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Rotenone and Paraquat do not Directly Activate Microglia or Induce Inflammatory Cytokine Release

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Abstract

Both epidemiological and pathological data suggest an inflammatory response including microglia activation and neuro-inflammation in the Parkinsonian brain. Treatments with lipopolysacchride (LPS), rotenone and paraquat have been used as models for Parkinson's disease, as they cause dopaminergic neuron degeneration in culture and in animals. Recent studies have suggested that rotenone and paraquat induce neuro-inflammation, however, it is not known if they can directly activate microglia. Here, we use primary cultured microglia to address this question. Microglia activation was analyzed by morphological changes and release of nitric oxide and inflammatory cytokines. Treatment with LPS was used as a positive control. While LPS induced morphological changes characteristic of microglial activation and release of nitric oxide and inflammatory cytokines, rotenone and paraquat did not. Our results suggest that paraquat and rotenone do not act directly on microglia and that neuro-inflammation and microglial activation in animals treated with these agents is likely non-cell autonomous, and may occur as a result of dopaminergic neuron damage or factors released by neurons and other cells.

Keywords

microglia; rotenone; paraquat; Parkinson's disease

Introduction

Brain inflammation has emerged as a possible risk factor in sporadic Parkinson's disease. Severe head trauma has been associated with increased risk of developing Parkinson's disease [5, 13]. While not directly proven, inflammation caused during such injury may be a contributing factor in the development of Parkinson's disease later in life. Moreover, non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin use is negatively correlated with disease risk suggesting that suppression of the inflammatory response may be beneficial in the treatment of Parkinson's disease [4, 7, 36].

From 1916–1927 a pandemic of Encephalitis lethargica swept through Europe and North America. Many of those who recovered from this disease developed post-encephalitic

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Parkinsonism [9,^{25,31}]. The cause of this outbreak of encephalitis has not been positively identified. However, as encephalitis is inflammatory in nature it is possible that this increase in brain inflammation may have contributed to the later development of Parkinsonism. West Nile Virus (WNV) could prove to be a contemporary example of the connection between neuro-inflammation and Parkinsonism. Humans that have been infected with WNV exhibit encephalitis, neuro-inflammation and transient Parkinsonism [20,³³].

Lipopolysaccharide (LPS) is a bacterial coat protein and is used as an inflammatory model for Parkinson's disease. LPS causes dopaminergic cell death and decreases locomotor activity in rodents [2,¹⁹]. During an inflammatory response in the brain, microglia become activated, releasing interleukin (IL) -1 β , IL-6, IL-8, tumor necrosis factor (TNF) - α , nitric oxide (NO) and superoxide. Postmortem studies examining the substantia nigra of Parkinson's disease patients have revealed the presence of reactive microglia [26]. These studies suggest that inflammation may be a key feature of Parkinson's disease.

Epidemiological studies have suggested a potential link between general pesticide exposure and increased risk for Parkinson's disease [3,^{11,14,16}]. Both rotenone and paraquat are pesticides used worldwide [10]. Treatments with rotenone and paraquat induce selective dopaminergic neuron degeneration and have been used to model sporadic Parkinson's disease [6,^{17,18,28}]. While LPS has been used as an inflammatory model for Parkinson's disease, less is known about the inflammatory response resulting from rotenone or paraquat exposure. It has been reported that rotenone induces microglial activation *in vitro* and *in vivo* [12,³⁸]. Indeed, microglia were determined to be a key component in rotenone-induced dopaminergic cell death *in vitro* [12]. Another study demonstrated that a single exposure to paraquat in mice induces activation of microglia even in the absence of observable dopaminergic neuron damage [29]. However, these studies did not determine if rotenone or paraquat act directly on microglia to induce an inflammatory response.

Here, we use primary cultured microglia to determine the role rotenone and paraquat play in the inflammatory process. We analyzed microglia for morphological changes and nitric oxide and cytokine release and found that while LPS generates a significant inflammatory response, rotenone and paraquat did not produce similar results. These results suggest that rotenone and paraquat do not directly activate microglia or induce microglia-mediated inflammation.

Materials and methods

Chemicals and reagents

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. Rotenone was dissolved in dimethyl sulfoxide (DMSO). LPS was dissolved in phosphate buffered saline (PBS). Paraquat was dissolved in water. Mouse β -III tubulin monoclonal antibody was purchased from Promega (Madison, WI). Rabbit ionized calcium-binding adaptor molecule 1 (Iba-1) polyclonal antibody was purchased from Wako (Richmond, VA). Rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody was purchased from Dako (Carpinteria, CA). Secondary Alexa Fluor[®] antibodies were purchased from Invitrogen (Carlsbad, CA).

Primary microglia culture

All animal work was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Timed-pregnant C57BL/6 mice were purchased from Charles River. We cultured cortices from 3–4 day old mice under conditions selective for glia proliferation followed by isolation of microglia to be used for microglia-enriched experiments [22,²³]. Post-natal day 3–4 (P3-P4) pups were sacrificed and the cortex was isolated in Hank's Balanced Salt Solution (Invitrogen). Cells were dissociated with 0.125% trypsin at 37°C for

25 minutes, followed by 50 µg/mL DNase (Worthington Biochemical Corporation, Lakewood, NJ) and mechanical trituration. Dissociated cells were then spun at 1000 rpm for 5 minutes and resuspended in D10C (Dulbecco's modified eagle medium (DMEM), 10% F-12, 10% horse serum (Invitrogen), 2 mM glutamine (Invitrogen), 10 mM HEPES, 50 µg/mL penicillin/streptomycin) and plated in poly-D-lysine (PDL) coated T75 flasks overnight. The following day, media was changed to D10C plus 5% L929 conditioned media and incubated for 1 week.

Microglia were isolated by shaking the flask, collecting the media and spinning at 1000 rpm for 5 minutes. Cells were resuspended in DMEM-F12 (Invitrogen), 1% N2 supplement (Invitrogen), and 0.1% bovine serum albumen (BSA) and plated on PDL-coated 24-well plates. After overnight incubation, cells were treated with LPS, TNF- α , rotenone or paraquat for 24 or 72 hours. Microglia-enriched cultures were immunostained with Iba-1, β -III tubulin and GFAP to determine purity of microglia culture. Cultures were determined to be at least 95% pure microglia by determining the number of β -III tubulin-positive neurons and GFAP-positive astrocytes relative to Iba-1-positive microglia.

Measurement of nitric oxide

Culture medium from treated microglia was added to Griess reagent in a 1:1 ratio. After 15 minutes, optical density (OD) was measured at 540 nm using a colormetric plate reader. Nitric oxide concentration ([NO]) was determined by use of a nitrite standard reference curve. A 100 µM sodium nitrate solution and six 2-fold serial dilutions were used to generate the standard reference curve. Sample nitric oxide concentrations were then calculated from a 4-parameter equation based on this standard curve.

$$[\text{NO}] = ((.003 - 3.594) / (1 + (\text{OD}/658.898) \wedge 1.232)) + 3.594 \text{ where } R^2 = 0.999.$$

Cytokine Measurement

Cytokine levels of IL-1 β , IL-2, IL-4, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (INF) - γ , and TNF- α were measured in culture medium using the Bio-Plex mouse cytokine 8-plex Panel A (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Briefly, 50 µL of each standard from a series of 4-fold dilutions and samples were incubated with target capturing beads on a 96-well plate for 30 minutes, followed by 30 minutes of incubation with specific biotinylated detection antibodies. Next, streptavidin R-phycoerythrin was added and incubated for 10 minutes. Filtration and washing were incorporated after each incubation step with wash buffer using vacuum manifold. Sample plate was incubated at room temperature with shaking. Samples were re-suspended in assay buffer prior to reading using the Luminex 100™ (Austin, TX) suspension array reader. All samples were analyzed in duplicates.

Immunocytochemistry

This was performed as previously described [17]. Briefly, cells were fixed with 4% paraformaldehyde/4% sucrose at room temperature for 30 minutes and permeabilized with 0.5% Igepal® (Sigma) in PBS for 30 minutes. To block non-specific binding, cells were treated for two hours with 2.5% BSA, 5% horse serum, and 5% goat serum in PBS/0.1% Triton X-100. Cells were then incubated with mouse β -III tubulin (1:200) monoclonal antibody or rabbit Iba-1 (1:500), anti-GFAP (1:500) polyclonal antibodies overnight at 4°C. Secondary antibody (goat anti-mouse Alexa Fluor® 488 or 594, goat anti-rabbit Alexa Fluor® 488 or 594) was then added for one hour followed by nuclear staining with Hoechst 33258 (2.5 µg/ml) for 10 minutes.

Statistics

For all experiments, data were results from at least three independent experiments, each with at least duplicate determinations. Statistical analysis of the data was performed using one-way analysis of variation (ANOVA) with statistically significant values representing an alpha level of 0.05 or below. Error bars represent standard error of means (SEM). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not statistically significant ($p > 0.05$). ND, data was below detectable levels and set at the lowest value of the dynamic range of detection.

Results

LPS, but not rotenone or paraquat, caused morphological changes characteristic of microglia activation

Resting microglia are characterized by long bipolar or unipolar processes and elongated cell bodies, whereas activated microglia display rounded, amoeboid cell bodies [38]. Morphology of microglia was analyzed immunocytochemically using ionized calcium-binding adaptor molecule 1 (Iba-1), a protein expressed selectively in microglia [1,³⁰]. Mouse primary cultured microglia were treated with vehicle control (DMSO or PBS), 1 ng/ml LPS, 10–50 nM rotenone or 5–50 μ M paraquat. These concentrations of rotenone and paraquat were chosen because dopaminergic neuron degeneration in culture has been reported at rotenone and paraquat concentrations as low as 5 nM and 10 μ M, respectively [17,²¹].

LPS stimulated changes in microglial morphology, whereas no appreciable morphological changes were found in primary cultured microglia stimulated with rotenone or paraquat (Fig 1). Rotenone at levels as high as 50 nM did not have any effect on microglia morphologically. While paraquat-treated microglia underwent cell death at high levels (50 μ M), there was no evidence of any morphological changes at lower doses. These data suggest that rotenone and paraquat do not directly activate primary cultured microglia.

LPS, but not rotenone or paraquat, stimulated nitric oxide release in primary cultured microglia

Nitric oxide (NO) release from microglia is an indicator of an inflammatory response (as reviewed in [15,³⁵]). In addition, nitric oxide has been identified as toxic to dopaminergic neurons [12,^{32,37}]. Here, LPS stimulated release of high levels of nitric oxide from microglia 24 and 72 hours post-treatment (Fig 2). Paraquat- or rotenone-stimulated microglia did not release significant amounts of nitric oxide, suggesting that these compounds do not activate one of the key inflammatory responses by microglial.

Unlike LPS, paraquat or rotenone treatment in primary cultured microglia does not induce release of inflammatory cytokines

Upon activation, microglia release high levels of interleukins and other cytokines which are part of the inflammatory response (as reviewed in [24]). We measured the levels of IL-10, IL-1 β , IL-2, IL-4, IL-5, TNF- α , GM-CSF and INF- γ released by primary cultured microglia after treatment with LPS, rotenone or paraquat. LPS treatment generated high levels of IL-10, IL-1 β and TNF- α release at 24 and 72 hours post-treatment (Fig 3 A, B, and Fig 4 A). Increases in IL-2, GM-CSF and INF- γ release were also found following LPS stimulation, though to a lesser degree (Fig 3 C, Fig 4 B and C). Conversely, very little of any of these cytokines was detectable in the media of microglia cultures after rotenone or paraquat treatment. These data suggest that while LPS induces an inflammatory response in primary cultured microglia, rotenone and paraquat do not directly act on microglia to induce inflammation.

Discussion

We analyzed microglia for morphological changes along with nitric oxide and cytokine release and discovered that while LPS generates a significant inflammatory response, rotenone and paraquat do not. LPS treatment of primary cultured microglia resulted in rounded, amoeboid-like cells that released high levels of NO, IL-10, IL-1 β , and TNF- α . No significant changes in morphology or increase in inflammatory mediators were observed with rotenone or paraquat treatment. Our findings are important and suggest that rotenone and paraquat do not activate microglia directly.

Microglia activation occurs *in vivo* after rotenone treatment [38]. Microglia play a key role in rotenone-induced dopaminergic cell death in mixed rat neuron/glia cultures [12]. Rotenone-treated microglia generate high levels of superoxide, contributing to dopaminergic cell death. However, it is still unclear whether rotenone can directly cause microglial activation. Using primary cultured microglia from rats, Gao *et al.* failed to observe significant accumulation of NO, IL-1 β or TNF- α after rotenone treatment [12]. However, a later study, using the same type of culture preparation and treatment, found morphological changes characteristic of microglial activation and increased levels of TNF- α , cyclooxygenase 2 and prostaglandin E2, although no explanation was provided for this apparent discrepancy [38]. Data presented in this study using highly enriched microglia preparation from mice showed that rotenone does not cause morphological changes of microglial or production and release of inflammatory cytokines, consistent with the findings of Gao *et al.* [12].

A single exposure of paraquat in mice induces activation of microglia even in the absence of observable dopaminergic neuron damage [29]. This study was performed *in vivo* and did not identify if paraquat was specifically and directly affecting microglia or if paraquat was causing sub-lethal effects in other cells which resulted in subsequent microglial activation. Our results suggest that paraquat does not directly activate microglia.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which specifically targets dopaminergic neurons via dopamine transporter uptake, is thought to also cause neuro-inflammation and microglia activation. Mice lacking inducible nitric oxide synthase, TNF receptors 1 and 2, or INF- γ receptor were protected from MPTP-induced reactive gliosis [8, 27, 34]. Since MPP⁺, the toxic metabolite of MPTP, acts specifically on dopaminergic neurons, perhaps MPP⁺-damaged neurons release TNF- α and INF- γ , which are known to activate inducible nitric oxide synthase in microglia. Thus, it seems likely that rotenone, paraquat, or MPTP, neurotoxins used to induce models for sporadic Parkinson's disease *in vivo*, induce neuro-inflammation and microglial activation indirectly through factors released from neurons or astrocytes.

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References

1. Arai H, Furuya T, Yasuda T, Miura M, Mizuno Y, Mochizuki H. Neurotoxic effects of lipopolysaccharide on nigral dopaminergic neurons are mediated by microglial activation, interleukin-1 β , and expression of caspase-11 in mice. *J Biol Chem* 2004;279:51647–51653. [PubMed: 15383538]

2. Bakos J, Duncko R, Makatsori A, Pirnik Z, Kiss A, Jezova D. Prenatal immune challenge affects growth, behavior, and brain dopamine in offspring. *Ann N Y Acad Sci* 2004;1018:281–287. [PubMed: 15240379]
3. Baldi I, Cantagrel A, Lebailly P, Tison F, Dubroca B, Chrysostome V, Dartigues JF, Brochard P. Association between Parkinson's disease and exposure to pesticides in southwestern France. *Neuroepidemiology* 2003;22:305–310. [PubMed: 12902626]
4. Bower JH, Maraganore DM, Peterson BJ, Ahlskog JE, Rocca WA. Immunologic diseases, anti-inflammatory drugs, and Parkinson disease: a case-control study. *Neurology* 2006;67:494–496. [PubMed: 16894114]
5. Bower JH, Maraganore DM, Peterson BJ, McDonnell SK, Ahlskog JE, Rocca WA. Head trauma preceding PD: a case-control study. *Neurology* 2003;60:1610–1615. [PubMed: 12771250]
6. Brooks AI, Chadwick CA, Gelbard HA, Cory-Slechta DA, Federoff HJ. Paraquat elicited neurobehavioral syndrome caused by dopaminergic neuron loss. *Brain Res* 1999;823:1–10. [PubMed: 10095006]
7. Chen H, Jacobs E, Schwarzschild MA, McCullough ML, Calle EE, Thun MJ, Ascherio A. Nonsteroidal antiinflammatory drug use and the risk for Parkinson's disease. *Ann Neurol* 2005;58:963–967. [PubMed: 16240369]
8. Dehmer T, Lindenau J, Haid S, Dichgans J, Schulz JB. Deficiency of inducible nitric oxide synthase protects against MPTP toxicity in vivo. *J Neurochem* 2000;74:2213–2216. [PubMed: 10800968]
9. Dourmashkin RR. What caused the 1918–30 epidemic of encephalitis lethargica? *J R Soc Med* 1997;90:515–520. [PubMed: 9370993]
10. Ecobichon, DJ. Toxic effects of pesticides. In: Klaassen, CD., editor. *Casarett & Doull's Toxicology: The Basic Science of Poisons*. The McGraw-Hill Companies, Inc; New York, NY: 2001. p. 763-810.
11. Engel LS, Checkoway H, Keifer MC, Seixas NS, Longstreth WT Jr, Scott KC, Hudnell K, Anger WK, Camicioli R. Parkinsonism and occupational exposure to pesticides. *Occup Environ Med* 2001;58:582–589. [PubMed: 11511745]
12. Gao HM, Hong JS, Zhang W, Liu B. Distinct role for microglia in rotenone-induced degeneration of dopaminergic neurons. *J Neurosci* 2002;22:782–790. [PubMed: 11826108]
13. Goldman SM, Tanner CM, Oakes D, Bhudhikanok GS, Gupta A, Langston JW. Head injury and Parkinson's disease risk in twins. *Ann Neurol* 2006;60:65–72. [PubMed: 16718702]
14. Gorell JM, Peterson EL, Rybicki BA, Johnson CC. Multiple risk factors for Parkinson's disease. *J Neurol Sci* 2004;217:169–174. [PubMed: 14706220]
15. Hartmann A, Hunot S, Hirsch EC. Inflammation and dopaminergic neuronal loss in Parkinson's disease: a complex matter. *Exp Neurol* 2003;184:561–564. [PubMed: 14769349]
16. Hertzman C, Wiens M, Bowering D, Snow B, Calne D. Parkinson's disease: a case-control study of occupational and environmental risk factors. *Am J Ind Med* 1990;17:349–355. [PubMed: 2305814]
17. Hsuan SL, Klintworth HM, Xia Z. Basic fibroblast growth factor protects against rotenone-induced dopaminergic cell death through activation of extracellular signal-regulated kinases 1/2 and phosphatidylinositol-3 kinase pathways. *J Neurosci* 2006;26:4481–4491. [PubMed: 16641227]
18. Inden M, Kitamura Y, Takeuchi H, Yanagida T, Takata K, Kobayashi Y, Taniguchi T, Yoshimoto K, Kaneko M, Okuma Y, Taira T, Ariga H, Shimohama S. Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone. *J Neurochem* 2007;101:1491–1504. [PubMed: 17459145]
19. Iravani MM, Leung CC, Sadeghian M, Haddon CO, Rose S, Jenner P. The acute and the long-term effects of nigral lipopolysaccharide administration on dopaminergic dysfunction and glial cell activation. *Eur J Neurosci* 2005;22:317–330. [PubMed: 16045485]
20. Kleinschmidt-DeMasters BK, Marder BA, Levi ME, Laird SP, McNutt JT, Escott EJ, Everson GT, Tyler KL. Naturally acquired West Nile virus encephalomyelitis in transplant recipients: clinical, laboratory, diagnostic, and neuropathological features. *Arch Neurol* 2004;61:1210–1220. [PubMed: 15313837]
21. Klintworth H, Newhouse K, Li T, Choi WS, Faigle R, Xia Z. Activation of c-Jun N-Terminal Protein Kinase Is a Common Mechanism Underlying Paraquat-and Rotenone-Induced Dopaminergic Cell Apoptosis. *Toxicol Sci* 2007;97:149–162. [PubMed: 17324951]

22. Kong LY, McMillian MK, Hudson PM, Jin L, Hong JS. Inhibition of lipopolysaccharide-induced nitric oxide and cytokine production by ultralow concentrations of dynorphins in mixed glia cultures. *J Pharmacol Exp Ther* 1997;280:61–66. [PubMed: 8996182]
23. Liu B, Du L, Hong JS. Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. *J Pharmacol Exp Ther* 2000;293:607–617. [PubMed: 10773035]
24. Liu B, Gao HM, Hong JS. Parkinson's disease and exposure to infectious agents and pesticides and the occurrence of brain injuries: role of neuroinflammation. *Environ Health Perspect* 2003;111:1065–1073. [PubMed: 12826478]
25. McCall S, Henry JM, Reid AH, Taubenberger JK. Influenza RNA not detected in archival brain tissues from acute encephalitis lethargica cases or in postencephalitic Parkinson cases. *J Neuropathol Exp Neurol* 2001;60:696–704. [PubMed: 11444798]
26. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* 1988;38:1285–1291. [PubMed: 3399080]
27. Mount MP, Lira A, Grimes D, Smith PD, Faucher S, Slack R, Anisman H, Hayley S, Park DS. Involvement of interferon-gamma in microglial-mediated loss of dopaminergic neurons. *J Neurosci* 2007;27:3328–3337. [PubMed: 17376993]
28. Peng J, Mao XO, Stevenson FF, Hsu M, Andersen JK. The herbicide paraquat induces dopaminergic nigral apoptosis through sustained activation of the JNK pathway. *J Biol Chem* 2004;279:32626–32632. [PubMed: 15155744]
29. Purisai MG, McCormack AL, Cumine S, Li J, Isla MZ, Di Monte DA. Microglial activation as a priming event leading to paraquat-induced dopaminergic cell degeneration. *Neurobiol Dis* 2007;25:392–400. [PubMed: 17166727]
30. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 2007;55:453–462. [PubMed: 17203472]
31. Reid AH, McCall S, Henry JM, Taubenberger JK. Experimenting on the past: the enigma of von Economo's encephalitis lethargica. *J Neuropathol Exp Neurol* 2001;60:663–670. [PubMed: 11444794]
32. Ruano D, Revilla E, Gavilan MP, Vizuet ML, Pintado C, Vitorica J, Castano A. Role of p38 and inducible nitric oxide synthase in the in vivo dopaminergic cells' degeneration induced by inflammatory processes after lipopolysaccharide injection. *Neuroscience* 2006;140:1157–1168. [PubMed: 16713109]
33. Sejvar JJ, Haddad MB, Tierney BC, Campbell GL, Marfin AA, Van Gerpen JA, Fleischauer A, Leis AA, Stokic DS, Petersen LR. Neurologic manifestations and outcome of West Nile virus infection. *Jama* 2003;290:511–515. [PubMed: 12876094]
34. Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI, O'Callaghan JP. Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. *FASEB J* 2002;16:1474–1476. [PubMed: 12205053]
35. Teismann P, Schulz JB. Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. *Cell Tissue Res* 2004;318:149–161. [PubMed: 15338271]
36. Ton TG, Heckbert SR, Longstreth WT Jr, Rossing MA, Kukull WA, Franklin GM, Swanson PD, Smith-Weller T, Checkoway H. Nonsteroidal anti-inflammatory drugs and risk of Parkinson's disease. *Mov Disord* 2006;21:964–969. [PubMed: 16550541]
37. Watanabe Y, Kato H, Araki T. Protective action of neuronal nitric oxide synthase inhibitor in the MPTP mouse model of Parkinson's disease. *Metab Brain Dis* 2008;23:51–69. [PubMed: 18030609]
38. Zhou F, Wu JY, Sun XL, Yao HH, Ding JH, Hu G. Iptakalim alleviates rotenone-induced degeneration of dopaminergic neurons through inhibiting microglia-mediated neuroinflammation. *Neuropsychopharmacology* 2007;32:2570–2580. [PubMed: 17356569]

Figure 1. LPS-treated microglia display activated amoeboid morphology while rotenone and paraquat treated microglia do not

Mouse primary cultured microglia were treated with either vehicle (DMSO or PBS), 1 ng/ml LPS, 20 ng/ml TNF- α , 50 nM rotenone or 50 μ M paraquat for 24 hours. Microglia were immunostained with Iba-1 to visualize cell morphology.

Figure 2. Rotenone or paraquat did not stimulate NO release in primary cultured microglia
 Mouse primary cultured microglia were treated with either vehicle control (DMSO or PBS), 1 ng/ml LPS, rotenone or paraquat for 24 or 72 hours. Nitric oxide concentration in the medium was measured using Griess reagent and calculated using a standard curve. ***, $p < 0.001$ compared to vehicle control.

D.

Figure 3. Rotenone or paraquat did not stimulate interleukin release in primary cultured microglia
 Microglia were treated with either vehicle control (DMSO or PBS), 1 ng/ml LPS, rotenone or paraquat for 24 or 72 hours. Levels of released IL-10 (A), IL-1 β (B), IL-2 (C), and IL-4 (D) in the medium were measured using the Bio-Plex mouse cytokine panel. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to vehicle control. ND, values below detectable levels. Data is set to the lowest concentration in the dynamic range of detection. IL-1 β ND=1.95.

Figure 4. Rotenone or paraquat did not stimulate inflammatory cytokine release in primary cultured microglia

Microglia were treated with either vehicle control (DMSO or PBS), 1 ng/ml LPS, rotenone or paraquat for 24 or 72 hours. Levels of released TNF- α (A), GM-CSF (B), and INF- γ (C) in the culture medium were measured using the Bio-Plex mouse cytokine panel. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to vehicle control. ND, values below detectable levels. Data is set to the lowest concentration in the dynamic range of detection. GM-CSF ND=1.94.