NADPH Oxidase Mediates Lipopolysaccharide-induced Neurotoxicity and Proinflammatory Gene Expression in Activated Microglia*

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Liya Qin‡, Yuxin Liu‡, Tongguang Wang‡, Sung-Jen Wei‡, Michelle L. Block‡, Belinda Wilson‡, Bin Liu‡, and Jau-Shyong Hong‡

From the ‡Neuropharmacology Section, Laboratory of Pharmacology and Chemistry and the §National Center for Toxicogenomics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

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Fax: 919-541-0841; E-mail: Hong3@niehs.nih.gov.

P.O. Box 12233, Research Triangle Park, NC 27709. Tel.: 919-541-5203;

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§ To whom correspondence should be addressed: MD F1–01 NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709. Tel.: 919-541-5203; Fax: 919-541-0841; E-mail: Hong3@niehs.nih.gov.

¶ Present address: Department of Pharmacodynamics, Box 100487, College of Pharmacy, University of Florida, Gainesville, FL 32610.

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1 The abbreviations used are: ROS, reactive oxygen species; LPS, lipopolysaccharide; TNF, tumor necrosis factor; TH, tyrosine hydroxylase; DA, dopamine; IR, immunoreactive; GFAP, glial fibrillary acidic protein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; SN, substantia nigra; PBS, phosphate-buffered saline; HBSS, Hank’s balanced salt solution; RT, reverse transcriptase.

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Parkinson’s disease is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra. We have previously reported that lipopolysaccharide (LPS)-induced degeneration of dopaminergic neurons is mediated by the release of proinflammatory factors from activated microglia. Here, we report the pivotal role of NADPH oxidase in inflammation-mediated neurotoxicity, where the LPS-induced loss of nigral dopaminergic neurons in vivo was significantly less pronounced in NADPH oxidase-deficient (PHOX−/−) mice when compared with control (PHOX+/+) mice. Dopaminergic neurons in primary mesencephalic neuron-glia cultures from PHOX−/− mice were significantly more sensitive to LPS-induced neurotoxicity in vitro when compared with PHOX+/+ mice. Further, PHOX+/+ neuron-glia cultures chemically depleted of microglia failed to show dopaminergic neurotoxicity with the addition of LPS. Neuron-enriched cultures from both PHOX+/+ mice and PHOX−/− mice also failed to show any direct LPS-induced dopaminergic neurotoxicity. However, the addition of PHOX+/+ microglia to neuron-enriched cultures from either strain resulted in reinstatement of LPS-induced dopaminergic neurotoxicity, supporting the role of microglia as the primary source of NADPH oxidase-generated insult and neurotoxicity. Immunostaining for F4/80 in mesencephalic neuron-glia cultures revealed that PHOX−/− microglia failed to show activated morphology at 10 h, suggesting an important role of reactive oxygen species (ROS) generated from NADPH oxidase in the early activation of microglia. LPS also failed to elicit extracellular superoxide and produced low levels of intracellular ROS in microglia-enriched cultures from PHOX−/− mice. Gene expression and release of tumor necrosis factor α was much lower in PHOX−/− mice than in control PHOX+/+ mice. Together, these results demonstrate the dual neurotoxic functions of microglial NADPH oxidase: 1) the production of extracellular ROS that is toxic to dopamine neurons and 2) the amplification of proinflammatory gene expression and associated neurotoxicity.

The pathogenesis of several neurological disorders, including Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, and the AIDS dementia complex, has been closely associated with localized inflammatory responses in the brain (1–4). Glial cell activation and oxidative stress have been frequently observed during the course of the disease processes (5, 6). Microglia, the resident immune cells in the brain, are extremely responsive to environmental stress or immunological challenges and have been implicated as the predominant cell type governing inflammation-mediated neuronal damage (3, 6–11). In particular, activated microglia exert cytotoxic effects by releasing inflammatory mediators, such as reactive oxygen species (ROS), tumor necrosis factor (TNF) α, interleukin-1, nitric oxide, arachidonic acid metabolites, and quinolinic acid (12–14). Although these immunotoxic factors are necessary for normal function, the microglia response must be tightly regulated to avoid overactivation and disastrous neurotoxic consequences (3).

ROS generated by phagocytic cells, such as superoxide anion, hydroxyl radical, lipid hydroperoxides, and their by-products (e.g. hydrogen peroxide), are reported to have dual functions in the inflammatory response. First, microglia respond to toxic agents, such as the bacterial endotoxin, lipopolysaccharide (LPS), human immunodeficiency virus, type 1 coat protein gp120, and β-amyloid peptides, and release ROS, which in turn induce neuronal degeneration (6, 15–17). Specifically, both in vitro and in vivo studies support the possibility that microglia-mediated neurotoxicity is influenced by the release of superoxide and other ROS (3, 9, 17, 18). Alternatively, there is increasing evidence that ROS can also function as second messengers to regulate several downstream signaling molecules, including protein kinase C, mitogen-activated protein kinase, and NF-κB (19–21). In phagocytic cells such as macrophages, activation of the NADPH oxidase pathway initiates an intracellular ROS signaling pathway that can amplify the production of proinflammatory cytokines, such as TNFα (22). In neutrophils, NADPH oxidase is an important source of ROS generation for both oxidative burst (23) and cell signaling pathways (24). Recent work suggests that intracellular ROS mediate β-amyloid peptide-induced microglial activation, whereas inhibition of intracellular ROS results in the reduction of β-amyloid-induced NF-κB activation and lowered interleukin-1β expression (25).

LPS is one of the most common inflammmogens used to investigate the impact of inflammation on neuron death. The intracellular signaling mechanisms related to the effects of LPS have been well studied in several types of cells including macrophage, microglia, and astrocytes (26–28). Briefly, LPS is...
known to activate protein kinase C, protein-tyrosine kinases, mitogen-activated protein kinases, and NF-kB, which have been implicated in the release of immune-related cytotoxic factors, such as NO and proinflammatory cytokines (26–30). Given the reported influence of intracellular ROS signaling on several kinase-mediated pathways (31), this common LPS signaling pathway in microglia presents a likely target for downstream ROS signaling.

The production of intracellular ROS results from the process of normal cellular function and metabolism and may originate from multiple cellular sources, such as xanthine oxidase, mitochondrial electron transport, NAPDH oxidase, peroxisomes, and the endoplasmic reticulum (32). The identification of ROS as a primary factor in LPS-mediated neurodegeneration, the localization of the source of ROS causing the neurotoxic effects, and the elucidation of the signaling pathway driving microglial overactivation is of paramount importance to understanding the molecular mechanisms of the neurodegenerative disease state. In effort to answer these questions, the following group of experiments sought to discern the role of NAPDH oxidase in microglia-mediated neurotoxicity as both a source of neurotoxic ROS and as a critical cellular mediator responsible for the amplification of the microglia proinflammatory response.

In this paper, we have performed both an in vivo study by using mesencephalic neuron-glia and an in vitro study using mesencephalic neuron-glia or enriched microglial cultures from both PHOX-deficient mice and PHOX+/− control mice in effort to delineate the role of NAPDH oxidase-generated ROS in LPS-induced dopaminergic neurotoxicity and to discern the underlying mechanism.

EXPERIMENTAL PROCEDURES

Animals—Eight-week-old male (25–30 g) and female (25–30 g) B6.129S6-Cbyh+/− (PHOX+/−) and C57BL/6J 000664 (PHOX+/+) mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6.129S6-Cbyh+/− PHOX−/− mice are lacking a functional gp91 protein, the catalytic subunit of the NAPDH oxidase complex. The PHOX−/− mutation is maintained in the C57BL/6J 000664 background; therefore, C57BL/6J 000664 (PHOX−/−) mice were used as control animals. Breeding of the mice was performed to achieve accurate timed pregnancy within ± 0.5 days.

Reagents—Lipopolysaccharide (strain O111:B4) was purchased from Calbiochem (San Diego, CA). Cell culture ingredients were obtained from Cell Systems Inc. (Minneapolis, MN). All other reagents came from Sigma. NADPH Oxidase, Neurotoxicity, and TNFα Gene Expression

H2O2-diaminobenzidine and 3% hydrogen peroxide for 10 min. The cultures were again washed (three times) with PBS before being incubated for 1 h with the Vectastain ABC reagents diluted in PBS containing 1% bovine serum albumin, 0.4% Triton X-100, and 4% appropriate secondary antibody: normal horse serum for Neu-N, or F4/80 and normal goat serum for TH or GFAP staining. The cultures were incubated overnight at 4 °C with the primary antibody diluted in DAKO antibody diluent, and then the cells were washed (three times) for 10 min each time with PBS. The cultures were then incubated with 1:250 dilution of secondary antibody: Neu-N, mouse anti-rabbit antibody, 1:227; TH or GFAP, goat anti-rabbit antibody, 1:227; F4/80, mouse anti-rat antibody, 1:1000.

After washing (three times) with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents diluted in PBS containing 0.3% Triton X-100 and the appropriate biotinylated secondary antibody (Neu-N, horse anti-mouse antibody, 1:227; TH or GFAP, goat anti-rabbit antibody, 1:227; F4/80, mouse anti-rat antibody, 1:1000).

After washing (three times) with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents diluted in PBS containing 0.3% Triton X-100. After washing (three times) with PBS, the cultures were incubated for 1 h with with light microscopy. The intensity of the specific staining in mesencephalic neuron-glia cultures was determined by immunocytochemistry. The mouse mesencephalic neuron-glia cultures contained 10–11% F4/80-IR microglia, 40% Neu-N-IR neurons, and 1% TH-IR dopaminergic neurons. The remaining cells were presumed to be astrocytes. Mesencephalic Microglia-depleted Cultures—Mesencephalic neuron-glia were seeded at 5 × 10^5/well in 24-well plates. Microglia were depleted by 0.5 mM leucine methyl ester for 72 h. The cultures stained with F4/80 antibody showed less than 0.1% microglia.

Mesencephalic Neuron-enriched Cultures—Mesencephalic neuron-glia were seeded at 5 × 10^5/well in 24-well plates with 1 μM cytosine β−δ-arabinofuranoside at 48 h. Two days later, the cytosine β−δ-arabinofuranoside was removed. The cultures stained with F4/80 and GFAP antibodies showed a purity of >98%.

Microglia-enriched Cultures—Primary microglia were prepared from whole brains of 2-day-old control (PHOX+/+) or PHOX−/− pups following a previously described protocol (34). The cells were seeded at 5 × 10^5 well in 96-well plates for TNFα or superseroxide assays. The purity of microglia was >98%.

Analysis of Neurotoxicity—The degeneration of dopaminergic neurons was assessed by measuring the ability of cultures to take up [3H]DA and counting the number of TH-IR neurons following immunostaining of mesencephalic neuron-glia cultures. For in vivo study, 24 consecutive brain slices (35-μm thickness), which encompassed the entire substantia nigra, compact, were collected. A normal distribution of the number of TH-immunoreactive (TH-IR) neurons in the substantia nigra, compact was constructed based on the counts of 24 slices from PHOX−/− mutant mice and PHOX+/− mice. The distribution curves from these two noninjected groups superimpose and show no difference in shape and number of the curves. Eight evenly spaced brain slices from saline or LPS-injected animals were immunostained with an antibody against TH and counted. The distribution of the cell numbers from each animal was matched with the normal distribution curve to correct for errors resulting from the cutting. With stereology equipment, measurements of individuals and up to 1000 stereological points (Olympus Danmark A/S, Albertslund, Denmark) system performed counting in a double-blind manner. Conclusions were drawn only when the difference was within 5%.

Uptake Assay—The cells were incubated for 20 min at 37 °C with 1 μM [3H]DA in Krebs-Ringer buffer (16 mM NaH2PO4, 16 mM NaHCO3, 119 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.3 mM EDTA, pH 7.4). Nonspecific uptake was measured in the presence of 10 μM mazindol. After the cells were washed (three times) with ice-cold Krebs-Ringer buffer (1 ml/well) and lysed with 1 N NaOH (0.5 ml/well), the lysate was mixed with 15 ml of scintillation fluid, and radioactivity was determined with a liquid scintillation counter. The specific [3H]DA uptake was calculated by subtracting the value obtained in the presence of mazindol from that obtained in the absence of mazindol.

Immunostaining—Neurons were stained with the antibody against Neu-N, for neuronal cell bodies but not for neurites. Microglia were stained with rat monoclonal antibody raised against F4/80 antigen, and astrocytes were stained with the antibody against GFAP, an intermediate filament protein whose expression is restricted to astrocytes. Dopamine neurons were detected with the polyclonal antibody against TH. Briefly, the cells were fixed for 20 min at room temperature in 3.7% paraformaldehyde in phosphate-buffered saline (PBS). After washing (two times) with PBS, the cultures were treated with 1% hydrogen peroxide for 10 min. The cultures were again washed (three times) with PBS before being incubated for 1 h with blocking solution (PBS containing 1% bovine serum albumin, 0.4% Triton X-100, and 4% appropriate secondary antibody: normal horse serum for Neu-N, or F4/80 and normal goat serum for TH or GFAP staining). The cultures were incubated overnight at 4 °C with the primary antibody diluted in DAKO antibody diluent, and then the cells were washed (three times) for 10 min each time with PBS. The cultures were then incubated with 1:500 dilution of secondary antibody: Neu-N, mouse anti-rabbit antibody, 1:227; TH or GFAP, goat anti-rabbit antibody, 1:227; F4/80, mouse anti-rat antibody, 1:1000. After washing (three times) with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents diluted in PBS containing 0.3% Triton X-100 and the appropriate biotinylated secondary antibody (Neu-N, horse anti-mouse antibody, 1:227; TH or GFAP, goat anti-rabbit antibody, 1:227; F4/80, mouse anti-rat antibody, 1:1000). After washing (three times) with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents diluted in PBS containing 0.3% Triton X-100. After washing (three times) with PBS, the cultures were incubated with 1:1500 dilution of secondary antibody: Neu-N, mouse anti-rabbit antibody, 1:227; TH or GFAP, goat anti-rabbit antibody, 1:227; F4/80, mouse anti-rat antibody, 1:1000. After washing (three times) with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents diluted in PBS containing 0.3% Triton X-100. After washing (three times) with PBS, the cultures were incubated with 1:1500 dilution of secondary antibody: Neu-N, mouse anti-rabbit antibody, 1:227; TH or GFAP, goat anti-rabbit antibody, 1:227; F4/80, mouse antirat antibody, 1:1000. After washing (three times) with PBS, the cultures were incubated with 1:1500 dilution of secondary antibody: Neu-N, mouse anti-rabbit antibody, 1:227; TH or GFAP, goat anti-rabbit antibody, 1:227; F4/80, mouse anti-rat antibody, 1:1000.


**RESULTS**

**PHOX**<sup>+/−</sup> Mice Show Reduced LPS-induced DA Neurotoxicity in the Substantia Nigra in Vivo—To investigate the role of NADPH oxidase-generated ROS in LPS-induced neurotoxicity, LPS (5 μg in 2 μl of saline) was stereotactically injected into the SN of PHOX<sup>+/−</sup> and PHOX<sup>+/+</sup> mice. Fourteen days later, the brains were removed, and coronal sections through the nigral complex were taken. The sections were immunostained with an antibody against TH. Eight animals were used for each strain. Dopaminergic neurons were counted by three individuals blindly and by the Computer-Assisted Stereology Toolbox system. A value of p < 0.05 was considered statistically significant.

Mice—To further understand the mechanisms underlying the difference in LPS-induced dopaminergic neurotoxicity between PHOX<sup>+/−</sup> and PHOX<sup>+/+</sup> mice observed in vivo, mixed mesencephalic neuron-glia cultures from PHOX<sup>+/−</sup> and PHOX<sup>+/+</sup> mice were treated with either vehicle or LPS (1–100 ng/ml). Neurotoxicity was assessed by DA uptake, morphological analysis and TH-IR neuron number. The cultures from PHOX<sup>+/−</sup> and PHOX<sup>+/+</sup> mice showed a concentration-dependent decrease in both [3H]HDA uptake and the number of tyrosine hydroxylase-immunoreactive (TH-IR) neurons. However, in neuron-glia cultures from PHOX<sup>−/−</sup> mice, the decrease in DA uptake and the number of TH-IR neurons was significantly less than that in neuron-glia cultures from PHOX<sup>+/−</sup> mice (Fig. 2, A and B). Morphological analysis showed that TH-IR neurons in the LPS-treated cultures from PHOX<sup>+/−</sup> mice displayed shorter and less elaborate TH-IR dendrites compared with those from PHOX<sup>+/−</sup> mice (Fig. 2C). This difference in the LPS-induced neurotoxicity of dopaminergic neurons between the cultures from PHOX<sup>+/−</sup> and PHOX<sup>+/+</sup> mice suggests an important role for ROS in LPS-induced neuron death. NADPH oxidase mediates LPS-induced morphological changes associated with the activation of microglia.

Earlier work from our laboratory demonstrates that LPS is not directly toxic to dopaminergic neurons and that microglia, rather than astroglia, are pivotal to LPS-induced neurotoxicity (39). To address whether microglia were the source of NADPH oxidase and consequent neurotoxicity for LPS-treated cells, the PHOX<sup>+/−</sup> and PHOX<sup>−/−</sup> mesencephalic neuron-glia, neuron-astrocyte, and neuron-enriched cultures were treated with vehicle or 100 ng/ml LPS for 7 days, respectively. In neuron-glia cultures, LPS caused a significant decrease in DA uptake (Fig. 3A). In neuron-astrocyte cultures, the PHOX<sup>+/−</sup> and PHOX<sup>−/−</sup> mesencephalic neu-
ron-glia were treated with L-leucine methyl ester to chemically deplete the microglia. L-Leucine methyl ester-treated PHOX−/− and PHOX−/− cultures devoid of microglia did not show significant neurotoxicity (Fig. 3B). The result was further confirmed by the PHOX−/− and PHOX−/− neuron-enriched cultures treated with LPS (Fig. 3C), which also failed to show LPS-induced neurotoxicity. To further support the importance of microglia, PHOX++ enriched microglia were added back to both PHOX++ and PHOX−/− neuron-enriched cultures. The addition of PHOX++ microglia reinstated LPS neurotoxicity in both PHOX++ and PHOX−/− neuron-enriched cultures (Fig. 3C). Taken together, these data indicate that LPS is not directly toxic to dopaminergic neurons and that microglia are the source of NADPH oxidase-induced neurotoxicity in the LPS model of inflammation mediated neurodegeneration.

Mesencephalic neuron-glia cultures from PHOX++ mice treated with LPS displayed the characteristics of activated microglia: increased cell size, irregular shape, and intensified F4/80 immunoreactivity, which is a specific marker for mouse microglia. In contrast, the LPS-stimulated activation of microglia was significantly less pronounced in neuron-glia cultures in PHOX−/− mice (Fig. 4). These results suggest that ROS generated from NADPH oxidase is associated with the activation of microglia and the subsequent death of dopaminergic neurons.

**Microglial NADPH Oxidase Is the Major Source of LPS-generated ROS**—To identify the mechanism of NADPH oxidase-mediated neurotoxicity, the production of several proinflammatory factors was measured in PHOX−/− and PHOX++ cultures treated with LPS. First, we examined the generation of superoxide by measuring superoxide dismutase-inhibitable reduction of WST-1, which measures the extracellular production of superoxide anions. As predicted, LPS caused significant production of superoxide in microglia from PHOX−/− mice but not from PHOX−/− mice (Fig. 5A). The data support that NADPH oxidase is the key enzyme for the LPS-elicited produc-
WST-1 as described under was measured as the superoxide dismutase-inhibitable reduction of extracellular ROS with vehicle or LPS for 30 min. Production of extracellular superoxide in microglia was measured by increased cell size, irregular shape, and intensified F4/80 staining. The images presented are representative of three independent experiments.

Intracellular oxidative stress was measured via DCFH oxidation. DCFH-DA enters cells passively and is deacetylated by esterase to nonfluorescent DCFH. DCFH reacts with ROS to form DCF, the fluorescent product. Intracellular ROS was significantly produced in microglia-enriched cultures from both PHOX–/– and PHOX+/+ mice following LPS treatment. However, the level of intracellular ROS induced by LPS was 68–76% (LPS 1–100 ng/ml) higher in PHOX–/– compared with PHOX+/+ mice (Fig. 5B), suggesting that NADPH oxidase is one of the primary components contributing to the LPS-induced intracellular ROS increase in microglia. Nitric oxide was examined in mesencephalic neuron-glia cultures and microglia-enriched cultures from both PHOX–/– and PHOX+/+ mice. However, there was no significant difference in the production of NO in LPS-stimulated cultures from PHOX–/– and that from PHOX–/– mice (data not shown).

**NADPH Oxidase Mediates LPS-induced Gene expression of TNFα**

To examine and analyze the molecular mechanisms for LPS-stimulated ROS production and their roles in the regulation of TNFα expression, real time RT-PCR analysis was performed. As shown in Fig. 6A, TNFα gene expression was significantly increased after LPS treatment, compared with corresponding vehicle controls. However, the levels of TNFα gene expression in mesencephalic neuron-glia cultures from PHOX–/– mice were 39–45% (1–100 ng/ml LPS) lower compared with PHOX+/+ mice (data not shown).
pared with PHOX/−/− mice. Secretion of TNFα in the supernatant of mesencephalic neuron-glia cultures from both PHOX/−/− and PHOX/+/− mice was also measured. Consistent with the real time RT-PCR analysis, levels of TNFα were increased in both cultures from PHOX/−/− and PHOX/+/− mice (Fig. 6B), compared with corresponding vehicle controls. Further, levels of TNFα secreted from mesencephalic neuron-glia cultures from PHOX/−/− mice were 31–38% (LPS 1–100 ng/ml) lower than those in the cultures from PHOX/+/− mice, suggesting that NADPH oxidase mediates the LPS-induced TNFα response in microglia. These data demonstrate that NADPH oxidase is the key enzyme for the LPS-elicited production of ROS and supports that intracellular ROS play a role as signaling molecules for the induction of TNFα expression in LPS-treated mesencephalic neuron-glia cultures.

**DISCUSSION**

In the present study, we have conducted both in vivo and in vitro studies using NADPH oxidase-deficient mice and have clearly demonstrated reduced dopaminergic neurotoxicity induced by LPS in PHOX/−/− mice. These observations strongly indicate that activation of NADPH oxidase and the subsequent release of superoxide are critical for LPS-induced neurotoxicity. The importance of this finding is reflected by the fact that DA neurons possess reduced antioxidant capacity, as evidenced by low intracellular glutathione (40). Thus, DA neurons are inherently more vulnerable to oxidative stress and microglia activation, when compared with other cell types (40). To our knowledge, this is the first report using PHOX/−/− mutants that clearly shows the role of NADPH oxidase-generated superoxide in LPS-induced dopaminergic toxicity.

Another factor highlighted by this work is the mandatory presence of microglia to influence LPS-induced neurotoxicity through NADPH oxidase (Fig. 3). These findings further define NADPH oxidase as one of the major mechanisms contributing to inflammation-mediated neurodegeneration. These results not only support the role of microglia in LPS-induced neurodegeneration, but they also emphasize the critical role of microglia in other models of dopaminergic neurodegeneration and Parkinson’s disease. Specifically, NADPH oxidase has been implicated in the neurotoxic mechanisms of several other dopaminergic neurotoxic compounds such as rotenone (41), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (42), and β-amyloid (17). Further, increased expression of NADPH oxidase has been reported in the post-mortem brains of Parkinson’s disease patients (43). Together, these results suggest the importance of targeting microglia and NADPH oxidase in the treatment of Parkinson’s disease and further validate LPS as an appropriate model for the investigation of the mechanisms of inflammation-mediated neurodegeneration.

In vitro studies revealed that the production of ROS from NADPH oxidase is also essential for the morphological activation of microglia, as shown by the scanty increase in F4/80 immunostaining in microglia prepared from PHOX/−/− mutants, compared with the control mice (Fig. 4). The smaller degree of activation of microglia from PHOX/−/− mutants was further reflected in a much smaller increase in LPS-induced TNFα release in these mutant mice (Fig. 6).

Microglia are considered the resident immune cells in the brain (44, 45), where NADPH oxidase activation is known to be associated with neuroinflammation (46). Under normal conditions, microglia serve a crucial role in immune surveillance. However, overactivation of microglia has been associated with neurodegeneration through the production of neurotoxic factors, such as proinflammatory cytokines and free radicals (47–49). Among the factors produced by activated microglia, ROS play a special role in neurodegeneration by serving dual functions. First, high concentrations of ROS released extracellularly may exert direct toxicity to dopaminergic neurons, and second, the increase in intracellular concentrations of ROS may modify the signaling events leading to the activation of microglia. The importance of ROS in LPS-induced inflammatory signaling in the microglia is supported by the drastic reduction of microglial activation in PHOX/−/− mice, demonstrated by the low expression of the microglial marker, F4/80, and the minimal release of TNFα in response to LPS. These findings suggest that ROS generated from NADPH oxidase play a role in the signaling events leading to microglia activation.

It is well documented that in the macrophage, LPS produces proinflammatory factors, such as TNFα and nitric oxide, by combining with the LPS-binding protein and binding to CD14-TLR4 receptors, which in turn activate mitogen-activated protein kinases and NF-κB. Findings from this study and other reports have revealed a PHOX-ROS signaling pathway in addition to this well described pathway. It has been reported that ROS mediate the expression of interleukin-1 and TNFα following LPS challenge in macrophage cell lines (30, 50). Using RAW cells transfected with Rac1, which activates NADPH oxidase and produces superoxide, Sanlioglu et al. (30) demonstrated enhanced expression of the TNFα gene after LPS treatment. Our results using microglia from PHOX/−/− mice are consistent with their finding and further extend this premise. Specifically, the decreased release of TNFα from PHOX/−/− mice in response to LPS may also be associated with decreased neurotoxicity.

In general, this work suggests that the PHOX-ROS and CD14-TLR4 pathways may serve different functions in mediating the LPS-induced activation of microglia. Time course analysis from this study indicates a temporal difference between PHOX-ROS and CD14-TLR4 pathways. The production of ROS happens within minutes after LPS treatment, and the early activation of microglia, as shown by the increase in F4/80 immunoreactivity and TNFα release, may be attributed to the PHOX-ROS pathway. We have also shown that the removal of functional NADPH oxidase results in a reduction of ~40% of the microglial inflammatory response to LPS, implicating that the PHOX-ROS pathway serves to amplify the classic TLR4 LPS signaling pathway. Further observations in our laboratory using TLR4 knockout mice have shown a minimal microglia inflammatory response to LPS (10 ng/ml) that can be increased to detectable levels with higher doses (>100 ng/ml LPS). Together these data suggest that although the PHOX-ROS pathway is not sufficient for the induction of the LPS-induced inflammatory response, it may be essential for normal LPS signaling function and inflammatory response.

In summary, this study has established the role of microglia-generated ROS in both LPS-induced neurotoxicity and the regulation of TNFα gene expression in primary cultures from PHOX/−/− and PHOX/+/− mice. The current study demonstrates that microglia are critical to NADPH oxidase-mediated neurotoxicity in LPS-induced inflammation-mediated neurodegeneration. The mechanism through which NADPH oxidase mediates LPS-induced neurotoxicity was shown to be through ROS-mediated microglial activation, the generation of microglial-derived extracellular neurotoxic ROS, and the amplification of microglial proinflammatory gene expression. Given the two-tiered impact of microglial NADPH oxidase on DA neurotoxicity as both a source of neurotoxic extracellular ROS and as a mediator of the microglial proinflammatory signaling pathway, inhibition of ROS production from microglia may provide
improved prevention and treatment for Parkinson’s disease and other neurodegenerative disorders.

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