ or PQ+MB treatment. Post-hoc analysis revealed significant difference (p<0.05) from: * saline treated non-transgenic mice; and # PQ treated non-transgenic mice.

Fig. 4. Paraquat concentration in the striatum and midbrain of nTg, GPx1 and SOD transgenic mice 1 hr after an acute exposure to PQ+MB. PQ concentration was determined using LC/ITMS. The SOD overexpressing mice had higher PQ levels in both regions compared to the nTg and GPx1 mice. Post-hoc analysis revealed significant difference (p<0.05) from: * C57BL/6 mice and # SOD transgenic mice.
Fig. 5. Effect of PQ+MB administration on lipid peroxidation levels in the striatum, midbrain and frontal cortex of nTg, GPx1 and SOD transgenic mice. Mice were sacrificed either one week after the chronic dosing paradigm (A) or 16 and 40 hrs after a single acute injection (B). Data indicate significant increases in the level of lipid peroxides as a measure of oxidative stress in the striatum and midbrain of non-transgenic mice after chronic PQ+MB administration (A), as well as in the striatum and midbrain after a single injection (B). No changes in the cortex were observed either with an acute or chronic exposure. Overexpression of both antioxidant enzymes provided significant protection against oxidative stress in all tested regions of transgenic mice. *, p<0.05 in comparison to saline treated animals.

Fig. 6. Expression profile of proapoptotic Bax in the midbrain and cortex of non-transgenic, GPx1 and SOD transgenic mice at 16 and 40 hrs after a single injection of saline or PQ+MB (n = 4 per treatment group). Expression of Bax as well as β-actin was analyzed by immunoblotting with specific antibodies. Representative blots are shown. Bands corresponding to Bax as well as actin were scanned, and the results expressed as Bax/actin ratio in arbitrary units ± SEM. Treatment with PQ+MB activated Bax expression at 16 hrs in the midbrain region of the non-transgenic mice. PQ+MB administration had no effect on Bax in the cortex of non-transgenic mice as well in both tested regions of animals overexpressing antioxidant enzymes. *, p<0.05 in comparison to saline treated animals.

Fig. 7. Expression profile of antiapoptotic Bcl-xL in the midbrain and cortex of non-transgenic, GPx1 and SOD transgenic mice at 16 and 40 hrs after a single injection of saline or PQ+MB (n = 4 per treatment group). Expression of Bcl-xL was analyzed by immunoblotting with specific antibodies. Representative blots are shown. Bands corresponding to Bcl-xL were scanned, and the results expressed as Bcl-xL/actin ratio (see Fig 6. legend) in arbitrary units ± SEM. Treatment with PQ+MB reduced level of Bcl-xL at 40 hrs only in the midbrain region of the non-transgenic mice. *, p<0.05 in comparison to saline treated animals.

Fig. 8. Expression profile of proapoptotic Bad in the midbrain of non-transgenic, GPx1 and SOD transgenic mice at 16 and 40 hrs after a single injection of saline or PQ+MB (n = 4 per treatment group). Expression of Bad was analyzed by immunoblotting with antibodies against Bad as well as Phospho-Bad (Ser112). Bands corresponding to Bad were scanned, and the results expressed as Bad or p-Bad/actin ratio (see Fig 6. legend) in arbitrary units ± SEM. Treatment with PQ+MB did not affect total Bad expression in all animal groups. Phosphorylation of Bad was induced in the midbrain of non-transgenic mice, both at 16 and 40 hrs after PQ+MB administration. *, p<0.05 in comparison to saline treated animals.

Fig. 9. Effect of a single injection of saline or PQ+MB on the levels of phosphorylated P44/42 MAP kinase in the midbrain and cortex of non-transgenic, GPx1 and SOD transgenic mice (n = 4 per treatment group). Expression was analyzed by immunoblotting with specific antibodies. Representative blots are shown. Bands corresponding to P44 and P42 were scanned, and the results expressed as MAPK/actin ratio (see Fig 6. legend) in arbitrary units ± SEM. Treatment with PQ+MB induced p44 as well as p42 phosphorylation at 16 hrs in the midbrain region of the non-transgenic mice. No effect on MAPK in the cortex of non-transgenic mice as well as in both tested regions of animals overexpressing antioxidant enzymes was detected. *, p<0.05 in comparison to saline treated animals.
Figure 1
Figure 2

Graphs showing the levels of dopamine, DOPAC, HVA, and serotonin in different conditions. The x-axis represents different conditions (SAL, PQ, PQ-MB) and the y-axis represents the concentration in ng/mg of protein. The graphs are color-coded for different groups.
Figure 3
Figure 4

[Graph showing the concentration of PQ in the Striatum and Midbrain with comparisons between nTg, GPx1, and SOD]
Figure 5

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Figure 6

Bax /actin ratio, arbitrary units

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Midbrain

Bax /actin ratio, arbitrary units

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Bax /actin ratio, arbitrary units

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Figure 7

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Bars represent the mean ± SEM, n = 4, and *p < 0.05 compared to control.
**Figure 8**

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**Bad**

midbrain

P-Bad

midbrain

**Midbrain**

Bad /actin ratio

arbitrary units

**Midbrain**

P-Bad /actin ratio

arbitrary units

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Figure 9
Overexpression of superoxide dismutase or glutathione peroxidase protects against the paraquat+maneb-induced Parkinson's disease phenotype
Mona Thiruchelvam, Olga Prokopenko, Deborah A Cory-Slechta, Eric K Richfield, Brian Buckley and Oleg Mirochnitchenko

J. Biol. Chem. published online April 11, 2005

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