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Differential Contribution of the Mitochondrial Respiratory Chain Complexes to Reactive Oxygen Species Production by Redox Cycling Agents Implicated in Parkinsonism

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Exposure to environmental pesticides can cause significant brain damage and has been linked with an increased risk of developing neurodegenerative disorders, including Parkinson's disease. Bipyridyl herbicides, such as paraquat (PQ), diquat (DQ), and benzyl viologen (BV), are redox cycling agents known to exert cellular damage through the production of reactive oxygen species (ROS). We examined the involvement of the mitochondrial respiratory chain in ROS production by bipyridyl herbicides. In isolated rat brain mitochondria, H_2O_2 production occurred with the following order of potency: BV > DQ > PQ in accordance with their measured ability to redox cycle. H_2O_2 production was significantly attenuated in all cases by antimycin A, an inhibitor of complex III. Interestingly, at micromolar (≤ 300μM) concentrations, PQ-induced H_2O_2 production was unaffected by complex I inhibition via rotenone, whereas DQ-induced H_2O_2 production was equally attenuated by inhibition of complex I or III. Moreover, complex I inhibition decreased BV-induced H_2O_2 production to a greater extent than with PQ or DQ. These data suggest that multiple sites within the respiratory chain contribute to H_2O_2 production by redox cycling bipyridyl herbicides. In primary midbrain cultures, H_2O_2 differed slightly with the following order of potency: DQ > BV > PQ. In this model, inhibition of complex III resulted in roughly equivalent inhibition of H_2O_2 production with all three compounds. These data identify a novel role for complex III dependence of mitochondrial ROS production by redox cycling herbicides, while emphasizing the importance of identifying mitochondrial mechanisms by which environmental agents generate oxidative stress contributing to parkinsonism.

Key Words: **paraquat; redox cycling; mitochondria; Parkinson's disease.**

Environmental factors have been linked in the etiology of Parkinson's disease (PD), an age-related neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. (Baldereschi *et al*., 2003; Gorell *et al*., 1998; Semchuk *et al*., 1992; Tanner *et al*., 1989). Specifically, a comprehensive review of epidemiology and toxicology studies has associated pesticide exposure with an increased risk for developing PD (Brown *et al*., 2006). While increased steady-state levels of reactive oxygen species (ROS) are implicated in the disease pathogenesis, the mechanisms by which pesticides and other environmental factors contribute to dopaminergic cell death remain unresolved.

Paraquat (PQ) is a widely applied herbicide that also produces a PD-like neurodegeneration model in rodents (Brooks *et al*., 1999; McCormack *et al*., 2002). Although PQ toxicity is typically associated with the lung, significant brain damage has also been observed in individuals exposed to lethal doses (Grant *et al*., 1980; Hughes, 1988). Furthermore, epidemiological studies suggest an increased risk for developing PD following chronic PQ exposure (Hertzman *et al*., 1990; Liou *et al*., 1997). Whereas the mechanisms of PQ-induced neurodegeneration remain to be determined, they are likely related to the compound's ability to generate ROS via redox cycling. The parent dication (PQ^{2+}) accepts a single electron from an appropriate reductant, forming the monocationic radical (PQ^{+}). The radical rapidly reacts with oxygen to produce superoxide ($O_2^{\cdot-}$) while regenerating the parent compound, PQ^{2+} (Bus and Gibson, 1984). Enzymes capable of donating an electron to initiate the PQ redox cycling process have been identified in microsomal, plasma membrane, mitochondrial, and cytosolic components of various organs (Drechsel and Patel, 2008).

The potential role of mitochondria in PQ-induced ROS production is relatively unexplored but has gained momentum based on observations that mitochondria can be a major source of PQ-induced ROS production (Castello *et al*., 2007), and mitochondrial expression of antioxidant enzymes, such as catalase or peroxiredoxin 5, protects against PQ toxicity more effectively than cytosolic expression (Mockett *et al*., 2003; Tien Nguyen-nhu and Knoops, 2003). Additionally, deficiencies in mitochondrial-localized manganese superoxide dismutase has been shown to increase the sensitivity of flies (Kirby *et al*., 2002) and mice (Van Remmen *et al*., 2004) to PQ. Although mitochondria seem an unlikely source for the

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oxygen-dependent toxicity of PQ due to relatively low-oxygen tension levels found in subcellular regions, the complexes of the respiratory chain serve as rich electron sources with close proximity to the site of oxygen reduction in complex IV. Therefore, these complexes represent strong candidates as contributors to the PQ redox cycling process.

Since environmental agents are important risk factors of neurodegeneration, there is a critical need to understand the mechanisms by which they cause neurotoxicity. The goal of this study was to characterize mitochondrial mechanisms of ROS production in response to PQ. Specifically, the contribution of the respiratory chain complexes in PQ-induced ROS production was examined and compared with structurally related redox cycling herbicides, diquat (DQ) and benzyl viologen (BV). The resulting data provide evidence that multiple sites within the respiratory chain are capable of contributing to ROS generation by redox cycling bipyridyl herbicides, such as PQ, with complex III serving as the major mediator.

MATERIALS AND METHODS

Chemical reagents. Redox cycling herbicides, PQ (1,1′-dimethyl-4,4′-bypyridinium dichloride; methyl viologen), DQ (diquat dibromide monohydrate), and BV (1,1′-dibenzyl-4,4′-bypridinium dichloride), and all other chemicals unless otherwise noted were obtained from Sigma (St Louis, MO). Cell culture reagents were obtained from Gibco/Invitrogen (Carlsbad, CA). Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) used in the detection of H_2O_2 was obtained from Molecular Probes (Eugene, OR).

Isolation of purified rat brain mitochondria. Brain mitochondria were isolated from male Sprague-Dawley rats (2–3 months of age) using Percoll density gradient centrifugation (Sims and Anderson, 2008) with slight modification as described (Castello *et al*., 2007). Purity of mitochondrial fractions was assessed via Western blot analysis using cytochrome oxidase subunit IV and lactate dehydrogenase antibodies as mitochondrial and cytosolic markers, respectively. Cytochrome oxidase was robustly expressed in mitochondrial fractions, while lactate dehydrogenase was undetectable, indicating the presence of highly purified mitochondria (data not shown). Mitochondrial viability was determined by oxygen consumption rates. Mitochondria with respiratory control ratios greater than 5 were used in all experiments.

Measurement of mitochondrial H_2O_2 production. ROS production was measured as H_2O_2 using a horseradish peroxidase (HRP)-linked fluorometric assay coupled to the oxidation of Amplex Red. Mitochondria (0.1 mg/ml) were suspended in respiration buffer (100mM KCl, 75mM mannitol, 25mM sucrose, 10mM Tris-HCl, and 10mM KH_2PO4, pH 7.4) with 0.1 U/ml HRP, 50μM Amplex Red, supplemented with 2.5mM malate + 10mM glutamate, or 10mM succinate as respiration substrates. The following inhibitors of the respiratory chain were used: 5 μg/ml rotenone (complex I—NADH:ubiquinone oxidoreductase [EC 1.6.5.3]), 10μM 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TTFA; complex II—succinate dehydrogenase [EC 1.3.5.1]), 10μM antimycin A (complex III—ubiquinone:cytochrome *c* oxidoreductase [EC 1.10.2.2]), 10μM myxothiazol (complex III), 10μM stigmatellin (complex III), 1mM potassium cyanide (KCN; complex IV—cytochrome oxidase [EC 1.7.2.1]), 1μM oligomycin (ATP synthase [EC 3.6.3.14]), and 1μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; mitochondrial membrane potential uncoupler). The reaction was initiated by the addition of PQ, DQ, or BV at indicated concentrations. H_2O_2 production rates were measured over a period of 10–15 min during incubation with redox cycling compounds. Fluorescence was measured at excitation/emission wavelengths of 530/590 nm, respectively, using a Gemini fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

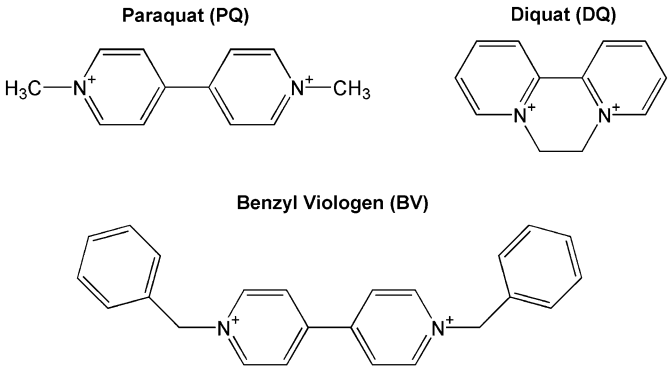

FIG. 1. Chemical structures of bipyridyl herbicides: PQ, DQ, and BV.

Measurement of H_2O_2 in primary midbrain cultures. Primary midbrain cell cultures were prepared from embryonic day 16 rats according to previously described methods (Castello *et al*., 2007). Cells were cultured in poly-*D*-lysine coated 48-well plates at a density of 2.5×10^5 cells per well. Primary midbrain cultures were maintained for 5–7 days before treatment. To measure H_2O_2 production, cells were incubated for 8 h at 37°C in medium containing 1.8mM $CaCl_2$, 5.4mM KCl, 0.8mM $MgSO_4$, 116mM NaCl, 14.7mM $NaHCO_3$, 1mM NaH_2PO_4, 10mM HEPES, and 5.5mM *D*-glucose, pH 7.4 containing 0.1 U/ml HRP and 50μM Amplex Red reagent. PQ, DQ, or BV was added at indicated concentrations and inhibitors used at concentrations described above.

Mitochondrial dysfunction and cell death assessment. Loss of cell viability due to mitochondrial dysfunction was analyzed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Primary midbrain cultures were incubated with 5 mg/ml MTT added to cell culture medium for 30 min. Cells were washed with PBS and formazan crystals dissolved using isopropanol containing 4mM hydrochloric acid. Absorbance values were read at 590 nm with a Versamax microplate reader (Molecular Devices).

Statistics. Statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, CA).

RESULTS

Comparison of Mitochondrial H_2O_2 Production by Redox Cycling Herbicides

In order to examine the contributions of redox cycling to ROS production, the magnitude of mitochondrial H_2O_2 production was determined for PQ and two structurally related bipyridyl herbicides: DQ and BV (Fig. 1). DQ and BV are also capable of redox cycling, albeit to a greater degree than PQ. As shown in Figure 2, all three bipyridyl compounds generated H_2O_2 in a concentration-dependent manner in malate + glutamate-supplemented mitochondria (Fig. 2A). Based on these concentration curves, half maximal effective concentration (EC_{50}) values were calculated to determine the potency of each compound. Comparison of H_2O_2 production rates in malate + glutamate-fed mitochondria (Fig. 2A) revealed that BV was the most effective (EC_{50} = 0.508mM), followed by DQ (EC_{50} = 1.133mM), while PQ was the least potent compound (EC_{50} = 1.675mM). Similar results were obtained

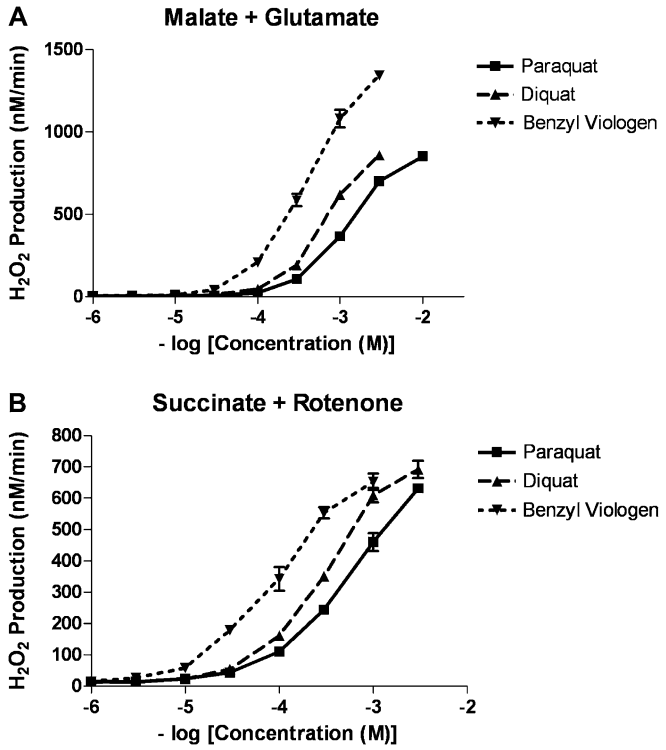

FIG. 2. Dose-response curves of H_2O_2 production in isolated rat brain mitochondria stimulated by redox cycling bipyridyl herbicides. Mitochondria were stimulated with (A) malate + glutamate or (B) succinate + rotenone and treated with PQ (solid line), DQ (dashed line), or BV (dotted line) at increasing concentrations. EC_{50} values were calculated to determine the potency of each compound in terms of H_2O_2 production (see text).

in mitochondria respiring on succinate, a complex II substrate, in combination with rotenone to block complex I–mediated reverse electron transport (RET): BV − EC_{50} = 0.104mM, DQ − EC_{50} = 0.369mM, and PQ − EC_{50} = 0.689mM (Fig. 2B). Mitochondria-free controls failed to produce any significant ROS levels (data not shown).

Contribution of Respiratory Chain Complexes to Mitochondrial H_2O_2 Production by Redox Cycling Herbicides

Our laboratory has shown in brain mitochondria that PQ-induced H_2O_2 production can be attenuated with antimycin A, a specific inhibitor of electron flow through complex III (Castello *et al.*, 2007). However, studies in nonneuronal tissues have implicated complex I as the major site of mitochondrial ROS production by PQ (Cocheme and Murphy, 2008; Fukushima *et al.*, 1993). Therefore, the contribution of each individual respiratory chain complex was tested in response to H_2O_2 production by redox cycling herbicides in isolated rat brain mitochondria. Using malate + glutamate as respiration substrates to feed electrons at the level of complex I, TTFA, KCN, and oligomycin (inhibitors of complex II, complex IV, and ATP synthase, respectively) had no significant effect on PQ-, DQ-, or BV-induced mitochondrial H_2O_2 production.

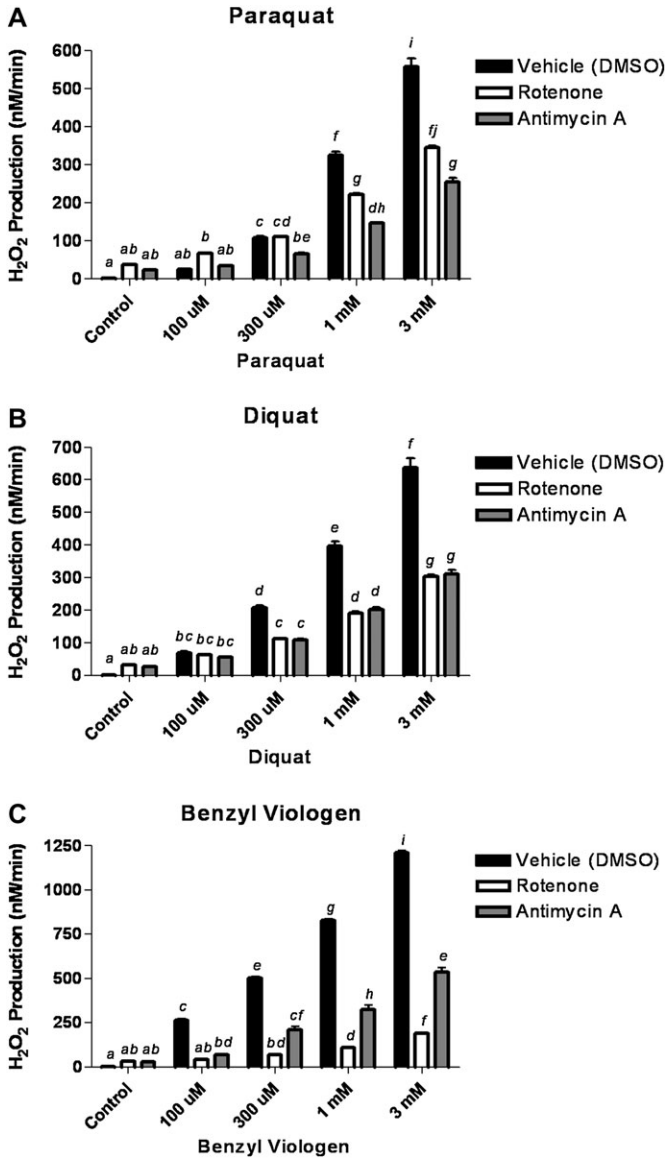

FIG. 3. Role of complexes I and III in redox cycling herbicide-induced mitochondrial H_2O_2 production. Isolated rat brain mitochondria were supplemented with malate + glutamate in the presence of (A) PQ, (B) DQ, or (C) BV. Inhibitors of the respiratory chain, rotenone (5 μg/ml; white bars) or antimycin A (10μM; gray bars), were added as indicated. Data expressed as H_2O_2 production rates in nanomolar per minute + SEM, n = 3–4. Bars with different letters indicate statistical significance from one another ($p < 0.05$, one-way ANOVA with Tukey's posttest).

However, dissipation of the mitochondrial membrane potential by the addition of FCCP completely abolished H_2O_2 production by all three herbicides (data not shown). By inhibiting electron flow through complex I or III by rotenone or antimycin A, respectively, bipyridyl herbicide–induced H_2O_2 production was inhibited to varying degrees. PQ-induced H_2O_2 production was inhibited by 60–70% consistently in the presence of antimycin A (Fig. 3A). However, rotenone had no effect on rates of H_2O_2 production at PQ concentrations $\leq$ 300μM. Interestingly,

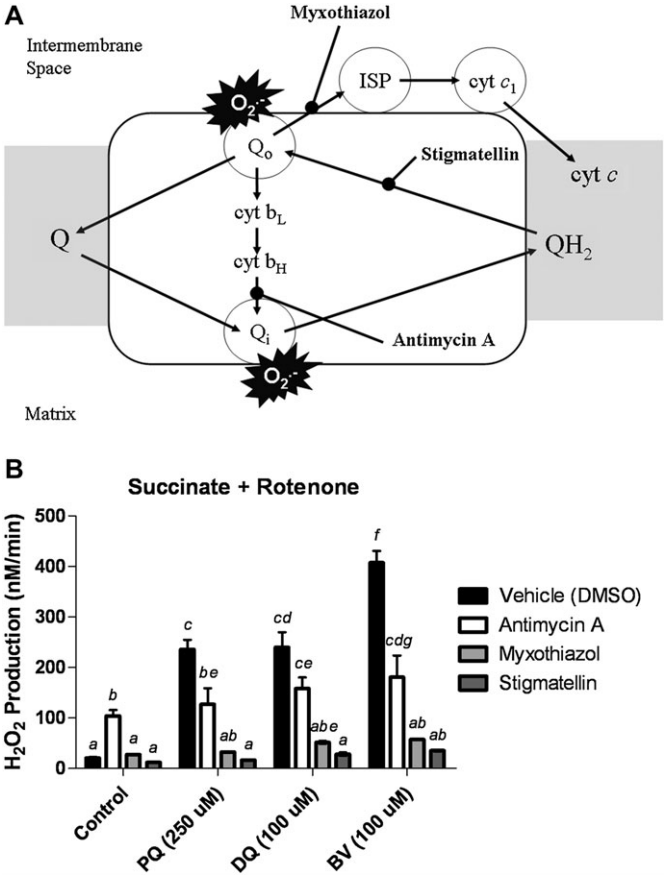

FIG. 4. Role of complex III in bipyridyl herbicide–induced H_2O_2 production in isolated mitochondria. (A) Scheme of electron flow through complex III. The sites of inhibition by antimycin A, myxothiazol, and stigmatellin are indicated. Electron leak can occur at either of the ubiquinone-binding sites (Q_o or Q_i) to reduce molecular oxygen and form $O_2^{\cdot-}$. (B) Isolated rat brain mitochondria were supplemented with succinate in the presence of rotenone to direct electron flow through complex III. PQ, DQ, and BV were used to stimulate H_2O_2 production where indicated in the presence of vehicle/dimethyl sulfoxide (DMSO) or complex III inhibitors. Data expressed as mean + SEM, $n = 3$. Bars with different letters indicate statistical significance from one another ($p < 0.05$, one-way ANOVA with Tukey's posttest).

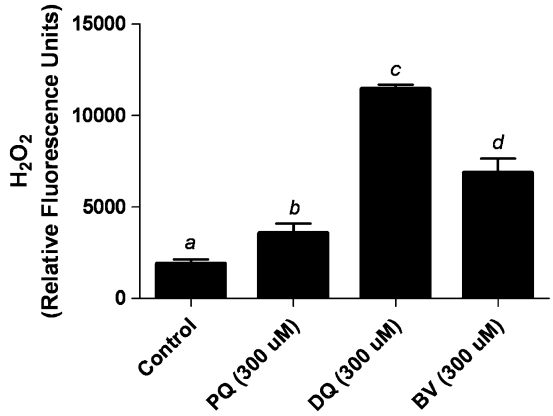

FIG. 5. H_2O_2 production by bipyridyl herbicides in primary midbrain cultures. Cultures were exposed to PQ, DQ, or BV for 8 h at 300μM. Data expressed as mean + SEM, $n = 3$. Bars with different letters indicate statistical significance from one another ($p < 0.05$, one-way ANOVA with Tukey's posttest).

rotenone-dependent inhibition of PQ-induced H_2O_2 production was only observed at concentrations of PQ $\geq$ 1mM, albeit to a lesser degree (35–40% inhibition) than observed with antimycin A (70%) (Fig. 3A). In the case of DQ, the addition of rotenone or antimycin A had similar attenuating effects (~50% inhibition) on H_2O_2 production at all concentrations of the herbicide (Fig. 3B). Finally, BV-induced H_2O_2 production was blocked to a greater extent by rotenone (~85%) than antimycin A (~60%) (Fig. 3C). These data suggest that multiple sites within the respiratory chain, particularly complexes I and III, differentially contribute to H_2O_2 production by bipyridyl herbicides. Notably, the effect of complex I inhibition on rates of H_2O_2 production by PQ appears to be concentration dependent.

To further explore the potential role of complex III in ROS production by bipyridyl herbicides, mitochondria were supplemented with succinate to feed electrons at the level of complex II in combination with rotenone to minimize the contributions of complex I–dependent ROS production via RET. Antimycin A, myxothiazol, and stigmatellin were utilized as specific inhibitors at different sites within complex III (Fig. 4A). Antimycin A and myxothiazol inhibit electron flow downstream of ubiquinone-binding domains (Q_o and/or Q_i) and thus allowing for electron leak to occur at these sites. Therefore, antimycin A and myxothiazol generated significant levels of H_2O_2 in control mitochondria. In contrast, stigmatellin prevents binding of ubiquinol to the Q_o site limiting the amount of leak and ROS produced from the complex (Fig. 4B). In this system, PQ-, DQ-, or BV-induced H_2O_2 production was substantially blocked by all three inhibitors of complex III (Fig. 4B).

Bipyridyl Herbicide–Induced H_2O_2 Production in Primary Midbrain Cultures

To further explore the mechanisms involved in bipyridyl herbicide–induced ROS production, primary midbrain cultures from embryonic rats were utilized. These cultures provide a more physiologically relevant whole-cell model to investigate the neurodegenerative mechanisms of PD. H_2O_2 production was measured after 8 h of incubation with bipyridyl herbicides in the presence of respiratory chain inhibitors, while MTT reduction was used to assess loss of cell viability due to mitochondrial dysfunction. No significant changes in cell viability were observed in response to PQ (300μM), DQ (100μM), or BV (100μM) and respiratory chain inhibitors (data not shown). Consistent with their effectiveness in isolated brain mitochondria, DQ and BV generated H_2O_2 at significantly greater rates than PQ (Fig. 5). By contrast, DQ was the most potent generator of H_2O_2, followed by BV; PQ was the least effective.

In this model, H_2O_2 production by PQ, DQ, or BV was completely inhibited only in the presence of antimycin A or

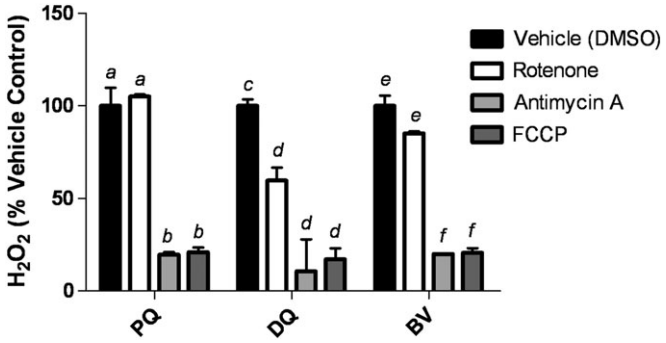

FIG. 6. Role of respiratory chain complexes in bipyridyl herbicide–induced H_2O_2 production in primary midbrain cultures. Cultures were exposed to PQ, DQ, or BV at 300μM for 8 h in the presence of respiratory chain inhibitors. Data expressed as percent control of vehicle for each bipyridyl herbicide group, mean + SEM, $n = 3$. Bars with different letters indicate statistical significance from one another ($p < 0.05$, one-way ANOVA with Tukey's posttest).

FCCP (Fig. 6). Similarly, myxothiazol and stigmatellin also significantly inhibited H_2O_2 production by all three bipyridyl herbicides (data not shown). However, the effect of complex I inhibition via rotenone varied with each redox cycling agent. Whereas rotenone did not alter PQ-induced H_2O_2 production in midbrain cultures, it significantly decreased DQ-induced H_2O_2 production (~40%). Furthermore, BV-induced H_2O_2 production was slightly attenuated (~15%) by rotenone, although this change was not statistically significant. Other respiratory chain inhibitors such as TTFA (complex II), KCN (complex IV), or oligomycin (ATP synthase) had no significant effect on H_2O_2 generated by PQ, DQ, or BV (data not shown).

DISCUSSION

This study illustrates three key points regarding mechanisms of mitochondrial ROS production by PQ and related herbicides: (1) The degree to which bipyridyl herbicides generate mitochondrial ROS is dependent upon their ability to redox cycle; (2) differential sensitivity to inhibition of respiratory chain components indicates that multiple sites, notably complexes I and III, contribute to ROS production by PQ, DQ, or BV; and (3) complex III serves as a major mediator of bipyridyl herbicide–induced ROS production.

While associations have been made between exposure to pesticides, such as PQ and increased of risk of neurodegenerative disease, the cellular/molecular mechanisms underlying the toxicity of such environmental agents in the brain remain undefined. The involvement of various enzymes localized to the cytosol, microsome, and plasma membrane has been implicated in redox cycling and ROS production by PQ (Bonneh-Barkay *et al*., 2005a; Clejan and Cederbaum, 1989; Day *et al*., 1999; Dicker and Cederbaum, 1991; Gray *et al*., 2007; Liochev *et al*., 1994). However, the role of mitochondrial systems in PQ-induced ROS production is relatively unexplored. We have shown that mitochondria are a major subcellular source of PQ-induced H_2O_2 production (Castello *et al*., 2007). More specifically, complexes of the respiratory chain have been proposed to participate in PQ redox cycling (Castello *et al*., 2007; Cocheme and Murphy, 2008; Fukushima *et al*., 1993; Gray *et al*., 2007). Here, we demonstrate that multiple respiratory chain complexes contribute to PQ-induced mitochondrial ROS production and compare its effect with structurally related bipyridyl herbicides (DQ and BV).

The bipyridyl herbicides tested in these studies are capable of generating ROS via redox cycling by a process that involves electron acceptance from an appropriate source with subsequent reduction of oxygen to produce $O_2^{\cdot-}$ and concomitant regeneration of the parent compound. A compound's standard reduction potential (E^0) is a measure of electron affinity and can be indicative of the ability to redox cycle. A more positive E^0 value indicates a greater tendency to accept an electron. Of the three bipyridyl herbicides used in these studies, DQ ($E^0 = -0.33$ V) is most likely to accept to an electron, followed by BV ($E^0 = -0.35$ V) and PQ ($E^0 = -0.45$ V). Therefore, DQ and BV were expected to generate greater amounts of ROS compared to PQ at equivalent concentrations in a given system. This is consistent with the data presented here and other neurotoxicity studies utilizing microglial (Bonneh-Barkay *et al*., 2005b) or primary mesencephalic cultures (Bonneh-Barkay *et al*., 2005a). However, the rank order potency of H_2O_2 generation by the herbicides DQ and BV differed in our models. Despite having a slightly less positive E^0 value than DQ, BV generated H_2O_2 to a greater extent (two to three times more potent based on EC_{50} values) in isolated mitochondria compared to primary midbrain cultures in which DQ was the most effective. In all cases, PQ was the least effective of the bipyridyl herbicides in generating H_2O_2. These data demonstrate that mitochondrial ROS production by bipyridyl herbicides was dependent on their ability to redox cycle. Given the discrepancy between DQ and BV, which possess similar E^0 values, other factors in addition to redox cycling ability, such as uptake or molecular interactions, may also contribute to rates of ROS production.

The potential involvement of the mitochondrial respiratory chain in PQ-induced ROS production was first identified in yeast, bacteria, and higher organisms (Aoki *et al*., 2002; Blaszczynski *et al*., 1985; Castello *et al*., 2007). These observations have been supported in mitochondria isolated from various mammalian organs, although the contribution of sites within the respiratory chain remains controversial. Assessing the role of the individual complexes in bipyridyl herbicide–induced ROS production in isolated rat brain mitochondria revealed that complex II, complex IV, or ATP synthase has no significant effect on H_2O_2 production by PQ, DQ, or BV, whereas complexes I and III contributed to H_2O_2 production at varying degrees. In mitochondria supplemented with malate + glutamate, PQ-induced H_2O_2 production was blocked by antimycin A to a greater degree than rotenone.

Interestingly, the effects of rotenone on PQ-induced H_2O_2 production were concentration dependent, occurring only at high ($\geq$ 1mM) concentrations. In the case of DQ, inhibition by either rotenone or antimycin A showed similar effects in decreasing rates of mitochondrial H_2O_2 production. Lastly, BV-induced H_2O_2 production was attenuated by rotenone to a greater degree than antimycin A. These data suggest that complexes I and III show differential contributions to mitochondrial H_2O_2 production by bipyridyl herbicides, which may be associated with differences in the standard reduction potentials of the redox cycling compounds. The standard reduction potential for complex I ranges from -0.32 to -0.03 V. Therefore, the likelihood of direct interaction with complex I by DQ or BV is greater than that by PQ. In agreement, both DQ- and BV-induced H_2O_2 production is inhibited to a greater degree by complex I inhibition via rotenone than PQ in isolated mitochondria and primary midbrain cultures.

While these data show variable contributions of the respiratory chain complexes to bipyridyl herbicide–induced ROS production in brain mitochondria, further investigation revealed that complex III serves a more significant role than complex I. In brain mitochondria supplemented with succinate and rotenone to limit the contributions of RET through complex I, blocking electron flow through complex III significantly reduced H_2O_2 production by PQ, DQ, or BV. Furthermore, the results from primary midbrain cultures also support a role for complex III–dependent ROS production in the presence of bipyridyl herbicides. Redox cycling agents and respiratory chain inhibitors did not alter cell viability in primary midbrain cultures assessed by the MTT assay at a time point where significant changes in H_2O_2 production were observed. This suggests that H_2O_2 production by redox cycling agents was not due to mitochondrial dysfunction or loss of cell viability. Since primary midbrain cultures are a physiologically more relevant preparation than isolated mitochondria, the latter in which conditions supporting a high electrochemical potential may favor electron leak and RET, it can be concluded that complex III serves as the major mediator of ROS production induced by bipyridyl herbicides, such as PQ.

The role for complex III in PQ-induced ROS production is in contrast with studies in which complex I has been suggested as the major site of ROS production (Cocheme and Murphy, 2008; Fukushima *et al*., 1993). However, the latter studies were conducted in mitochondria from nonneuronal tissues and may not accurately reflect the mechanisms underlying the neurotoxicity of PQ. Evidence shows that different mitochondrial populations, even those from the same tissue, may vary in terms of metabolic properties and pharmacological response (Lai and Clark, 1976; Michelakis *et al*., 2002). Therefore, the differential contributions of complex I versus III may be attributable to the unique properties of brain mitochondria that differ from that of heart or liver. Additionally, it has been shown that ROS production in isolated brain mitochondria preparations is not proportional to levels of complex I, suggesting that contributions of complex I to ROS production in brain mitochondria may be overestimated (Chinta *et al*., 2009). Finally, recent studies have shown that PQ neurotoxicity is distinct from that of complex I inhibitors (Richardson *et al*., 2005), and dopaminergic cell death induced by PQ treatment is independent of complex I inhibition (Choi *et al*., 2008). Our studies using isolated mitochondria demonstrate some dependence of PQ-induced ROS production on complex I, although this only occurs when PQ $\geq$ 1mM. PQ is capable of inhibiting complex I, which may explain the observed decreased H_2O_2 production in the presence of rotenone. However, in comparison to rotenone or 1-methyl-4-phenyl-pyridinium, which inhibit complex I at nanomolar or micromolar concentrations, respectively, PQ shows only weak inhibitory effects at millimolar concentrations (Richardson *et al*., 2005). Furthermore, limited accumulations of PQ in actively respiring mitochondria and the inability of rotenone to inhibit H_2O_2 production at micromolar PQ concentrations suggests that complex I may play only a minor role in PQ neurotoxicity.

Although the data from this study might suggest that PQ, DQ, and BV interact directly with complex III in generating H_2O_2, the electron carriers of complex III possess standard reduction potential values ranging from 0 to $+0.25$ V compared to those of PQ (-0.45 V), DQ (-0.33 V), and BV (-0.33 V), which makes it unlikely that these redox cycling compounds would be directly reduced by complex III. If these compounds were in fact being reduced by complex III, upstream block of electron flow at complex I via rotenone would exhibit similar attenuation on H_2O_2 production as complex III inhibition via antimycin A. We did not observe this, which suggests that bipyridyl herbicide–induced complex III–dependent ROS production arises via indirect alternate mechanisms. Since H_2O_2 generation by complex III has been observed following a variety of *in vitro* stress, including Ca^{2+} overload and cytochrome *c* depletion (Gyulkhandanyan and Pennefather, 2004), it is plausible that PQ and other bipyridyl herbicides cause similar effects leading to increased complex III–dependent ROS generation. In addition to its major function as an electron transporter, complex III has been recently identified as a mediator of both apoptotic and hypoxic signaling pathways (Childs *et al*., 2008; Klimova and Chandel, 2008). It therefore seems plausible that complex III may affect other mitochondrial pathways.

The role of complex III in environmental toxicant–induced PD is at odds with the known association between complex I deficiency in the etiology of PD (Schapira *et al*., 1990). This may be because even mild inhibition of complex I is a potent source and target of ROS in comparison to other respiratory chain complexes (Zhang *et al*., 1990). However, a potential role for complex III in PD has already been established with the pesticides, maneb and dieldrin (reviewed in Drechsel and Patel, 2008). Therefore, we propose that complex III–dependent ROS induced by PQ and other redox cycling bipyridyl herbicides under physiologically relevant conditions induces multiple

deleterious consequences leading to a further increase in ROS, including inhibition and inactivation of complex I. Ultimately, the dopaminergic neurons of the substantia nigra pars compacta that are uniquely sensitive to complex I inhibition (Betarbet *et al*., 2000) are damaged.

Exposure to PQ, DQ, or mixed preparations produces extensive damage to the central nervous system and is linked with increased risk for developing PD (Grant *et al*., 1980; Hertzman *et al*., 1990; Hughes, 1988; Liou *et al*., 1997; Price *et al*., 1995; Sechi *et al*., 1992). Although *in vitro* concentrations used in this study were in the high micromolar range, the results have important implications in predicting neurodegeneration following environmental or accidental exposure to redox cycling herbicides. Our concentration-response studies showed that 10–30μM of PQ, or as little as 3μM of DQ and BV, was sufficient to produce significant levels of H_2O_2 (Fig. 2). Considering that PQ concentrations reaching up to ~10μM were recently observed in mouse brain following repeated exposures (Prasad *et al*., 2009) and that the brain relies almost exclusively on oxidative metabolism, the mitochondrial ROS production and neurodegeneration by bipyridyl herbicides shown here may be particularly relevant following prolonged exposures.

In summary, these data provide evidence that generation of mitochondrial ROS by bipyridyl herbicides, including PQ, is dependent upon their redox cycling ability. Whereas multiple sites of the respiratory chain serve as sites of ROS production, complex III is the major mediator. Complex III–dependent mechanism of bipyridyl herbicide–induced ROS production is therefore a novel finding, which warrants further investigation. In addition, we demonstrate that mitochondria are an important source of ROS production that should be considered when examining the toxicity of PQ and other redox cycling agents. Overall, these studies identify potential neurotoxic mechanisms of mitochondrial ROS production by environmental toxins leading to oxidative stress and development of PD.

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