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Toxicity of Redox Cycling Pesticides in Primary Mesencephalic Cultures

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ABSTRACT

A loss of nigrostriatal dopaminergic neurons is the primary neurodegenerative feature of Parkinson's disease. Paraquat, a known redox cycling herbicide, has recently been shown to kill selectively nigrostriatal dopaminergic cells in the mouse model. The purpose of this study was to test the ability of paraquat and other redox cycling pesticides to damage dopaminergic neurons in primary mesencephalic cultures. Addition of paraquat, diquat, or benzyl viologen to mesencephalic cultures induced morphological changes (*e.g.,* dystrophic neuronal processes) consistent with dopaminergic cell injury. The three pesticides also caused cell death as assessed by a reduction of the number of tyrosine hydroxylase-immunoreactive neurons and a dose-dependent decrease in [^{3}H]dopamine uptake. Quite interestingly, diquat and benzyl viologen were significantly more toxic than paraquat, probably reflecting their more pronounced ability to trigger redox cycling reactions. The data support a role of redox cycling as a mechanism of dopaminergic cell degeneration and suggest that the property of redox cycling should be taken into consideration when evaluating putative environmental risk factors for Parkinson's disease. *Antioxid. Redox Signal.* 7, 649–653.

INTRODUCTION

NIGROSTRIATAL DOPAMINERGIC NEURONS are the primary targets of the neurodegenerative process of Parkinson's disease (PD). Although the precise mechanisms that underlie dopaminergic cell degeneration remain uncertain, epidemiological and experimental evidence suggests that exposure to neurotoxicants could contribute to the pathogenesis of PD and that oxidative damage may play an important role in neuronal demise (4, 5). We have recently characterized a model of PD-like pathology caused by treatment of mice with the herbicide paraquat. In this model, three weekly injections of paraquat resulted in a significant loss of dopaminergic neurons in the substantia nigra pars compacta (11). This neurodegenerative effect was selective because paraquat exposure did not decrease the number of GABAergic and cholinergic cells in the substantia nigra pars reticulata and hippocampus. Another intriguing feature of paraquat neurotoxicity is its interaction with the protein α-synuclein. α-Synuclein is a major component of the intraneuronal inclusions called Lewy bodies that are typical of PD (16). After exposure to paraquat, levels of α-synuclein are markedly enhanced in the mouse brain, and α-synuclein-immunoreactive deposits are observed within dopaminergic neurons (10).

The mechanisms by which paraquat causes nigrostriatal cell degeneration and α-synuclein aggregation are presently unknown. It has long been recognized, however, that paraquat is capable of undergoing a toxic process of redox cycling (2, 3). The one-electron reduction of paraquat forms a radical species, which can then react with molecular oxygen to form superoxide and to regenerate the parent molecule (Fig. 1). This redox cycling process could ultimately lead to oxidative stress and cytotoxicity because of a disproportionate consumption of oxygen and cellular reducing equivalents and the production of reactive oxygen species (ROS). Redox cycling and ROS formation could also explain, at least in part, the greater vulnerability of dopaminergic neurons to paraquat neurotoxicity because the interaction of ROS with dopamine may generate additional toxic products, such as 6-hydroxydopamine (6, 12).

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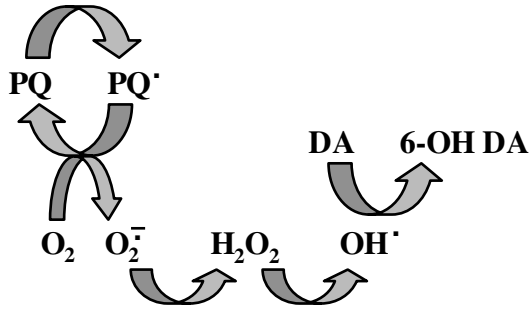

FIG. 1. Redox cycling as a mechanism of paraquat toxicity. The one-electron reduction of compounds like paraquat (PQ) generates ROS. Dopaminergic cell injury could be enhanced by the interaction of oxygen radicals with dopamine (DA). H_2O_2, hydrogen peroxide; $O_2^{\cdot -}$, superoxide; $OH^{\cdot}$, hydroxyl radical.

In this study, the role of redox cycling in toxicant-induced dopaminergic cell death was assessed in primary mesencephalic cultures. Morphological observations and biochemical measurements were used to document the damaging effects of paraquat toward dopaminergic neurons, and these effects were compared with those of diquat and benzyl viologen, two other redox cycling bipyridyl pesticides. The data show a correlation between the efficiency of redox cycling and the severity of dopaminergic cell injury caused by bipyridyl derivatives.

MATERIALS AND METHODS

Materials

Paraquat, diquat, benzyl viologen, and mazindol were purchased from Sigma (St. Louis, MO, U.S.A.). Cell culture media and serum were obtained from Hyclone (Logan, UT, U.S.A.), and a monoclonal mouse antibody against tyrosine hydroxylase (TH) was from Chemicon International (Temecula, CA, U.S.A.). Vectastain ABC kit was purchased from Vector Laboratories (Burlingame, CA, U.S.A.). [^{3}H]Dopamine and Optiphase SuperMix Cocktail were obtained from PerkinElmer–New England Nuclear (Boston, MA, U.S.A.).

Primary rat ventral mesencephalic cultures

Cultures were prepared from the ventral mesencephalon of Sprague–Dawley rats of 14-days gestation (14). Experimental protocols were in accordance with the NIH guidelines for animal use and were approved by the Institutional Animal Care and Use Committee. Dissected tissues were incubated in calcium-free Hanks' balanced salt solution (HBSS) containing trypsin (0.1%) and DNase (0.5 mg/ml) for 6 min. The trypsinization process was terminated by addition of HBSS containing soybean trypsin inhibitor (0.1 mg/ml) and DNase (0.5 mg/ml). Cells were dissociated by gentle trituration using a fire-polished Pasteur pipette and were then centrifuged at 100 *g* for 10 min. The supernatant was discarded, and the cells were resuspended in Dulbecco's modified Eagle medium (DMEM):F12 medium supplemented with 10% fetal bovine serum (FBS). Cultures were plated in 96-well plates coated with poly-D-lysine (100 μg/ml) at a density of 100,000 cells/well in DMEM:F12 containing 10% FBS, penicillin, and streptomycin. After 72 h, the medium was changed to DMEM-low glucose supplemented with 10% FBS.

[^{3}H]Dopamine uptake

Cultures were rinsed with DMEM:F12 containing 0.2 mg/ml ascorbic acid and incubated for 30 min at 37°C with the same buffer containing 1 μCi/ml [^{3}H]dopamine. After rinsing with phosphate-buffered saline (PBS; pH 7.4), the radioactivity was extracted with Optiphase SuperMix Cocktail and measured using a liquid scintillation counter. Nonspecific binding was determined in cultures treated with the dopamine uptake inhibitor mazindol (10 μM).

Immunohistochemistry

Cultures were fixed with 4% formaldehyde and 4% sucrose in PBS for 20 min. After fixation, they were washed with PBS and incubated with permeabilization buffer containing 0.3% Triton and 1% bovine serum albumin in PBS for 30 min at room temperature. Cultures were washed and incubated overnight at 4°C with monoclonal anti-TH. Vectastain ABC and 3,3′-diaminobenzidine peroxidase kits were used to complete the immunostaining.

Statistical analysis

Differences among means were analyzed using a one-way ANOVA. Fisher's *post-hoc* analysis was used when differences were observed in the ANOVA testing ($p < 0.05$).

RESULTS

Paraquat-induced damage to dopaminergic neurons

In agreement with previously reported findings (8), ~5% of neurons in our primary mesencephalic cultures were dopaminergic and showed TH-immunoreactive cell bodies and a network of TH-positive fibers (Fig. 2A). After paraquat treatment, the number of TH-immunoreactive neurons declined, and damaged dopaminergic cells showed typical morphological changes, such as spherical cell bodies and progressively dysmorphic neuronal processes (Fig. 2B). For quantification of dopaminergic cell injury, cultures were exposed to vehicle or different concentrations of paraquat (10–70 μM) and, after 24 h, the uptake of [^{3}H]dopamine was measured (Fig. 3). Paraquat caused a dose-dependent reduction of dopamine uptake, with a 50% loss at a concentration of ~40 μM.

Neurotoxicity of diquat and benzyl viologen

Diquat and benzyl viologen are structurally related to paraquat (Fig. 4) and, like paraquat, are capable of generating free radicals through a redox cycling mechanism (13). We

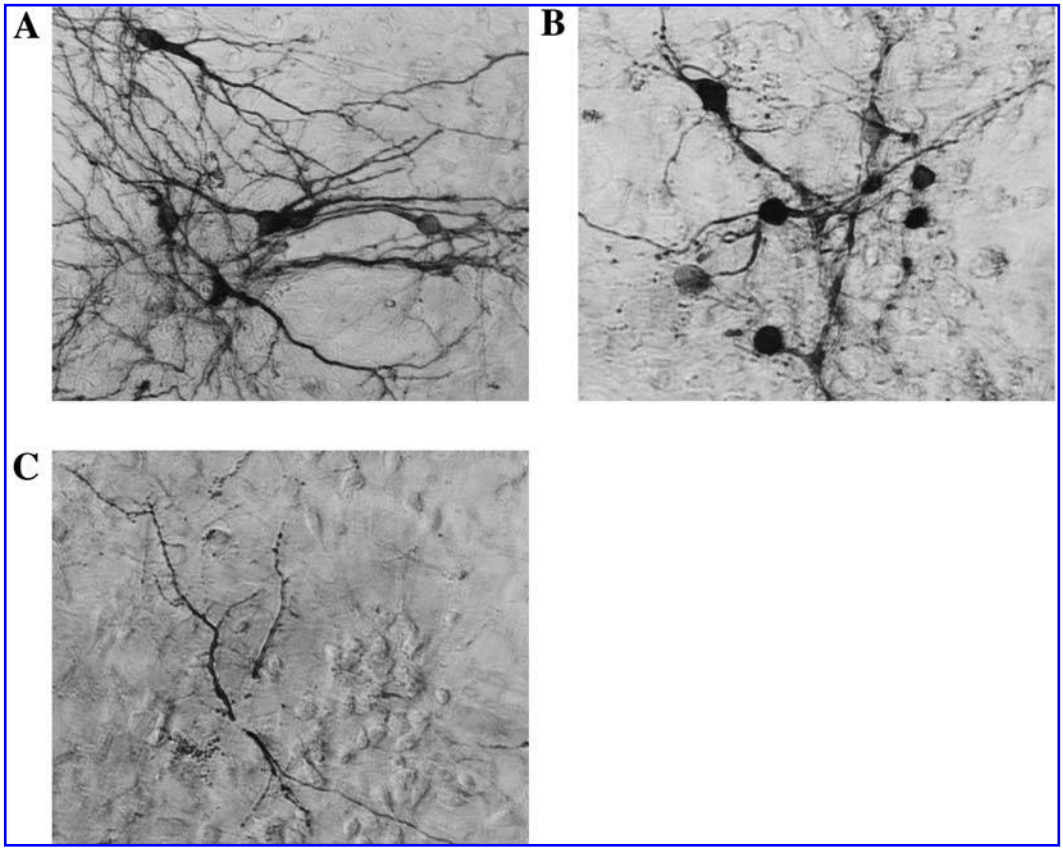

FIG. 2. Paraquat and diquat neurotoxicity in rat mesencephalic cultures. Mesencephalic cultures were prepared as described in Materials and Methods. On day 6 *in vitro,* vehicle (**A**), 30 *μM* paraquat (**B**), or 30 *μM* diquat (**C**) was added and, after 24 h, dopaminergic cells were visualized by immunostaining with an antibody against TH.

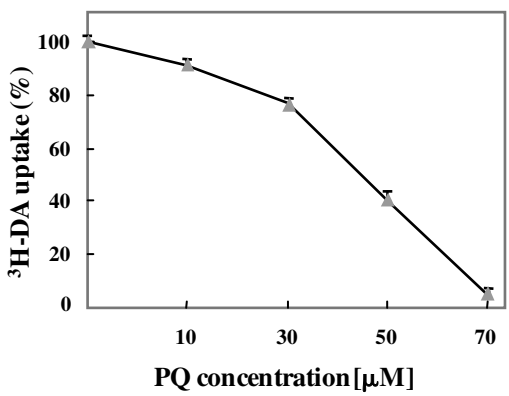

FIG. 3. Paraquat-induced reduction of [^{3}H]dopamine uptake. On day 6 *in vitro,* mesencephalic cultures were exposed to vehicle or different concentrations of paraquat (PQ). After 24 h, dopamine (DA) uptake was measured as described in Materials and Methods. Data are means ± SEM and are expressed as % of control values measured in vehicle-treated cultures.

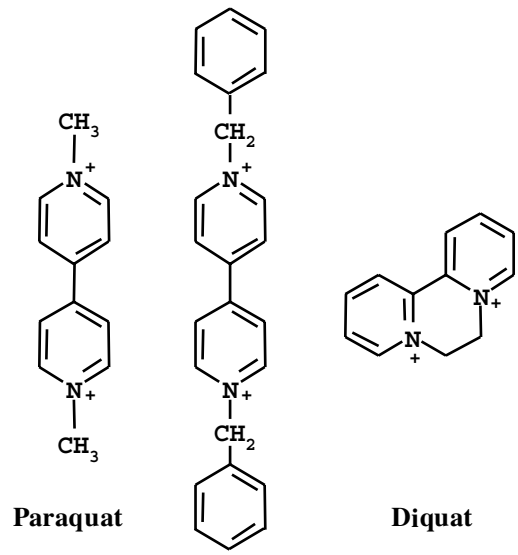

FIG. 4. Chemical structures of paraquat, benzyl viologen, and diquat.

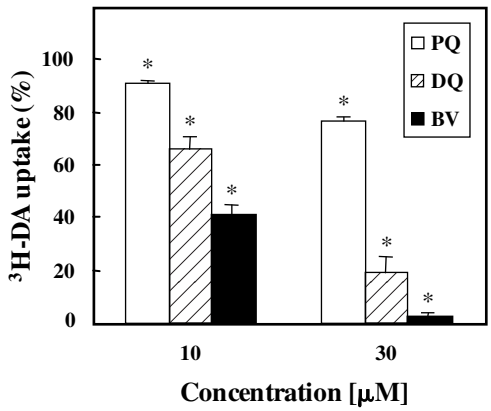

FIG. 5. Comparison of the effects of paraquat, diquat, and benzyl viologen on [^{3}H]dopamine uptake. On day 6 *in vitro,* mesencephalic cultures were treated with vehicle, paraquat (PQ; empty bars), diquat (DQ; striped bars), or benzyl viologen (BV; black bars). After 24 h, dopamine (DA) uptake was measured as an indicator of neurotoxicity. Data are means ± SEM and are expressed as % of control values measured in vehicle-treated cultures. $^*p < 0.001$ compared with the other treatment groups at each concentration.

therefore compared the effects of these bipyridyl derivatives on the survival of dopaminergic neurons in culture. Similar to paraquat, diquat and benzyl viologen induced marked changes in the morphology and number of TH-immunoreactive cells. At equal concentrations of the three bipyridyl compounds, toxic changes caused by diquat and benzyl viologen were more pronounced than those induced by paraquat. In cultures treated for 24 h with 30 μM diquat (Fig. 2C) or benzyl viologen (data not shown), only a few dopaminergic neurons were observed. These cultures were mostly characterized by the remains of dystrophic fragmented processes. In contrast, changes in the number and morphology of TH-immunoreactive neurons were significantly less severe in cultures exposed to 30 μM paraquat (Fig. 2B).

Differences in dopaminergic cell damage caused by paraquat, diquat, and benzyl viologen were confirmed by measurements of dopamine uptake. When chemicals were added at a concentration of 30 μM, dopamine uptake was reduced by ~25% in the presence of paraquat. In contrast, a loss of >80% was measured in cultures treated with either diquat or benzyl viologen (Fig. 5). The ranking of neurotoxicity as assessed by the reduction of dopamine uptake was paraquat < diquat < benzyl viologen (Fig. 5).

DISCUSSION

Exposure to environmental agents has been suggested to play a role in the pathogenesis of PD (5). Evidence in support of this hypothesis includes the discovery of the parkinsonism-inducing toxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and, more recently, the characterization of the neurotoxic effects of the pesticides rotenone and paraquat (1, 9, 11). MPTP, rotenone, and paraquat selectively injure nigrostriatal dopaminergic cells, thus affecting the same neuronal population that is targeted by the degenerative process of PD. The chemical characteristics and mechanisms of action that account for the ability of PD-related toxicants to target the nigrostriatal system remain subjects of speculation. A long-standing hypothesis concerning mechanisms of neurodegeneration in PD is that dopaminergic cells may be particularly vulnerable to oxidative processes (6). Thus, the property of neurotoxicants to generate ROS could play an important role in their selective action toward nigrostriatal neurons.

Toxicant-induced ROS formation could conceivably occur through different mechanisms. For example, the nigrostriatal damage that follows exposure of rats to rotenone has been proposed to arise from a partial blockage of electron flow at the level of mitochondrial complex I and the consequent stimulation of superoxide production (1). Another mechanism by which neurotoxicants could induce an enhanced formation of ROS is through their redox cycling with molecular oxygen (Fig. 1). This mechanism has been suggested to explain, at least in part, the ability of paraquat to kill dopaminergic neurons when administered to mice (11).

Results of this study provide the first *in vitro* evidence of a relationship between redox cycling and dopaminergic cell injury. Paraquat, diquat, and benzyl viologen, three known redox cycling compounds, were all found to kill dopaminergic neurons in primary mesencephalic cultures. Perhaps more importantly, the neurotoxic effects of paraquat, diquat, and benzyl viologen seemed to be correlated with their relative abilities to redox cycle. Redox cycling compounds must undergo a one-electron reduction catalyzed by cellular reductases. The rate of this reaction, which affects the overall rate of the redox cycling process, is dependent on the electrochemical properties and, in particular, the one-electron reduction potentials of individual compounds (3, 7). A more negative potential is characteristic of agents that accept electrons less readily and are therefore poorer redox cyclers. The reduction potential of paraquat (−0.44 V versus a normal hydrogen electrode) is more negative than the one of diquat (−0.35 V), predicting that paraquat would be a less potent redox cycling toxicant (7). Our findings in mesencephalic cultures are consistent with this prediction. They show that paraquat is significantly less neurotoxic than diquat and suggest that ROS production through a redox cycling mechanism underlies the damaging effects of these compounds on dopaminergic neurons.

In a previous study in which microsomal preparations were used to assess superoxide generation as an indicator of redox cycling, the rate of ROS formation induced by benzyl viologen was intermediate between that of paraquat and diquat (*i.e.,* paraquat < benzyl viologen < diquat) (13). Our current results indicate, however, that benzyl viologen was the most toxic against cultured dopaminergic neurons. This apparent discrepancy is likely to stem from differences in the bioavailability of the three bipyridyl derivatives in cellular as compared with subcellular preparations. In particular, the benzyl substituents confer relative lipophilicity to benzyl viologen as compared with diquat and paraquat (Fig. 4). In cellular systems, this would allow for its easier permeabilization across cell membranes and greater availability for redox cycling.

The identification of neurotoxicants that affect dopaminergic neurons bears critical implications for PD because environmental agents, together with genetic and age-related factors, are likely to contribute to the disease process (5). The results of this study support a role of redox cycling as a mechanism of dopaminergic cell injury. Besides bipyridyl pesticides like paraquat and diquat, a variety of naturally occurring compounds, such as quinones, possess the property of redox cycling (7, 15). This property should therefore be taken into consideration when evaluating putative environmental risk factors for PD in the experimental and epidemiological settings.

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ABBREVIATIONS

DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; HBSS, Hanks' balanced salt solution; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate-buffered saline; PD, Parkinson's disease; ROS, reactive oxygen species; TH, tyrosine hydroxylase.

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