Interleukin-10 Protects Lipopolysaccharide-Induced Neurotoxicity in Primary Midbrain Cultures by Inhibiting the Function of NADPH Oxidase

Li Qian, Michelle L. Block, Sung-Jen Wei, Chiou-feng Lin, Jeffrey Reece, Hao Pang, Belinda Wilson, Jau-Shyong Hong, and Patrick M. Flood

ABSTRACT

The role of anti-inflammatory cytokines in Parkinson’s disease is not completely understood. In this study, using mesencephalic neuron-glia cultures, we report that both pretreatment and post-treatment of rat mesencephalic neuron-glia cultures with interleukin (IL)-10, a natural immune modulator, reduced lipopolysaccharide (LPS)-induced DA neurotoxicity. The main purpose of this study was to elucidate the molecular mechanism underlying IL-10-elicited neuroprotection. IL-10 significantly inhibited LPS-induced production of tumor necrosis factor-α, nitric oxide, and extracellular superoxide in microglia cells. In addition, using reconstituted neuron and glia cell cultures, IL-10 was shown to be neuroprotective only in the presence of microglia. More importantly, IL-10 failed to protect DA neurons in cultures from mice lacking NADPH oxidase (PHOX), a key enzyme for extracellular superoxide production in immune cells, suggesting the critical role of PHOX in IL-10 neuroprotection. This conclusion was further supported by the finding that IL-10 inhibited LPS-induced translocation of the cytosolic subunit of NADPH oxidase p47phox to the membrane. When the Janus tyrosine kinase (JAK) 1 signaling pathway was blocked, IL-10 failed to attenuate LPS-induced superoxide production, indicating that the JAK1 signaling cascade mediates the inhibitory effect of IL-10. Together, our results suggest that IL-10 inhibits LPS-induced DA neurotoxicity through the inhibition of PHOX activity in a JAK1-dependent mechanism.

The pathogenesis of several neurological disorders, including Parkinson’s disease (PD), Alzheimer’s disease, multiple sclerosis, and the AIDS dementia complex, has been closely associated with localized inflammatory responses in the brain (McGeer et al., 1988; Liu and Hong, 2003; Rosi et al., 2005). It has been suggested that this inflammation may play a role in a delayed and progressive degeneration of dopaminergic (DA) neurons in the substantia nigra, which is a hallmark of PD (McGeer et al., 1988). Recent studies have implicated a critical role of inflammation induced by microglia in neurodegenerative diseases, including PD. Although activation of microglia serves an important protective function in immune surveillance by removing foreign microorganisms (Aloisi, 1999), overactivation of microglia followed by overproduction of proinflammatory factors has been shown to result in neuronal death in the brain (McGuire et al., 2001; Sriram et al., 2002). The midbrain region that encompasses the substantia nigra is particularly rich in microglia (Kim et al., 2000); therefore, activation of nigral microglia and release of these proinflammatory neurotoxic factors may be a crucial component of the degenerative process of DA neurons in PD.

ABBREVIATIONS: PD, Parkinson’s disease; DA, dopaminergic; NO, nitric oxide; PHOX, NADPH oxidase; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; CNS, central nervous system; JAK, Janus tyrosine kinase; OX-42, CR3 complement receptor; DCFH-DA, dichlorodihydrofluorescein diacetate; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TH-IR, tyrosine hydroxylase-immunoreactive; PG, prostaglandin; SOD, superoxide dismutase; WST, water-soluble tetrazolium salt; HBSS, Hank’s balanced salt solution; ROS, reactive oxygen species; PCR, polymerase chain reaction; iNOS, inducible nitric-oxide synthase; COX, cyclooxygenase; STAT, signal transducer and activator of transcription; NF, nuclear factor; HAPI, highly aggressively proliferating immortalized.
Because these proinflammatory neurotoxic factors also exhibit immunoregulatory functions necessary for normal immune responses, the microglial response to inflammatory stimuli must be tightly regulated to avoid overactivation and disastrous neurotoxic consequences (Liu and Hong, 2003). It has been shown that DA neurons are particularly sensitive to the lethal effects of oxidative stress, including nitric oxide (NO) and superoxide (Jenner and Olanow, 1996; Greenamyre et al., 1999). Previous reports from our laboratory have shown that inhibition of microglial production of extracellular superoxide was most effective in protecting DA neurons, suggesting that superoxide was the dominant neurotoxic factor for these DA neurons (Liu et al., 2000a). NADPH oxidase (PHOX), an enzyme that is a major source of superoxide in immune cells, is highly expressed in microglia. Both in vivo and in vitro studies using PHOX-deficient mice have clearly demonstrated reduced DA neurotoxicity induced by lipopolysaccharide (LPS) in PHOX−/− compared with PHOX+/+ wild-type mice (Qin et al., 2004). Moreover, PHOX activity may also regulate the levels of tumor necrosis factor (TNF)-α production by microglia after LPS stimulation (Qin et al., 2004), indicating that PHOX not only mediates superoxide production but also controls the levels of other proinflammatory neurotoxic factors produced by activated microglia. Therefore, the neurotoxicity mediated by microglia is a complex puzzle that potentially involves a number of distinct proinflammatory responses by microglia. The exact mechanism and kinetics by which microglia mediate neurotoxicity and the signals that control their proinflammatory response have yet to be determined.

Interleukin (IL)-10 is a pleiotropic cytokine that plays a critical role in the regulation of inflammatory responses and immune reactions, acting on both hematopoietic and nonhematopoietic cells (Kremlev and Palmer, 2005). Increasing evidence indicates that IL-10 has the ability to improve neurological outcome after CNS injury, and this ability relies on its anti-inflammatory effects (Grilli et al., 2000; Kremlev and Palmer, 2005). For example, IL-10 has neuroprotective properties against glutamate-induced (Bachis et al., 2001) or hypoxic-ischemic (Dietrich et al., 1999) neuronal death and against LPS- or interferon-activated oligodendrocyte cell death (Molina-Holgado et al., 2001). It was also reported that IL-10 counteracts acute effects of endotoxin on cerebral metabolism, microcirculation, and oxygen tension during hypoxia-ischemia in the perinatal brain (Froen et al., 2002). Although it is well known that IL-10 inhibits known inflammatory responses of microglia (Hu et al., 1999; Kremlev and Palmer, 2005), the interplay among glial cells, neurons, and induced proinflammatory mediators leading to CNS pathology is complex and incompletely understood. Therefore, further assessment of the functional contribution of IL-10 critically depends on the elucidation of downstream secondary signaling mechanisms.

The main purpose of this study was to elucidate the molecular mechanism underlying IL-10-elicited neuroprotection. We show that IL-10 has significant protective effects on LPS-induced DA neurotoxicity through its inhibition of microglia activation. The finding that IL-10 is acting to inhibit PHOX activity, which then results in the inhibition of a wide array of proinflammatory mediators produced by activated microglia, offers new insights into our understanding of the etiology and eventual treatment of neurodegenerative diseases such as PD.

**Materials and Methods**

**Animals.** NADPH oxidase-deficient (gp91phox−/−) and wild-type C57BL/6J (gp91phox+/+) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Breeding of the mice was performed to achieve timed pregnancy with the accuracy of ± 0.5 days. Timed-pregnant Fisher F344 rats were obtained from Charles River Laboratories (Raleigh, NC). Housing and breeding of the animals were performed in strict accordance with the National Institutes of Health guidelines.

**Reagents.** The recombinant rat IL-10 was obtained from R&D Systems (Minneapolis, MN). JAK inhibitor I and LPS (strain O111:B4) were purchased from Calbiochem (San Diego, CA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). [3H]DA (30 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA), and the monoclonal antibody against the CR3 complement receptor (OX-42) was purchased from Chemicon International (Temecula, CA). The polyclonal anti-tyrosine hydroxylase antibody was a generous gift from Dr. John Reinhard (GlaxoSmithKline, Research Triangle Park, NC). The Vectastain ABC kit was obtained from Vector Laboratories (Burlingame, CA). The fluorescence probe dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Calbiochem. Rabbit anti-p47phox was obtained from Upstate (Lake Placid, NY). FITC-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Rabbit anti-GAPDH was obtained from Abcam (Cambridge, MA). Mouse anti-β-actin was purchased from BD Transduction Laboratories (San Jose, CA).

**Cell Lines.** The rat microglia HAP1 cells were a generous gift from Dr. James R. Connor (Pennsylvania State University, Hershey, PA) (Cheepsunthorn et al., 2001) and were maintained at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator with 5% CO2/95% air.

**Primary Mesencephalic Neuron-Glia Culture.** Neuron-glia cultures were prepared from the ventral mesencephalic tissues of embryonic day 14 to 15 rats or day 13 to 14 mice, as described previously (Liu et al., 2000a; Gao et al., 2002). In brief, dissociated cells were seeded at 1 × 105/well and 5 × 105/well to poly-L-lysine-coated 96- and 24-well plates, respectively. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air in minimal essential medium containing 10% fetal bovine serum, 10% horse serum, 1 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin. Seven-day-old cultures were used for treatment.

At the time of treatment, immunocytochemical analysis indicated that the rat neuron-glia cultures were made up of 11% microglia, 48% astrocytes, 41% neurons, and 1% tyrosine hydroxylase-immunoreactive (TH-IR) neurons. The composition of the neuron-glia cultures of NADPH oxidase-deficient mice was very similar to that of the wild-type mice consisting of 12% microglia, 48% astrocytes, 40% neurons, and 1% TH-IR neurons.

**Primary Mesencephalic Neuron-Enriched Cultures.** Midbrain neuron-enriched cultures were established as described previously (Gao et al., 2002). In brief, 24 h after seeding the cells, cytokine β-d-arabinoside was added to a final concentration of 10 μM to suppress glial proliferation. Three days later, cultures were changed back to maintenance medium and were used for treatment 7 days after initial seeding.

**Primary Microglia-Enriched Cultures.** Rat microglia-enriched cultures with a purity of >98% were prepared from whole brains of 1-day-old Fischer 344 rat pups as described previously (Liu et al., 2000b). For superoxide assays, 105 cells were grown overnight in 96-well culture plates before use.
Mesencephalic Microglia-Depleted Cultures. Mesencephalic neuron-glia were seeded at 5 × 10^6/well in 24-well plates. Microglia were depleted by 1 mM L-leucine methyl ester for 72 h (Liu et al., 2000b). The cultures stained with OX-42 antibody showed less than 0.1% microglia.

DA Uptake Assay. [3H]DA uptake assays were performed as described previously (Liu et al., 2000b). In brief, cells were incubated for 20 min at 37°C with 1 µM [3H]DA in Krebs-Ringer buffer (16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl_2, 1.2 mM MgSO_4, 1.3 mM EDTA, and pH 7.4). After washing three times with ice-cold Krebs-Ringer buffer, the cells were collected in 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Non-specific DA uptake observed in the presence of mazindol (10 µM) was subtracted.

Immunostaining. DA neurons were recognized with the anti-TH antibody, and microglia were detected with the OX-42 antibody, which recognizes the CR3 receptor as described previously (Gao et al., 2002). In brief, formaldehyde (3.7%)-fixed cultures were treated with 1% hydrogen peroxide (10 min) followed by sequential incubation with blocking solution for 30 min, primary antibody overnight at 4°C, biotinylated secondary antibody for 2 h, and ATP-binding cassette reagents for 40 min. Color was developed with 3,3'-diaminobenzidine. For morphological analysis, the images were recorded with an inverted microscope (Nikon, Tokyo, Japan) connected to a charge-coupled device camera (DAGE-MTI, Michigan City, IN) operated with the MetaMorph software (Universal Imaging Corporation, Downingtown, PA). For visual counting of TH-IR neurons, nine representative areas per well of the 24-well plate were counted under the microscope at 100× magnification by three individuals. The average of these scores was reported.

Nitrile and TNF-α Assays. The production of NO was determined by measuring the accumulated levels of nitrite in the supernatant with Griess reagent, and the release of TNF-α was measured with a rat TNF-α enzyme-linked immunosorbent assay kit from R&D Systems, as described previously (Liu et al., 2002).

Prostaglandin E₂ Production. PGE₂ in supernatant was measured with a prostaglandin (PG) E₂ EIA kit from Cayman (Ann Arbor, MI) according to the manufacturer’s instructions.

Superoxide Assay. The production of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of the tetrazolium salt WST-1 (Peskin and Winterbourn, 2000; Tan and Berridge, 2000). Neuron-glia or microglia-enriched cultures in 96-well culture plates were washed twice with HBSS without phenol red. Cultures were then incubated at 37°C for 30 min with vehicle control (water) or IL-10 in HBSS (50 µl/well). Then, 50 µl of HBSS with and without SOD (50 U/ml, final concentration) was added to each well along with 50 µl of WST-1 (1 mM) in HBSS and 50 µl of vehicle or LPS (10 ng/ml). Thirty minutes later, absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The difference in absorbance observed in the absence and presence of SOD was considered to be the amount of superoxide produced, and results were expressed as percentage of vehicle-treated control cultures.

Assay of Intracellular Reactive Oxygen Species. Intracellular oxidative stress was measured by DCFH-DA. DCFH-DA enters cells passively and is deacylated by esterase to nonfluorescent DCFH. DCFH reacts with reactive oxygen species (ROS) to form dichlorodifluorescein, the fluorescent product (Liu et al., 2001). DCFH-DA was dissolved in methanol at 10 mM and was diluted 500-fold in HBSS to give DCFH-DA at 20 µM. The cells were exposed to DCFH-DA for 1 h and then treated with HBSS containing the corresponding concentrations of LPS for 2 h. The fluorescence was read immediately at wavelengths of 485 nm for excitation and 530 nm for emission using a SpectraMax Gemini XS fluorescence microplate reader (Molecular Devices). The value subtracted by control group was viewed as the increase of intracellular ROS.

Real-Time Reverse Transcription-PCR Analysis. For real-time quantitative PCR, RNA was obtained using the RNeasy Mini Kit from QIAGEN (Valencia, CA). DNase (Ambion, Austin, TX) was used to digest DNA at 37°C for 30 min, and the integrity of the RNA was assessed by visualization of high 18S and 28S ribosomal RNA peaks as well as a small amount of 5S RNA. Quantity and quality assessment using a UV-visible spectrophotometer was performed at 260 and 280 nm. Optical density 260/280 ratio greater than 1.8 was an acceptable indicator of good RNA quality. Total RNA (1 µg) was reverse-transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase. The SYBR green DNA PCR kit (Applied Biosystems, Foster City, CA) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time values normalized to GAPDH. The relative differences between control and treatment groups were calculated and expressed by relative increases setting control as 100%.

The sequence of the oligonucleotide primers used were from rat: TNF-α, 5′-TGCTGACAAACCAACGACCA-3′ and 5′-CCCTTGAAGA-GAAACCTGGGAGTA-3′; inducible nitric-oxide synthase (iNOS), 5′-ACATCGAGTGGGACCTACATC-3′ and 5′-CGTACCGGATGAGC- TTGTAAT-3′; cyclooxygenase (COX)-2, 5′-CGACAGGCCTCCTAC-TGATAGGA-3′ and 5′-GAGGCTGTGCTCCGAATCTGG-3′; and GAPDH, 5′-CCTGGAGAATCCTGCTGAGTAT-3′ and 5′-AGCAGGTGGATCC TTGTAGT-3′.

Subcellular Fractionation and Western Blot Analysis. Subcellular fractionation were performed as described previously (Yuan et al., 1996). HAPI cells were lysed in hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 2 mM MgCl₂, 10 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of leupeptin, apro tinin, and pepstatin A), incubated on ice for 30 min, and then subjected to Dounce homogenization (~20–25 strokes, tight pestle A). The lysates were loaded onto sucrose in lysis buffer (final 0.5 M) and centrifuged at 1600g for 15 min, the supernatant above the sucrose gradient was used as cytosolic fractions after centrifugation at 100,000g for 30 min. The pellets solubilized in 1% Nonidet P-40 hypotonic lysis buffer were used as membranous fraction. Equal amounts of protein (20 µg/lane) were separated by 4 to approximately 12% Bis-Tris polyacrylamide gel electrophoresis gel and transferred to polyvinylidene difluoride membranes (Novex, San Diego, CA). Membranes were blocked with 5% nonfat milk and incubated with rabbit anti-p47(phox) antibody (1: 2000) or rabbit anti-GAPDH (1:2000) or mouse anti-p91(phox) (1:2000) for 1 h at 25°C. Horseradish peroxidase-linked anti-rabbit or mouse IgG (1:3000) for 1 h at 25°C and ECL + Plus reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK) were used as a detection system.

Confocal Microscopy. HAPI cells seeded in dish at 5 × 10⁴ cells/well were treated with LPS for 10 min in the absence or presence of IL-10 pretreatment for 1 h. Cells were fixed with 3.7% paraformaldehyde in PBS for 10 min. After being washed with PBS, cells were incubated with rabbit polyclonal antibody against p47(phox). Cells were then washed and incubated with FITC-conjugated goat anti-rabbit antibody. Focal planes spaced at 0.4–µm intervals were imaged with a Zeiss 510 laser scanning confocal microscope (63× PlanApo 1.4 numerical aperture objective; Carl Zeiss GmbH, Jena, Germany) equipped with LSM510 digital imaging software. The signal of p47(phox) (FITC-p47(phox); green) and the merge view of cell morphology and p47(phox) (Phase plus FITC-p47(phox)) are shown.

Statistical Analysis. The data were presented as the means ± S.E. For multiple comparisons of groups, analysis of variance was used. Statistical significance between groups was assessed by paired Student’s t test with Bonferroni’s correction. P < 0.05 was considered statistically significant.

Results

Effect of IL-10 on LPS-Induced Degeneration of DA Neurons. We first sought to determine whether LPS-induced inflammation by glial cells leading to destruction of DA neurons.
neurons could be inhibited by the anti-inflammatory cytokine IL-10. Mesencephalic neuron-glia cultures were pretreated with IL-10 for 1 h and then stimulated with LPS for 7 days. The degeneration of DA neurons was then determined by the [3H]DA uptake assay and numeration of the TH-IR neurons. The [3H]DA uptake assay showed that LPS treatment reduced the capacity of the cultures to take up DA to approximately 60% of the vehicle control, and this LPS-induced reduction was prevented by a dose-dependent pretreatment with IL-10 (Fig. 1A). At 10 and 30 ng/ml IL-10, LPS-induced decrease in DA uptake was completely restored, whereas IL-10 alone at this concentration range did not affect DA uptake levels in the cultures. A similar protective effect was obtained with IL-10 when counting the number of TH-IR neurons after immunostaining (Fig. 1B). Thus, LPS-induced loss of TH-IR neurons was prevented by IL-10 pretreatment in a concentration-dependent manner with a significant effect at 10 and 30 ng/ml. Morphological inspection revealed that LPS treatment not only decreased the number of TH-IR neurons but also caused a loss of neuronal process, and these characteristics were also reversed by 10 ng/ml IL-10 pretreatment (Fig. 1C).

Microglia, but Not Astroglia, Mediated IL-10’s Neuroprotective Effect against LPS-Induced DA Neurotoxicity. Our mesencephalic neuron-glia cultures contained ~10% microglia and ~50% astrocytes in addition to DA and other neurons; we sought to evaluate what cell type of glia mediated the neuroprotective effect of IL-10. The [3H]DA uptake assay showed that LPS treatment reduced the capacity of the cultures to take up DA to approximately 60% of the vehicle control, and this LPS-induced reduction was prevented by a dose-dependent pretreatment with IL-10 (Fig. 1A). At 10 and 30 ng/ml IL-10, LPS-induced decrease in DA uptake was completely restored, whereas IL-10 alone at this concentration range did not affect DA uptake levels in the cultures. A similar protective effect was obtained with IL-10 when counting the number of TH-IR neurons after immunostaining (Fig. 1B). Thus, LPS-induced loss of TH-IR neurons was prevented by IL-10 pretreatment in a concentration-dependent manner with a significant effect at 10 and 30 ng/ml. Morphological inspection revealed that LPS treatment not only decreased the number of TH-IR neurons but also caused a loss of neuronal process, and these characteristics were also reversed by 10 ng/ml IL-10 pretreatment (Fig. 1C).
cultures do not express detectable levels of IL-10 receptors (data not shown). Therefore, we investigated whether microglia or astroglia cells were the targets mediating the inhibitory effects of IL-10 on LPS-induced neurotoxicity by performing reconstitution experiments where we added either microglia or astroglia back to neuron-enriched cultures.

As shown in Fig. 2A, LPS alone, IL-10 alone, or the combination of LPS and IL-10 did not affect the DA uptake capacity in either neuron-enriched cultures or neuron-astroglia cultures. In contrast, the addition of 10% of microglia back to the neuron-enriched cultures reduced the capacity of DA uptake to 52% by LPS, similar to the original neuron-glia cultures, and this reduction can be reversed by IL-10. In addition, when 1-methyl-4-phenylpyridinium, which is known to damage DA neurons directly, was added to neuron-enriched or microglia-depleted cultures, IL-10 failed to show any protection (Fig. 2B), suggesting that the neuroprotective effect of IL-10 is not a direct effect on neurons. These results demonstrate that it is the microglia, not astroglia, that serve as the target of IL-10-mediated neuroprotection against LPS-induced neurodegeneration.

**IL-10 Pretreatment Suppresses LPS-Induced Release of Proinflammatory Mediators and Their Gene Expression.** Mesencephalic neuron-glia cultures treated with LPS displayed a significant enlargement of cell size from predominantly resting round and small cells to activated rod- and/or amoeboid-shaped cells (Kreutzberg, 1996) and intensified OX-42 immunoreactivity, a marker for the activation of microglia (Fig. 3A). The LPS-stimulated activation of microglia was suppressed in neuron-glia cultures pretreated with IL-10, whereas IL-10 alone did not show a significant effect on microglia activation. We also found that pretreatment with 3 to approximately 30 ng/ml IL-10 significantly blocked LPS-stimulated NO production and iNOS mRNA expression (Fig. 3B), TNF-α production and mRNA expression (Fig. 3C), and PGE₂ production and COX-2 mRNA expression (Fig. 3D). In addition, IL-10 at 10 and 30 ng/ml, but not 3 ng/ml, significantly attenuated the LPS-induced ROS production, which included extracellular superoxide and intracellular ROS production (Fig. 3, E and F). Interestingly, the two concentrations of IL-10 found to significantly inhibit both ROS and TNF-α production were identical to the concentrations that correspond with the DA neuroprotective effect of IL-10 in the LPS-induced mesencephalic neuron-glia cultures (Fig. 1), whereas the 3 ng/ml dose (which has little if any protective effect) shows no significant inhibition of the

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**Fig. 3.** Effect of IL-10 on LPS-induced production of proinflammatory factors and their gene expression in microglial. Mesencephalic neuron-glia cultures treated with LPS displayed the characteristics of activated microglia and intensified OX-42 immunoreactivity when analyzed on day 7 after LPS stimulation (A). The effect of IL-10 on LPS-induced production of NO, TNF-α, and PGE₂ (B–D, respectively, closed bars) or iNOS, TNF-α, and COX-2 mRNA expression (B–D, respectively, open bars) were shown. Protein levels of these factors are expressed as absolute values. Effects of IL-10 on LPS-induced production of superoxide (E) as percentage of control and intracellular ROS (F) as absolute absorbance. Enriched microglia cells were treated with the vehicle control or IL-10 for 1 h before the addition of LPS (10 ng/ml). For analysis of mRNA, total RNA was harvested 3 h after LPS treatment, followed by real-time reverse transcription-PCR analysis of iNOS, TNF-α, and COX-2 using specific primers. IL-10 alone has no effect on the protein or mRNA production of these inflammatory mediators. The results are the means ± S.E. of three experiments performed in triplicate. *, *P < 0.05; **, *P < 0.01; and ***, *P < 0.001 compared with the LPS-treated cultures.
PHOX Plays an Important Role in IL-10-Mediated Protection against LPS-Induced Neuron Degeneration. The above-mentioned results indicate that IL-10 reduced the LPS-induced production of superoxide through the inhibition of PHOX activity by blocking \( p47^{\text{phox}} \) translocation; thus, we further studied the role of ROS in IL-10-mediated neuroprotection by determining whether IL-10 could protect LPS-induced neurodegeneration in PHOX-deficient cells. Neuron-glia cultures were prepared from wild-type mice. As shown in Fig. 5A, LPS treatment of neuron-glia cultures prepared from PHOX\(^{-/-}\) mice reduced \( ^{3}\text{H} \)-DA uptake by 42\%, whereas IL-10 at 10 ng/ml significantly attenuated the decrease (Fig. 5A). In contrast, LPS treatment significantly reduced the uptake capacity by only 21\% in PHOX\(^{-/-}\) mice, but IL-10 failed to show any protective effect. Consistent with the results of DA uptake, although LPS-induced TNF-\( \alpha \) production in PHOX\(^{+/+}\) mice is significantly less than that in PHOX\(^{-/-}\) mice but not in PHOX\(^{-/-}\) mice (Fig. 5B).

IL-10 Inhibits Superoxide Production through the JAK-STAT Pathway. We further studied the mechanism underlying the inhibitory effect of IL-10 on LPS-induced increase in superoxide production. Because IL-10 receptor signaling functions by activating the JAK1/STAT3 pathway, an inhibitor of JAK1 was used for this purpose. As shown in Fig. 6, the addition of JAK1 inhibitor eliminated the inhibitory effect of IL-10 on superoxide production by LPS-stimulated microglia. The inhibitory effect of IL-10 on LPS-induced superoxide production was also significantly inhibited when anti-IL-10 receptor antibody was used to block the action of IL-10 in these cultures (data not shown). These results suggest that IL-10 binds to its receptor to activate the JAK1/STAT3 pathway, leading to the inhibition of superoxide production by microglia.

**Post-Treatment with IL-10 Was Still Effective in Protecting LPS-Induced DA Neuronal Damage.** Because the production of most inflammatory mediators occurred within a few hours after stimulation of glial cells with LPS, we sought to determine whether post-treatment with IL-10 was still effective in protecting DA neurons from LPS-induced toxicity. Neuron-glia cocultures were first treated with LPS...
for 3, 6, or 12 h and then IL-10 was added to the cultures and incubation was continued for another 7 days. We found that post-treatment with IL-10 up to 6 h after LPS exposure still showed a protective effect (Fig. 7A). However, when IL-10 was added at 12 h post-LPS treatment, no significant protective effect was seen. When we analyzed nitric oxide and TNF-α production, we found that they were consistently inhibited only when IL-10 was added 3 h after LPS treatment but not when added at 6 or 12 h after exposure to LPS (Fig. 7, B and C). In contrast, superoxide production was significantly inhibited by the addition of IL-10, even up to 6 h after LPS treatment (Fig. 8). Thus, among the proinflammatory factors released from microglia, the inhibition of superoxide by IL-10 was best correlated with its protection against LPS-induced DA neuron damage.

Discussion

LPS-induced degeneration of DA neurons in mesencephalic neuron-glia cultures is a useful in vitro model for studying the mechanism and identifying the potential therapeutic implications on inflammation-mediated neurodegeneration (Liu and Hong, 2003). Using this well characterized model, we sought to determine whether and how the major anti-inflammatory cytokine, IL-10, which regulates the production of proinflammatory mediators by macrophage and glial cells, could be effective in diminishing inflammation-induced neurodegeneration in vitro. Our results showed that IL-10 exerted potent effects in inhibiting LPS-induced inflamma-
tion and neuronal destruction. Three salient features of this protective role of IL-10 were observed in this study: both pretreatment and post-treatment with IL-10 showed strong neuroprotection in LPS-induced DA neuron degeneration, the neuroprotective effect of IL-10 was mediated through the inhibition of microglia activation, and PHOX is a major target of IL-10-mediated neuroprotection.

It has been reported that IL-10 has a major regulatory effect by inhibiting the production of a number of proinflammatory mediators by activated glial cells (Heyen et al., 2000). Our study demonstrates a neuroprotective effect of IL-10 in LPS-induced DA neurotoxicity. Further studies indicate that IL-10 has no direct effect on either neurons or astroglia; instead, the neuroprotective effect of IL-10 was mediated through the inhibition of microglial overactivation by LPS. This observation is novel and critical to further characterize the mechanisms by which microglia mediate neurotoxicity after LPS stimulation. These results confirm those previously obtained from our laboratory (Gao et al., 2002) and others (Tseng et al., 2005) that microglia are the key players responsible for inflammation-related neuronal damage. In addition to the neuroprotective effect seen when IL-10 was added before LPS, we also observed that IL-10 can inhibit inflammation-induced neuronal degeneration when added up to 6 h after LPS exposure. The effectiveness in neuroprotection with post-treatment of IL-10 suggests that the release of this immune modulator after brain inflammation may play a critical role in suppressing the overactivation of microglia and preventing the excessive damage of neurons.

Increasing evidence has shown that oxidative stress plays a very important role in PD (Smith and Zigmond, 2003; Jackson-Lewis and Smythe, 2005). DA neurons in the substantia nigra are uniquely vulnerable to oxidative stress (Jenner, 1998; Greenamyre et al., 1999). The higher sensitivity of DA neurons to oxidative damage so far has been attributed in large part to their known reduced antioxidant capacity, increased accumulation of iron, and the concentration of neurochemicals such as dopamine that are prone to oxidative modification. The fact that IL-10 significantly inhibits the production of superoxide induced by LPS within a few minutes after stimulation led us to examine this factor in greater details by using PHOX-deficient mutant mice. The findings that IL-10 could significantly lessen the LPS-induced DA uptake reduction in cells from wild-type mice but has no significant protective effect on cells from PHOX−/− mice (Fig. 5A) strongly support the contention that the protective effect of IL-10 is most probably mediated through the inhibition of PHOX activity. Since we are measuring ROS production from primary midbrain cultures, we cannot rule out the possibility that cells other than microglia are producing superoxide and that PHOX is not the only enzyme that may play a role in ROS production. However, only microglia express LPS receptors and are activated by LPS; therefore, no other cell is likely to produce ROS upon stimulation with LPS. In addition, no extracellular superoxide production has been detected in cultures from PHOX−/− mice (Qin et al., 2004), suggesting that PHOX is the only enzyme involved in superoxide production in these cultures. Activation of PHOX in microglia not only increases the production of superoxide but indirectly increases the intracellular ROS concentration, possibly through the conversion of superoxide to H2O2, which is membrane permeable. Increase of intracellular ROS can intensify the activation of NF-κB, which leads to higher TNF-α and PGE2 production (Liu and Hong, 2003; Qin et al., 2004). In addition, it was reported that PHOX inhibitors prevented LPS/IFNγ-induced degradation of IκBα and, thus, inhibited the activation of NF-κB (Pawate et al., 2004). However, the ability to activate NF-κB-dependent genes such as TNF-α in PHOX−/− cells suggests that PHOX plays an important but not exclusive role in regulating inflammation in microglial cells. These data are consistent with the notion that PHOX is a major effector of neurotoxicity as well as the target of IL-10.

It has been found by a number of laboratories that IL-10 is a potent inhibitor of NF-κB activity (Ehrlich et al., 1998), primarily by inhibiting the transcriptional binding of NF-κB to κB sites in inflammatory mediators (Baldwin, 1996). However, others found that the direct anti-inflammatory effect of IL-10 is independent of the activity of NF-κB (Clarke et al., 1998) and rather targets the JAK/STAT pathway to selectively inhibit transcription of inflammatory genes (Murray, 2005). So far, the target of the IL-10-activated inhibitory effect still remains controversial. By inhibiting JAK1, a primary signaling kinase for the IL-10 receptor signaling, we demonstrate that the inhibition of NADPH oxidase by IL-10 is mediated through the inhibition of the JAK1 signaling pathway, which in turn prevents the translocation of cytosolic subunit p47phox to the membrane, rather than a direct inhibition of PHOX activity. The inhibition of both LPS- and phorbol 12-myristate 13-acetate-induced (data not shown) superoxide production by IL-10 and reversal of this inhibition by a JAK1 inhibitor lend further support of this possibility.

Microglia play a critical role in the pathogenesis of various neurological disorders, not only because of their role as proinflammatory cytokine-producing cells, but also by playing a major regulatory role in limiting inflammation (Gehrman et al., 1995). For example, it is known that resident microglial cells in the CNS produce IL-10 and express the IL-10 receptor; therefore, IL-10 may play an important autocrine role in the regulation of acute brain inflammation (Ledeboer et al., 2002). The potent anti-inflammatory and immune regulatory property of IL-10 suggests that it has the potential to be of major therapeutic value in the treatment of PD. In addition, IL-6 has also been described as a neuroprotectant and is released mainly from astrocytes after LPS stimulation (Bolin et al., 2005). It is possible that IL-6 and IL-10 have a similar
role in neuroprotection, and they might even act in concert to regulate chronic inflammation in the brain. However, both IL-6 and IL-10 have been shown to be most effective anti-inflammatory cytokines only in acute inflammatory responses. It has yet to be determined what role IL-10 plays in regulating chronic CNS inflammation in PD. In addition, the level of the immune response in the brain necessary to effectively control infections without resulting in neuropathology is yet to be understood. Consequently, much work remains to determine whether IL-10, either therapeutically delivered or produced in the CNS, plays an important anti-inflammatory role by limiting the activation of microglia and promoting a well-regulated immune response that is lacking during the pathophysiology of CNS disorders.

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References

Address correspondence to: Dr. Patrick M. Flood, The Comprehensive Center for Inflammatory Disorders, University of North Carolina, Chapel Hill, NC 27599-7455. E-mail: pat_flood@dentistry.unc.edu