ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 5 & 6, 2005

Forum Original Research Communication

The Role of Microglia in Paraquat-Induced Dopaminergic Neurotoxicity

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ABSTRACT

The herbicide paraquat (PQ) has been implicated as a potential risk factor for the development of Parkinson's disease. In this study, PQ (0.5–1 *μM*) was shown to be selectively toxic to dopaminergic (DA) neurons through the activation of microglial NADPH oxidase and the generation of superoxide. Neuron-glia cultures exposed to PQ exhibited a decrease in DA uptake and a decline in the number of tyrosine hydroxylase-immunoreactive cells. The selectivity of PQ for DA neurons was confirmed when PQ failed to alter γ-aminobutyric acid uptake in neuron-glia cultures. Microglia-depleted cultures exposed to 1 *μM* PQ failed to demonstrate a reduction in DA uptake, identifying microglia as the critical cell type mediating PQ neurotoxicity. Neuron-glia cultures treated with PQ failed to generate tumor necrosis factor-α and nitric oxide. However, microglia-enriched cultures exposed to PQ produced extracellular superoxide, supporting the notion that microglia are a source of PQ-derived oxidative stress. Neuron-glia cultures from NADPH oxidase-deficient (PHOX$^{-/-}$) mice, which lack the functional catalytic subunit of NADPH oxidase and are unable to produce the respiratory burst, failed to show neurotoxicity in response to PQ, in contrast to PHOX$^{+/+}$ mice. Here we report a novel mechanism of PQ-induced oxidative stress, where at lower doses, the indirect insult generated from microglial NADPH oxidase is the essential factor mediating DA neurotoxicity. *Antioxid. Redox Signal.* 7, 654–661.

INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by the progressive and selective loss of dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta (28), where >90% of PD cases are sporadic with unknown etiology (15). Epidemiological studies indicate that rural residence, farming, well water drinking, and exposure to agricultural chemicals are associated with an increased risk of developing PD (18, 31, 35, 36). In addition, exposure to several classes of environmental toxins, such as paraquat (PQ; 1,1′-dimethyl-4,4′-bypyridinium), maneb, rotenone, and dieldrin, has resulted in DA neurotoxicity in animal models (20). Across this list of environmental neurotoxins, the common theme governing the selective DA neurotoxicity is the generation of oxidative stress. The following study sought to elucidate the source and the mechanism of PQ-induced oxidative stress at low concentrations (up to 1 *μM*) and the consequent DA neurotoxicity.

Initially, the herbicide PQ was speculated to be toxic to DA neurons because of its structural similarity to 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the well known

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parkinsonism-inducing agent discovered in synthetic heroin (16). Indeed, epidemiological studies have shown a strong correlation between the incidence of PD and exposure to PQ (18, 34). Studies using animal models have also supported the role of PQ and DA cell death, where intranigral injection of PQ induced dopamine (DA) depletion in the striatum, loss of DA neurons, glial reaction in the SN, and behavior abnormality (17). The effect of systemically administered PQ on the nigrostriatal system has been less consistent. Some studies have reported that PQ failed to damage DA neurons in SN and failed to deplete DA in striatum (29, 45, 46). However, more studies have indicated that systemic PQ exposure *in vivo* results in the selective degeneration of DA neurons in SN, DA depletion in striatum, and behavior abnormality (2, 6, 25, 26, 38, 39). Animals exposed to PQ combined with other pesticides also show selective DA neurotoxicity in the SN and behavioral deficits (1, 43). The abilities of PQ to induce oxidative stress in the brain (41, 47), to be taken up by DA neurons through the DA transporter (37, 38), and to upregulate α-synuclein expression and facilitate its fibrillation (25, 44) may contribute to its DA neurotoxicity. However, the detailed mechanism governing PQ-induced DA neurotoxicity remains unclear.

There is increasing evidence supporting the association of inflammation and PD (20, 27), where microglia have been identified as the pivotal cell type governing inflammation-mediated neurodegeneration (20). Microglia are the resident immune cells of the central nervous system and contribute to neurodegeneration through the release of a variety of neurotoxic factors, including cytokines, free radicals, inflammatory prostaglandins, and fatty acid metabolites (17, 20, 24, 26, 42). Previous studies have observed a glial response in the midbrain of animals exposed to PQ (17, 26). However, the specific role of inflammation in PQ-induced DA neurotoxicity has yet to be investigated.

In this study, selective DA neurotoxicity was observed in rat and mouse mesencephalic neuron-glia cultures exposed to low (up to 1 μM) concentrations of PQ. Here, we show that PQ-induced neurotoxicity was mediated through the presence of microglia and the activation of NADPH oxidase, which resulted in the production of the extracellular reactive oxygen species (ROS) responsible for DA neurotoxicity.

MATERIALS AND METHODS

Animals

All animals were treated in strict accordance with the National Institutes of Health (Bethesda, MD, U.S.A.) *Guide for Humane Care and Use of Laboratory Animals.* Timed pregnant (gestational day 14) adult female Fischer 344 rats were purchased from Charles River Laboratories (Raleigh, NC, U.S.A.). Eight-week-old male and female C57BL/6J ($PHOX^{+/+}$) and B6.129S6-*Cybb*^{tm1Din} ($PHOX^{-/-}$) mice were obtained from Jackson Laboratory (Bar Harbor, ME, U.S.A.) and maintained in a strict pathogen-free environment. Breeding of the mice was designed to achieve accurate timed pregnancy ± 0.5 days. The $PHOX^{-/-}$ mice lack the functional catalytic subunit of the NADPH oxidase complex, gp91. Because the $PHOX^{-/-}$ mutation is maintained in the C57BL background, the C57BL/6J ($PHOX^{+/+}$) mice were used as control animals.

Reagents

Lipopolysaccharide (LPS; strain O111:B4) was purchased from Calbiochem (San Diego, CA, U.S.A.). Cell culture ingredients were obtained from Life Technologies (Grand Island, NY, U.S.A.). [^{3}H]DA (28 Ci/mmol) and γ-[2,3-^{3}H]aminobutyric acid (GABA; 81 Ci/mmol) were purchased from NEN Life Science (Boston, MA, U.S.A.). The polyclonal antibody against tyrosine hydroxylase (TH) was a kind gift from Dr. John Reinhard of Glaxo Smith Kline (Research Triangle Park, NC, U.S.A.). The monoclonal antibody raised against the CR3 compliment receptor (OX42) was obtained from Pharmingen (San Diego, CA, U.S.A.). The biotinylated horse anti-mouse and goat anti-rabbit secondary antibodies were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). WST-1 was purchased from Dojindo Laboratories (Gaithersburg, MD, U.S.A.). Tumor necrosis factor-α (TNFα) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). All other reagents came from Sigma–Aldrich Chemical Co. (St. Louis, MO, U.S.A.).

Mesencephalic neuron-glia cultures

Rat and mouse ventral mesencephalic neuron-glia cultures were prepared using a previously described protocol (22). In brief, mesencephalic tissues were dissected from embryonic day 14 Fischer 344 rats and $PHOX^{+/+}$ or $PHOX^{-/-}$ mice. Cells were dissociated via gentle mechanical trituration in minimum essential medium, immediately seeded (5×10^5/well) in poly-D-lysine (20 μg/ml) precoated 24-well plates, and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were seeded in maintenance medium and treated with the treatment medium described previously (22). Three days after seeding, the cells were replenished with 0.5 ml of fresh maintenance medium. Cultures were treated 7 days after seeding.

Microglia-enriched cultures

Primary mixed microglia cultures were prepared from the whole brains of 1-day-old Fischer 344 rat pups, following the procedure described previously (23). Two weeks after seeding when the cells reached confluence, microglia were separated from astrocytes by shaking the flasks for 5 h at 150 rpm and replated at 1×10^5 in a 96-well plate. Enriched microglia were treated 24 h after seeding.

Mesencephalic microglia-depleted cultures

Microglia were depleted from mesencephalic neuron-glia cultures by the addition of 2 mM L-leucine methyl ester to the cultures 24 h after seeding. The cultures were exposed to L-leucine methyl ester for 72 h. Microglia-depleted cultures stained with OX-42 antibody showed <0.1% microglia.

Uptake assays (DA and GABA)

The DA and GABA uptake assays were performed as previously reported (8). In brief, after rinsing with warm Krebs–Ringer buffer, cultures were incubated for 20 min at 37°C with either 5 *μM* [^{3}H]GABA or 1 *μM* [^{3}H]DA. After incubation, cells were rinsed three times with 1 ml/well ice-cold Krebs–Ringer buffer, then lysed with 0.5 ml/well 1*M* NaOH, and mixed with 15 ml of scintillation fluid. Radioactivity was measured on a scintillation counter, where specific [^{3}H]GABA or [^{3}H]DA uptake was calculated by subtracting the mazindole or the NO-711 and β-alanine counts from the wells without the uptake inhibitors.

Immunostaining

Immunocytochemistry was performed using the conditions specified previously (21). DA neurons were identified by staining with the polyclonal antibody against TH. Microglia were stained with the monoclonal antibody raised against the CR3 receptor, OX-42. To quantify cell numbers, nine representative areas per well in the 24-well plate were counted under the microscope at 100× magnification by three individuals. The average of these scores was reported.

Superoxide assay

Extracellular superoxide ($O_2^{\bullet-}$) production from microglia was determined as reported previously (32) by measuring the superoxide dismutase (SOD)-inhibitable reduction of tetrazolium salt, WST-1 (19, 30, 40). In brief, immediately before treatment, enriched microglia were washed twice with Hanks' balanced salt solution (HBSS). To each well, 100 μl of HBSS with or without SOD (600 U/ml), 50 μl of vehicle, PQ, or LPS, and 50 μl of WST-1 (1 m*M*) in HBSS were added. The cultures were incubated for 30 min at 37°C, and the absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). The amount of SOD-inhibitable $O_2^{\bullet-}$ was calculated and expressed as percentage of vehicle-treated control cultures. Cell-free experiments determined that PQ treatment alone did not affect absorbance.

TNFα assay

The production of TNFα was measured with a commercial ELISA kit from R&D Systems, as described previously (21).

Nitrite assay

As an indicator of nitric oxide production, the amount of nitrite accumulated in culture supernatant was determined with a colorimetric assay using Griess reagent [1% sulfanilamide, 2.5% H_3PO_4, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride] (11), as previously reported (21).

Statistical analysis

The data are expressed as the means ± SEM, and statistical significance was assessed with an analysis of variance followed by Bonferroni's *t* test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

PQ (0.5–1 μM*) is toxic to DA neurons*

The DA neurotoxicity of PQ was measured in rat mesencephalic neuron-glia cultures. The ability of cells to take up [^{3}H]DA was measured to assess the effect of PQ on DA neuronal function. Exposure of neuron-glia cultures to PQ resulted in a significant ($p < 0.01$) and dose-dependent reduction in DA uptake (Fig. 1A), where 0.5 *μM* and 1 *μM* PQ caused 24% and 37% loss in DA neuronal function, respectively. To investigate the DA cell loss induced by PQ, neuron-glia cultures were exposed to PQ and stained with the TH antibody, and the number of TH-immunoreactive neurons was counted. PQ decreased the number of TH-immunoreactive neurons in a dose-dependent manner, with a 36% decrease in the cultures treated with 1 *μM* PQ ($p < 0.01$) (Fig. 1B). In addition to the reduction in cell numbers, TH-immunoreactive neurons in PQ-treated cultures showed a less extensive dendritic network compared with those in control cultures (Fig. 1C).

PQ neurotoxicity is selective for DA neurons

To examine the selectivity of PQ neurotoxicity, [^{3}H]GABA uptake assay was performed to assess the function of GABAergic neurons in the cultures exposed to PQ. Figure 2 demonstrates that only DA uptake was reduced significantly ($p < 0.01$) by the addition of PQ to neuron-glia cultures, whereas GABA uptake remained unaffected ($p > 0.05$). LPS was used as a positive control for microglia activation and selective DA neurotoxicity (9).

Microglia mediate PQ-induced DA neurotoxicity

To investigate the role of microglia in PQ-induced DA neurotoxicity, [^{3}H]DA uptake was compared in microglia-depleted cultures and neuron-glia cultures. Higher doses of PQ (4 *μM* and above) showed neurotoxicity in microglia-depleted cultures (data not shown), consistent with previous studies suggesting direct DA toxicity (39). However, whereas DA uptake was decreased in the neuron-glia culture exposed to 1 *μM* PQ, there was no toxicity in microglia-depleted cultures ($p < 0.05$) (Fig. 3), suggesting the critical role of microglia in low-dose PQ neurotoxocity. LPS was used as a positive control for microglia-mediated DA neurotoxicity (9).

PQ induces extracellular $O_2^{\bullet-}$ production by microglia

It has been well documented that the activation of microglia and the consequent production of neurotoxic factors, including TNFα, nitric oxide, and ROS, have been linked to DA neurotoxicity (33, 37, 38). In an effort to discern the mechanism through which microglia exposed to PQ exerted neurotoxicity, the supernatant from PQ-treated neuron-glia cultures was tested for the presence of classic proinflammatory factors. Analysis of supernatant collected at 3 h, 6 h, 12 h, 24 h, 4 days, and 7 days post PQ treatment in neuron-glia cultures revealed that no TNFα or nitrite (indicative of nitric oxide production) was produced (data not shown). However, PQ did induce a dose-dependent increase in extracellular

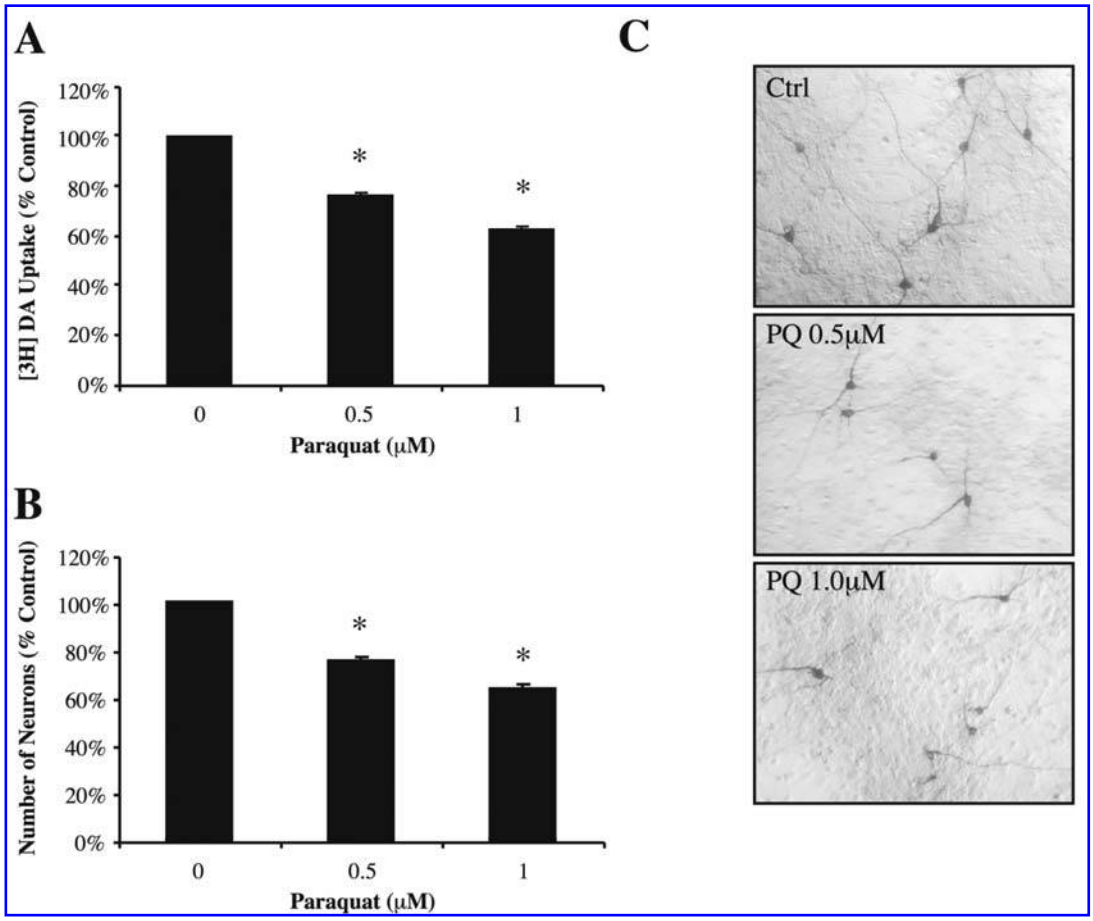

FIG. 1. PQ is toxic to DA neurons. Mesencephalic midbrain neuron-glia cultures were treated with either vehicle, 0.5 *μM* PQ, or 1 *μM* PQ. (**A**) DA neurotoxicity was measured at 6–7 days post treatment using the [^{3}H]DA uptake assay. (**B**) DA cell death was determined at 6–7 days post treatment with immunocytochemical staining by using the TH antibody and counting the number of TH-positive neurons present. The data are expressed as the percentage of the control cultures and are the means ± SEM from three independent experiments. An asterisk indicates a significant difference ($p < 0.01$) compared with control. (**C**) Cell morphology of TH-immunoreactive neurons in the neuron–glia cultures. The images shown are representative of three independent experiments.

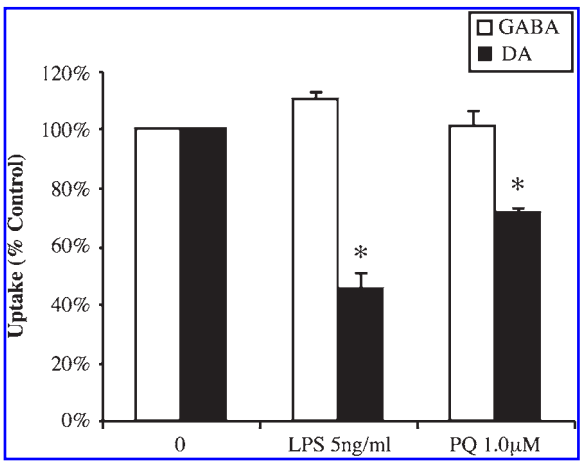

FIG. 2. PQ neurotoxicity is selective for DA neurons. Midbrain neuron-glia cultures were treated with either vehicle, 5 ng/ml LPS, or 1 *μM* PQ. LPS was used as a positive control for selective DA neurotoxicity. (**A**) DA neurotoxicity and GABA neurotoxicity were measured at 6–7 days post treatment by using the [^{3}H]DA uptake and [^{3}H]GABA uptake assays, respectively. The data are expressed as the percentage of the control cultures and are the means ± SEM from three independent experiments. An asterisk indicates a significant difference ($p < 0.01$) compared with control.

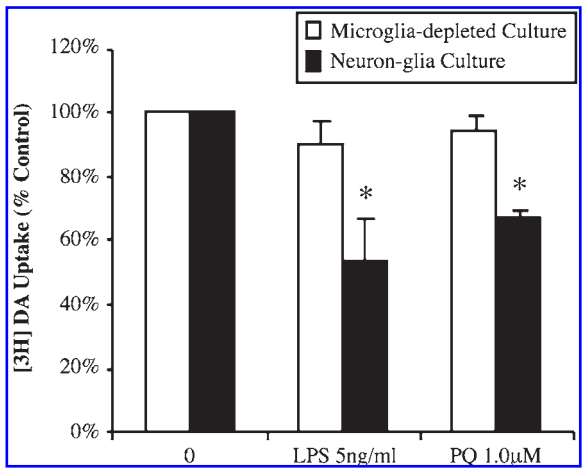

FIG. 3. Microglia mediate DA neurotoxicity induced by 1 *μM* PQ. Mesencephalic neuron-glia cultures and microglia-depleted cultures were treated with either vehicle, 5 ng/ml LPS, or 1 *μM* PQ. LPS was used as a positive control for microglia-mediated DA neurotoxicity. DA neurotoxicity was measured at 6–7 days post treatment by using the [^{3}H]DA uptake assay. The data are expressed as the percentage of the control cultures and are the means ± SEM from five independent experiments. An asterisk indicates a significant difference ($p < 0.05$) compared with control.

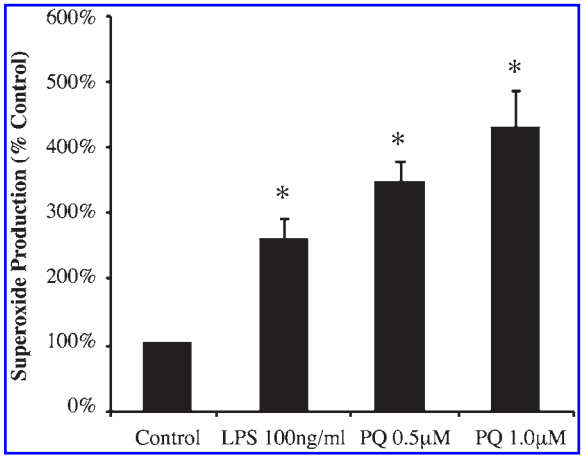

FIG. 4. PQ induces extracellular $O_2^{\bullet-}$ production in microglia. Primary microglia-enriched cultures were treated with either vehicle, 100 ng/ml LPS, 0.5 *μM* PQ, or 1 *μM* PQ. LPS was used as a positive control for microglial $O_2^{\bullet-}$ production. The production of extracellular $O_2^{\bullet-}$ was measured by the SOD-inhibitable reduction of tetrazolium salt, WST-1. The data are expressed as the percentage of the control cultures and are the means ± SEM from five independent experiments. An asterisk indicates a significant difference ($p < 0.05$) compared with control.

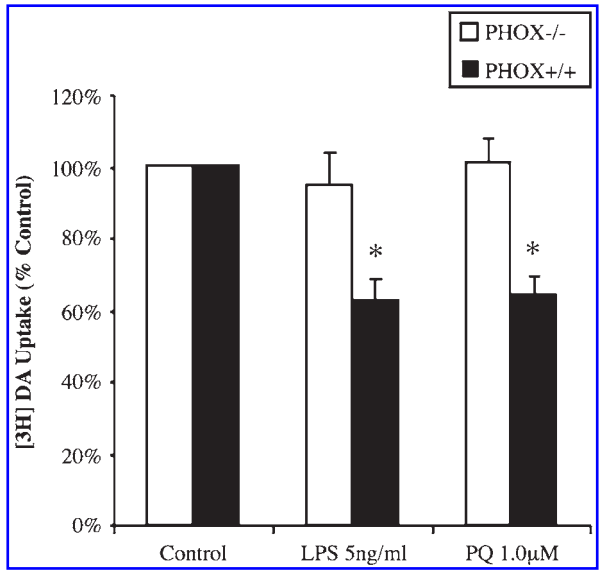

FIG. 5. NADPH oxidase mediates PQ DA neurotoxicity. $PHOX^{-/-}$ mice lack the functional catalytic subunit of the NADPH oxidase complex, gp91, and fail to produce the phagocytic respiratory burst. Mesencephalic neuron-glia cultures from $PHOX^{-/-}$ and $PHOX^{+/+}$ mice were treated with either vehicle, 5 ng/ml LPS, or 1 *μM* PQ. LPS was used as a positive control for microglial NADPH oxidase activation. DA neurotoxicity was measured at 6–7 days post treatment using the [^{3}H]DA uptake assay. The data are expressed as the percentage of the control cultures and are the means ± SEM from four independent experiments. An asterisk indicates a significant difference ($p < 0.01$) compared with control.

$O_2^{\bullet-}$ production in enriched microglia ($p < 0.05$) (Fig. 4), which suggests that extracellular $O_2^{\bullet-}$ may be the main mediator of the microglia-mediated neurotoxicity. Consistent with previous reports (9, 33), LPS, as a positive control, was shown to induce $O_2^{\bullet-}$ production by microglia.

PQ-induced microglia-dependent neurotoxicity is mediated by NADPH oxidase

NADPH oxidase has been documented as the predominant source of extracellular $O_2^{\bullet-}$ in phagocytic cells. $PHOX^{-/-}$ mice lack the functional gp91 protein, the catalytic subunit of the NADPH oxidase complex, and thus produce no extracellular $O_2^{\bullet-}$ in response to immunological stimuli. DA uptake was compared between mesencephalic neuron-glia cultures from $PHOX^{-/-}$ mice and $PHOX^{+/+}$ mice. Figure 5 shows that whereas DA neurons in $PHOX^{+/+}$ cultures lost 36% of uptake function after 7-day treatment with 1 *μM* PQ ($p < 0.01$), DA neurons in $PHOX^{-/-}$ cultures were completely resistant to the same dose of PQ. This suggests that, at 1 *μM,* PQ neurotoxicity is mediated through NADPH oxidase and supports the importance of $O_2^{\bullet-}$ in DA neurotoxicity. LPS was used as a positive control for NADPH oxidase-mediated neurotoxicity, where LPS showed less DA neurotoxicity in midbrain neuron-glia cultures from $PHOX^{-/-}$ mice compared with $PHOX^{+/+}$ mice (33).

DISCUSSION

Oxidative stress has been strongly linked to neurodegenerative disease, with particular emphasis on PD. Thus, the identification of multiple, potential triggers of oxidative insult in the brain and the elucidation of corresponding mechanisms are of paramount importance to both the understanding of disease progression and the development of neuroprotective compounds. In the present study, the neurotoxicity of PQ was examined in mesencephalic neuron-glia cultures, where 0.5–1 *μM* PQ was shown to damage DA neurons in a dose-dependent manner (Fig. 1) and to be selectively toxic to DA neurons (Fig. 2). This is consistent with several previously reported *in vivo* studies (25, 26, 39), supporting the notion that PQ is indeed selectively toxic to DA neurons.

More interestingly, microglia were identified as the cell type governing PQ toxicity at lower concentrations (0.5–1 *μM*). Originally, PQ was believed to be directly neurotoxic to DA neurons due to the structural similarity to MPP^+ and because both are actively taken into the DA neuron through the DA transporter receptor (37, 38). Further, PQ is known to be able to redox-cycle with various diaphorases (3–5, 7) and oxygen to produce $O_2^{\bullet-}$. Thus, once inside the DA neurons, PQ may induce an increase of intracellular ROS and consequently damage the neurons. However, this study reports that whereas higher concentrations of PQ may indeed be directly toxic to DA neurons, lower concentrations (up to 1 *μM*) require the presence of microglia in order to be toxic (Fig. 3). Microglia, the resident immune cells of the central nervous system, have been shown to play an important role in LPS- and rotenone-induced DA neuronal death, where microglial

NADPH oxidase-derived $O_2^{\bullet -}$ is the common mediator of cell death (8, 10, 33).

Similar to rotenone (40, 41), here we show that PQ activated microglia to produce ROS. The addition of PQ at any dose to the midbrain neuron-glia cultures failed to result in the production of soluble proinflammatory factors, a response commonly reported in the traditional microglial proinflammatory response. However, PQ did induce the production of extracellular $O_2^{\bullet -}$ from microglia (Fig. 4), identifying microglia as an additional source of PQ-induced oxidative stress. Although the exact species of ROS governing the neurotoxicity is unknown, there is a well established association between the microglial production of $O_2^{\bullet -}$ and DA neurotoxicity (35, 40, 41).

DA neurons are known to be particularly vulnerable to oxidative insult due to a reduced antioxidant capacity, a high content of iron and oxidation-prone DA, and a potential defect in mitochondrial complex I (12, 13). Thus, any compound inducing oxidative stress in the central nervous system is a potential candidate for selective DA neurotoxicity. However, the existence of a class of agrochemicals (rotenone and PQ) shown to induce oxidative stress at low concentrations solely through the production of microglial $O_2^{\bullet -}$ may provide novel insight into the mechanism governing the selective degeneration of DA neurons by brain region. Specifically, the SN is reported to contain 4.5 times as many microglia, compared with other regions of the brain (14), supporting the notion that the DA neurons localized there could be preferentially susceptible to microglia-mediated oxidative stress.

NADPH oxidase is the enzyme responsible for the microglial production of extracellular $O_2^{\bullet -}$ in response to LPS (35) and rotenone (40, 41). PHOX$^{-/-}$ mice are devoid of the gp91 protein, catalytic subunit of NADPH oxidase complex, and are unable to produce extracellular $O_2^{\bullet -}$ in response to stimuli. In this study, we show that neuron-glia cultures from PHOX$^{-/-}$ mice were also insensitive to 1 μM PQ (Fig. 5), suggesting that NADPH oxidase is critical for PQ-induced neurotoxicity at lower concentrations. Current studies in our laboratory are focused on identifying the mechanisms of how PQ and rotenone activate NADPH oxidase in microglia.

In summary, we demonstrate that the selective DA neurotoxicity of lower concentrations (up to 1 μM) of PQ is still mediated through the production of the $O_2^{\bullet -}$ radical. However, we report that at lower concentrations, the source of PQ-induced DA neurotoxic oxidative stress is initially extracellular and originates from the microglia, rather than from the neuron itself. Further, this microglia-mediated neurotoxicity is derived from the activation of NADPH oxidase. Together, these data suggest an emerging class of environmental agents (PQ and rotenone) that act at lower concentrations to provide an indirect source of oxidative insult through microglia, which is consequently selectively toxic to DA neurons.

ACKNOWLEDGMENTS

We thank Yu-Xiang Diao for his technical assistance. DISCLAIMER: This document has been reviewed by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute the endorsement of recommendation for use.

ABBREVIATIONS

DA, dopamine or dopaminergic; ELISA, enzyme-linked immunosorbent assay; GABA, γ-amino-*n*-butyric acid; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; MPP^+, 1-methyl-4-phenylpyridinium; $O_2^{\bullet -}$, superoxide; PD, Parkinson's disease; PQ, paraquat; ROS, reactive oxygen species; SN, substantia nigra; SOD, superoxide dismutase; TH, tyrosine hydroxylase; TNFα, tumor necrosis factor-α.

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Received for publication March 24, 2004; accepted November 30, 2004.

This article has been cited by:

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