Microglia-Mediated Neurotoxicity Is Inhibited by Morphine through an Opioid Receptor-Independent Reduction of NADPH Oxidase Activity

Li Qian, Kai Soo Tan, Sung-Jen Wei, Hung-Ming Wu, Zongli Xu, Belinda Wilson, Ru-Bin Lu, Jau-Shyong Hong and Patrick M. Flood

*J Immunol* 2007; 179:1198-1209; doi: 10.4049/jimmunol.179.2.1198

http://www.jimmunol.org/content/179/2/1198
Microglia-Mediated Neurotoxicity Is Inhibited by Morphine through an Opioid Receptor-Independent Reduction of NADPH Oxidase Activity

Li Qian,*† Kai Soo Tan,*§ Sung-Jen Wei,¶ Hung-Ming Wu,*∥ Zongli Xu,* Belinda Wilson,* Ru-Bin Lu,† Jau-Shyong Hong,‡ and Patrick M. Flood‡*  

Recent studies have shown that morphine modulates the function of glia cells through both opioid receptor dependent and independent mechanisms. However, the mechanism by which morphine regulates neuronal disorders through the alteration of microglia activity remains unclear. In this study, using rat primary mesencephalic neuron-glia cultures, we report that both l-morphine and its synthetic stereoisomerantior, d-morphine, an ineffective opioid receptor agonist, significantly reduced LPS- or 1-methyl-4-phenylpyridinium-induced dopaminergic neurotoxicity with similar efficacy, indicating a nonopioid receptor-mediated effect. In addition, using reconstituted neuron and glia cultures, subpicomolar concentrations of morphine were found to be neuroprotective only in the presence of microglia, and significantly inhibited the production of inflammatory mediators from LPS-stimulated microglia cells. Mechanistic studies showed that both l- and d- morphine failed to protect dopaminergic neurons in cultures from NADPH oxidase (PHOX) knockout mice and significantly reduced LPS-induced PHOX cytosolic subunit p47phox translocation to the cell membrane by inhibiting ERK phosphorylation. Taken together, our results demonstrate that morphine, even at subpicomolar concentrations, exerts potent anti-inflammatory and neuroprotective effects either through the inhibition of direct microglial activation by LPS or through the inhibition of reactive microgliosis elicited by 1-methyl-4-phenylpyridinium. Furthermore, our study reveals that inhibition of PHOX is a novel site of action for the mu-opioid receptor-independent effect of morphine. *The Journal of Immunology, 2007, 179: 1198 –1209.

Parkinson’s disease (PD)3 is a neurodegenerative movement disorder characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra and depletion of dopamine in the striatum, which leads to pathological and clinical abnormalities. The midbrain region that encompasses the substantia nigra is particularly rich in microglia (1), and recent studies have shown that dysregulated microglia activation can result in neurodegeneration (2, 3). Activation of nigral microglia and the subsequent release of proinflammatory neurotoxic factors, including IL-1β, TNF-α, PGE2, IL-6, NO, and superoxide, are considered key components of DA neuron degeneration in PD (2). The exact mechanism and kinetics by which microglia mediate neurotoxicity and the signals that control their proinflammatory response remain to be elucidated.

The naturally occurring form of morphine, l-morphine, is a potent narcotic analgesic and is used in the management of moderately severe to severe pain (4). The analgesic effect of l-morphine is believed to be mediated through the mu-opioid receptor, which is a member of the seven-transmembrane-spanning G-protein-coupled receptor family (4). Increasing in vitro and in vivo evidence have demonstrated wide-spectrum effects of morphine including anti-inflammatory, antifibrotic, antitumor, cardioprotective, and renoprotective (5–9). Morphine has also been shown to possess immunomodulation function, regulating the activities of immune cells including NK cells, T cells, B cells, monocytes, and polymorphonuclear leukocytes (10, 11). In addition, morphine has been reported to exert anti-inflammatory effects by reducing adhesion molecule expression, inhibiting cell trafficking and respiratory burst activity, as well as suppressing the activation of transcription factor NF-κB (12). Morphine can exert its potent anti-inflammatory effects at very low concentrations. Concentrations of morphine as low as 10−10–10−12 M can cause significant suppression of PMA or opsonized zymosan-stimulated superoxide release in monocytes (13, 14), whereas inhibition of phagocytosis of Cryptococcus neoforms by microglia was observed at 10−16 M (11).

These observations suggest that morphine has the potential to be used as an immune regulator to treat chronic inflammatory conditions, provided that these treatments do not lead to chronic dependence. Consequently, there is an urgent need to identify and understand the molecular mechanisms of morphine that function independently of the mu-opioid receptor, to prevent narcotic addiction resulting from treatment for these chronic inflammatory conditions. Recent studies have shown that morphine can work in

www.jimmunol.org

1 Abbreviations used in this paper: PD, Parkinson’s disease; MPP+, 1-methyl-4-phenylpyridinium; DA, dopaminergic; ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase; TH-IR, tyrosine hydroxylase immunoreactive; PHOX, NADPH oxidase; DCFH-DA, dichlorodihydrofluorescein diacetate; SOD, superoxide dismutase; MFI, mean fluorescent intensity.
both opioid receptor-dependent and -independent manners (11, 15–19). Further, our laboratory and others have found that naloxone and naltraxone, which are mu-opioid receptor antagonists, also show significant anti-inflammatory properties both in vitro and in vivo (20, 21). In addition, these compounds provide neuroprotection in ischemic injury, spinal cord injury, PD, and brain ischemia animal models (22–24). Although the analgesic effect of l-morphine is believed to be mediated through the mu-opioid receptor, whether the effects of the receptor antagonists naloxone and naltraxone are mediated through the mu-opioid receptor remains controversial. Therefore, we used both l-morphine, which has a high affinity to the mu-opioid receptor, and its synthetic enantiomer d-morphine, which does not bind to the mu-opioid receptor (25), to explore the underlying molecular mechanism of the protective effect of morphine on DA neurons in two in vitro models of PD. In this study, we show that submicromolar concentrations of both l-morphine and d-morphine significantly down-regulate LPS- or 1-methyl-4-phenylpyridinium (MPP+)-induced microglia activation with similar efficacy, clearly showing that the cellular mechanism of morphine-mediated neuroprotection is independent of the mu-opioid receptor pathway. Additionally, we show that the morphine-mediated molecular effects result in the inhibition of NADPH oxidase (PHOX) activity in activated microglia via the inhibition of the ERK signaling pathway.

**Materials and Methods**

**Animals**

NADPH oxidase-deficient (gp91phox−/−) and wild-type C57BL/6J (gp91phox+/+) mice were obtained from The Jackson Laboratory. The PHOX−/− mutation is maintained in the C57BL/6 background; thus, C57BL/6 mice were used as control animals. Breeding of the mice was performed to achieve timed pregnancy with the accuracy of ±0.5 days. Timed-pregnant F344 rats were obtained from Charles River Laboratories. Housing and breeding of the animals were performed in strict accordance with the National Institutes of Health guidelines.

**Reagents**

L-Morphine sulfate and d-morphine base were obtained from National Institute of Drug Abuse. LPS (Escherichia coli strain O111:B4) was purchased from Calbiochem. Cell culture reagents were obtained from Invitrogen (Life Technologies). [3H]DA (30 Ci/mmol) was obtained from PerkinElmer Life Sciences, and the mAb against the complement 3 receptor (OX42) was purchased from Chemicon International. The polyclonal anti-tyrosine hydroxylase Ab was a gift from Dr. John Reinhard (Glaxo, Durham, NC). The Vectastain ABC kit and biotinylated secondary Abs were purchased from Vector Laboratories. The fluorescence probe dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Calbiochem. Rabbit anti-phosphotyrosine was purchased from Upstate. FITC-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories. Rabbit anti-GAPDH was obtained from Abcam. Mouse anti-gp91phox was purchased from BD Transduction Laboratories.

**Microglial cell line**

The rat microglia HAPI cell line was a gift from Dr. James R. Connor (Department of Neuroscience and Anatomy, M. S. Hershey Medical Center, Hershey, PA) (26). Briefly, cells were maintained at 37°C in DMEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator with 5% CO2 and 95% air. The HAPI cell line was derived from rat primary microglia-enriched cultures and retains the phenotypic and morphological characteristics of microglia, including phagocytic activity, expression of the receptor for isocitrate B4 from *Griffonia simplicifolia*, as well as the specific microglial markers OX42 (complement 3 receptor) and glucose transport protein 5. Gene expression of TNF-α and inducible NO synthase (iNOS) can be induced by treatment with LPS.

**Primary mesencephalic neuron-glia cultures**

Neuron-glia cultures were prepared from the ventral mesencephalic tissues of embryonic day 14–15 rats or day 13–14 mice, as described previously (24, 27). Briefly, dissociated cells were seeded at 1 × 10^5/well and 5 × 10^2/well in poly-ornithine-coated 96-well and 24-well plates, respectively. Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air, in MEM containing 10% FBS, 10% horse serum, 1 g/l glucose, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 μM L-arginine, and 50 μM streptomycin. Seven-day-old cultures were used for drug treatments. 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, which consists 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 (28). Briefly, 24 h after seeding, cytosine β-arabinoside was added to a final concentration of 10 μM to suppress glial proliferation. Three days later, the medium was removed and replaced with maintenance medium. Cells were used for drug treatments 7 days after initial seeding. Routinely, the 7-day-old neuron-enriched cultures, which normally contain <0.1% microglia, and <3–5% astrocytes, were used for treatment. Among the neuronal population (Neu-N immunoreactive neurons), 2.7–3.9% were dopaminergic neurons (TH-IR-positive neurons).

**Primary microglia-enriched cultures**

Microglia-enriched cultures with a purity of >98%, were prepared from whole brains of 1-day-old F344 rat pups as described previously (29). For superoxide assays, 10^5 cells were grown overnight in 96-well culture plates before use.

**Rat astroglial cultures**

Mixed-glia cultures were first prepared from brains of 1-day-old F344 rat pups, as described previously (30). Briefly, mechanically dissociated brain cells (5 × 10^5) were seeded onto 150-cm2 culture flasks in DMEM containing 10% heat-inactivated FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 μM L-arginine, and 50 μg/ml streptomycin. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air, and medium was replenished 4 days after the initial seeding. Upon reaching confluence (usually 12–14 days later), microglia were detached from astrocytes by shaking the flasks at a speed of 180 rpm for 5 h. Astrocytes were then detached with trypsin-EDTA and seeded in the same culture medium. After five or more consecutive passages, cells were seeded onto 24-well plates (10^3/well) for experiments. Immunocytochemical staining of the astroglial cultures with either antilysosomal acidic protein or anti-OX42 Ab indicated an astrocyte purity of >98% and <2% of microglia contamination.

**[3H]DA uptake assay**

[3H]DA uptake assays were performed as described (29). Briefly, 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 CaCl2, 1.2 mM MgSO4, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). Cells were washed with mDivine and replaced with buffer three times, after which the cells were collected in 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Nonspecific DA uptake observed in the presence of mazindol (10 μM) was subtracted.

**Immunochemistry**

Immunostaining was performed as previously described (27). Briefly, formaldehyde (3.7%)-fixed cultures were treated with 1% hydrogen peroxide followed by sequential incubation with blocking solution, after which the cells were incubated overnight at 4°C with Abs against tyrosine hydroxylase (1/20,000). Cells were incubated with biotinylated secondary Ab for 2 h followed by incubation with ABC reagents for 40 min. Color development was performed as described (33), with diaminobenzidine for morphological analysis. The images were recorded with an inverted microscope (Nikon) connected to a charge-coupled device camera (DAGE-MTI) operated with the Meta-Morph software (Universal Imaging). For visual counting of TH-IR neurons, nine representative areas per well of the 24-well plate were counted under the microscope at ×100 by three individuals. The average of these scores was reported.

**Superoxide assay**

The production of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of the tetrazolium salt WST-1 (31, 32, 33).
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 morphine in HBSS (50 μM/well). Then, 50 μl of HBSS with and without SOD (50 U/ml, final concentration) were added to each well along with 50 μl of HBSS and with 50 μl of vehicle or LPS (10 ng/ml). To measure superoxide production induced by MPP⁺, 7-day-old mesencephalic neuron-glia cultures grown in 96-well plates were treated with morphine in the

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** L- and d-morphines protect neuron against LPS-induced neurotoxicity with similar efficacy. Rat primary mesencephalic neuron-glia cultures were seeded in a 24-well culture plate at 5 × 10⁵ cells/well and then pretreated with various concentrations of morphine for 30 min before the addition of 10 ng/ml LPS. Seven days later, the LPS-induced dopaminergic neurotoxicity was quantified by the [³H]DA uptake assay (A), the immunocytochemical analysis, including TH-IR neuron counts (B), and the representative pictures of immunostaining (C). Results were expressed as a percentage of the vehicle-treated control cultures and were the means ± SE. from three independent experiments in triplicate. *, p < 0.05; **, p < 0.01 compared with the LPS-treated cultures.

1200 MORPHINE PROTECTS DA NEURON AGAINST OXIDATIVE STRESS

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** L- and d-morphine protects neuron against MPP⁺ induced neurotoxicity. Different doses of morphine were added to the neuron-glial cultures for 30 min before the addition of 0.1 μM MPP⁺ treatment. The [³H]DA uptake measurements were performed 8 days after MPP⁺ treatment (A); the immunocytochemical analysis, including TH-IR neuron counts (B), and the representative pictures of immunostaining (C) were shown. Results were expressed as a percentage of the vehicle-treated control cultures and were the means ± SE. from four independent experiments in triplicate. *, p < 0.05; **, p < 0.01 compared with the LPS-treated cultures.
presence and absence of MPP⁺ or with vehicle alone in 150 µl of phenol red-free treatment medium. Four days after treatment, 50 µl of HBSS with and without SOD (50 U/ml, final concentration) were added to each well along with 50 µl of WST-1 (1 mM) in HBSS. Fifteen minutes later, OD₄₅₀ was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices). The difference in optical density observed in the presence and absence of SOD was considered to be the amount of superoxide produced, and results were expressed as the percentage of vehicle-treated control cultures.

**Intracellular reactive oxygen species (ROS) assay**

Intracellular ROS were determined by using a DCFH-DA assay as described previously with minor modifications (33). DCFH-DA enters cells passively and is deacetylated by esterase to nonfluorescent DCFH. DCFH reacts with ROS to form dichlorodihydrofluorescein; the fluorescent product 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 experimental value minus the value of the control group was used to determine the concentration of ROS.

**Real-time RT-PCR analysis**

For real-time quantitative PCR, DNA-free RNA was obtained using the RNeasy Mini Kit (Qiagen). We reverse-transcribed 1 µg of total RNA using random hexamers and Moloney murine leukemia virus reverse transcriptase. The SYBR green DNA PCR kit (Applied Biosystems) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time values normalized to GADPH. The relative differences between control and treatment groups were calculated and expressed as a percentage of the vehicle-treated control cultures and as the mean ± SE of three experiments performed in triplicate.

**Confocal microscopy**

HAPI cells seeded in dishes at 5 × 10⁴ cells/well were treated with LPS for 10 min in the presence and absence of morphine pretreatment for 30 min. Cells were fixed with 3.7% paraformaldehyde in PBS for 10 min. After a washing with PBS, cells were incubated with rabbit polyclonal Ab against p47⁰phox (0.5 µg/ml). Cells were then washed and incubated with FITC-conjugated goat anti-rabbit Ab. 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) equipped with LSM510 digital imaging software. The signal of p47⁰phox (FITC–p47⁰phox, green) and the merge view of cell morphology and p47⁰phox (phase plus FITC–p47⁰phox) are shown.

**Membrane fractionation and Western blot analysis**

Membrane fractionation was performed as described (34). 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 DTT, 1 mM PMSF, and 10 µg/ml each leupeptin, apro- tin, and pepstatin A), incubated on ice for 30 min, and then subjected to Dounce homogenization (20–25 stokes, tight pestle A). The lysates were

![FIGURE 3.](image-url) **FIGURE 3.** Microglia are essential for morphine-elicted neuroprotection. l- and d-morphines (10⁻¹⁴ M) were added to the following cultures before treatment with 0.1 µM MPP⁺. N, Neuron-enriched cultures; (N + 10%MG): reconstituted cultures by adding 10% of microglia to the neuron-enriched cultures; and (N + 50%AS): reconstituted cultures by adding 50% of astroglia to the neuron-enriched cultures. The [³H]DA uptake measurements were performed 8 days after the treatment. Results were expressed as a percentage of the vehicle-treated control cultures and were the means ± SE from four independent experiments in triplicate. *, p < 0.05 compared with the LPS-treated cultures.

![FIGURE 4.](image-url) **FIGURE 4.** Morphine inhibits both LPS- and MPP⁺-induced ROS production by activated microglia. Microglia-enriched cultures were seeded at a density of 1 × 10⁵ cells/well. Cells were pretreated with different concentrations of morphine for 30 min followed by the addition of LPS (10 ng/ml). The production of ROS included extracellular superoxide (A) and intracellular ROS (B). Extracellular superoxide was measured as SOD-inhibitable reduction of WST-1, and intracellular ROS was determined by probe DCFH-DA. Primary rat mesencephalic neuron-glia cultures were pretreated for 30 min with vehicle or morphine before treatment with 0.25 µM MPP⁺. Two and four days after MPP⁺ treatment, morphine was added again to the morphine-treated cultures. On day 4, the release of superoxide was determined as described above (C). Superoxide production was expressed as a percentage of the vehicle-treated control cultures and as the mean ± SE. of three experiments performed in triplicate.* p < 0.05; ** p < 0.01 compared with corresponding LPS or MPP⁺-treated cultures.
loaded onto sucrose in lysis buffer (final concentration, 0.5 M) and centrifuged at 1600 × g for 15 min; the supernatant above the sucrose gradient was centrifuged at 150,000 × g 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 4–12% Bis-Tris Nu-PAGE gel and transferred to polyvinylidene difluoride membranes.

**FIGURE 5.** Effects of morphine on LPS-induced mRNA expression of proinflammatory factors and microglial surface molecular expression. A, Microglia-enriched cultures seeded at 1 × 10⁶/well were pretreated with morphine for 30 min followed by the addition of LPS (5 ng/ml). The effect of morphine on LPS-induced iNOS, TNF-α, MIP-1α, IL-6, IL-1β, gp91 RNA mRNA expression was detected by real-time PCR 3 h after LPS stimulation as described in Materials and Methods. B, HAPI cells were pretreated with morphine (10⁻¹⁴ M) for 30 min followed by stimulation with LPS for 24 h. Expression of OX18 (MHC class I), OX6 (MHC class II), and costimulatory molecules CD40 and CD80 were detected by flow cytometry. The cells were analyzed on a FACS-Calibur, and the MFI of experimental groups was determined by subtracting the MFI of the isotype control from the MFI of the unstimulated or LPS-stimulated microglia. Data are representative of four separate experiments. *, p < 0.05; **, p < 0.01 compared with corresponding LPS-treated cultures; #, p < 0.05, ##, p < 0.01 compared with vehicle-treated control cultures.
considered statistically significant.

To discern whether both stereoenantiomers of morphine possess neuroprotective effect on DA neurons, we reconstituted these neuron-enriched cultures by adding 50% astrocytes in addition to DA and other neuronal processes, including the loss of neurites (Fig. 1). However, both l- and d-morphine (10−7 M) for 30 min and then stimulated with LPS for 7 days. The [3H]DA uptake assay showed that LPS treatment reduced the capacity of the cultures to take up [3H]DA by 47% compared with control cells treated with vehicle alone. This LPS-induced reduction in DA uptake was significantly inhibited by the addition of either the l or the d forms of morphine at concentrations as low as 10−14 M (Fig. 1A). Microscopic analysis of immunostained TH-IR neurons revealed that LPS treatment not only decreased the number of TH-IR neurons (Fig. 1C), which was reversed when cells were pretreated with either l- or d-morphine at concentrations of 10−7, 10−10, and 10−14 M. Taken together, these results indicate that both l- and d-morphines protect DA neurons from LPS-induced neuronal cell death and loss of function with similar efficacies.

To investigate whether morphine mediates its protective function directly on DA neurons, rather than inhibiting LPS-induced inflammatory response by glial cells, we treated neuron-glial cultures with MPP+, the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine which is known to kill DA neurons directly. Our previous work has shown that neuronal death induced by MPP+ is the result of both direct cytotoxic effects on the neuron and induced by MPP+ as well as reactive microgliosis induced by toxic factors released by dying neurons (2). After treatment with 0.2 μM MPP+ for 7 days, DA uptake was reduced to 30% in neuron-glial cultures (Fig. 2). However, both l- and d- forms of morphine were capable of significant but not complete protection from MPP+-mediated neurotoxicity (Fig. 2).

Microglia, but not astroglia, are the target of the neuroprotective effect of morphine on MPP+-induced neurotoxicity.

The results in Fig. 2 are consistent with the idea that the direct toxicity of MPP+ on DA neurons is resistant to the neuroprotective effects of morphine, whereas toxicity mediated by reactive microgliosis is inhibited by both the l and d forms of morphine with equal potency. To further investigate the cell type that mediates the neuroprotective effect of morphine, we treated cultures of rat midbrain lacking glial cells (neuron-enriched cultures, N) with MPP+ and measured DA uptake of neurons 7 days after treatment. DA uptake was reduced to ~45% in these neuron-enriched cultures (Fig. 3). However, both l- and d- forms of morphine did not significantly protect DA neurons from MPP+-induced toxicity. Because our standard mesencephalic neuron-glial cultures contain ~10% microglia and ~50% astrocytes in addition to DA and other neurons, we reconstituted these neuron-enriched cultures by adding either ~10% microglia (Fig. 3, N + 10% MG) or ~50% astrocytes (Fig. 3, N + 50% AS) back to these cultures (Fig. 3). We found that both l- and d-morphines failed to protect DA neurons from MPP+-induced toxicity in either neuron-enriched cultures or neuron-astroglia cultures. Interestingly, the presence of 50% astrocytes in neuron-enriched cultures showed some protective effects from MPP+-induced cytotoxicity when compared with cultures containing only neurons or neurons with only microglial cells, but this protective effect was morphine independent. In contrast, addition of either the l or d isomers of morphine to neuron-enriched cultures reconstituted with 10% of microglia showed

**Flow cytometry**

Microglial cells (HAPI) were used for the flow cytometry study. Cells were treated with LPS for 24 h in the presence and absence of morphine pretreatment for 30 min. The cells were removed from culture and washed twice with cold FACS buffer (saline with 5% normal goat serum). Microglia were incubated with FcR block for 30 min at 4°C and then incubated for 45 min at 4°C with FITC-conjugated Abs specific for OX18 (MHC class I), CD40, and PE-conjugated Abs specific for OX4 (MHC class II) and CD80 or the appropriate isotype control Abs (BD Pharmingen). After Ab binding, cells were washed and fixed. Fluorescence was analyzed on a FACSCalibur (BD Biosciences). The mean fluorescent intensity (MFI) of experimental groups was determined by subtracting the MFI of the isotype control from the MFI of the unstimulated or LPS-stimulated microglia.

**Neutralization of TNF-α or IL-6**

Neuron-glial cultures were used for Ab blocking experiments 7 days after initial seeding. Indicated concentrations of goat anti-rat TNF-α and anti-rat IL-6 Abs, or the isotype-matched goat IgG control Ab (R&D Systems) were added to the neuron-glial cultures concurrently with 10 ng/ml LPS, and [3H]DA uptake assays were then performed as described above 8 days after addition of Abs and LPS to measure the neurotoxicity.

**Statistical analysis**

Data were presented as the means ± SE. For multiple comparisons of groups, two-way ANOVA was used. Statistical significance between groups was assessed by paired Student’s t test, followed by Bonferroni correction using the JMP program (SAS Institute). A value of p < 0.05 was considered statistically significant.

**Results**

*Both l- and d-morphine are equipotent in protecting DA neurons against LPS- and MPP+ induced neurotoxicity*

To discern whether both stereoisomers of morphine possess neuroprotective properties, primary mesencephalic rat neuron-glial cultures were pretreated with either l- or d-morphine (10−18–10−7 M) for 30 min and then stimulated with LPS for 7 days. The [3H]DA uptake assay showed that LPS treatment reduced the capacity of the cultures to take up [3H]DA by 47% compared with control cells treated with vehicle alone. This LPS-induced reduction in DA uptake was significantly inhibited by the addition of either the l or the d forms of morphine at concentrations as low as 10−14 M (Fig. 1A). Microscopic analysis of immunostained TH-IR neurons revealed that LPS treatment not only decreased the number of TH-IR neurons (Fig. 1B) but also caused a loss of neuronal processes, including the loss of neurites (Fig. 1C), which was reversed when cells were pretreated with either l- or d-morphine at concentrations of 10−7, 10−10, and 10−14 M. Taken together, these results indicate that both l- and d-morphines protect DA neurons from LPS-induced neuronal cell death and loss of function with similar efficacies.

To investigate whether morphine mediates its protective function directly on DA neurons, rather than inhibiting LPS-induced inflammatory response by glial cells, we treated neuron-glial cultures with MPP+, the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine which is known to kill DA neurons directly. Our previous work has shown that neuronal death induced by MPP+ is the result of both direct cytotoxic effects on the neuron and induced by MPP+ as well as reactive microgliosis induced by toxic factors released by dying neurons (2). After treatment with 0.2 μM MPP+ for 7 days, DA uptake was reduced to 30% in neuron-glial cultures (Fig. 2). However, both l- and d- forms of morphine were capable of significant but not complete protection from MPP+-mediated neurotoxicity (Fig. 2).

Microglia, but not astroglia, are the target of the neuroprotective effect of morphine on MPP+-induced neurotoxicity.

The results in Fig. 2 are consistent with the idea that the direct toxicity of MPP+ on DA neurons is resistant to the neuroprotective effects of morphine, whereas toxicity mediated by reactive microgliosis is inhibited by both the l and d forms of morphine with equal potency. To further investigate the cell type that mediates the neuroprotective effect of morphine, we treated cultures of rat midbrain lacking glial cells (neuron-enriched cultures, N) with MPP+ and measured DA uptake of neurons 7 days after treatment. DA uptake was reduced to ~45% in these neuron-enriched cultures (Fig. 3). However, both l- and d- forms of morphine did not significantly protect DA neurons from MPP+-induced toxicity. Because our standard mesencephalic neuron-glial cultures contain ~10% microglia and ~50% astrocytes in addition to DA and other neurons, we reconstituted these neuron-enriched cultures by adding either ~10% microglia (Fig. 3, N + 10% MG) or ~50% astrocytes (Fig. 3, N + 50% AS) back to these cultures (Fig. 3). We found that both l- and d-morphines failed to protect DA neurons from MPP+-induced toxicity in either neuron-enriched cultures or neuron-astroglia cultures. Interestingly, the presence of 50% astrocytes in neuron-enriched cultures showed some protective effects from MPP+-induced cytotoxicity when compared with cultures containing only neurons or neurons with only microglial cells, but this protective effect was morphine independent. In contrast, addition of either the l or d isomers of morphine to neuron-enriched cultures reconstituted with 10% of microglia showed...
significant protective effects. These results demonstrate that microglia, but not astroglia or neurons, serve as the target of morphine-mediated neuroprotection against MPP+/H11001-induced toxicity, and that both \(l\)- and \(d\)-morphine are capable of inhibiting this reactive microgliosis.

Effects of morphine on the expression of proinflammatory factors and microglial surface molecules after LPS stimulation

Overactivation of microglia through the direct effects of LPS on microglia or by reactive microgliosis via the death of neurons produces an array of proinflammatory mediators, including ROS, which is the pivotal product mediating inflammation-related neurotoxicity (35, 36). To test the effect of \(l\)- and \(d\)-morphines on the generation of ROS by microglia, cells were pretreated with morphine, then exposed to either LPS or MPP+/H11001. Both \(l\)- and \(d\)-morphine significantly reduced LPS-mediated extracellular superoxide production (Fig. 4A) and intracellular ROS concentrations (Fig. 4B). Likewise, treatment of cultures with morphine significantly inhibited the MPP+/H11001-induced superoxide production resulting from reactive microgliosis (Fig. 4C). Taken together, these results demonstrated that morphine can inhibit ROS free radical production by microglial cells induced either directly by LPS stimulation or indirectly by MPP+/H11001-mediated reactive microgliosis.

We also investigated the effect of morphine on other inflammatory markers by measuring mRNA expression of proinflammatory mediators in microglia-enriched cultures stimulated with LPS. We used LPS to activate microglia because reactive microgliosis induced by MPP+/H11001 does not result in measurable NO or proinflammatory cytokine production in neuron-glial mixed cultures. Both \(l\)- and \(d\)-morphine at subpicomolar concentrations significantly inhibited LPS-induced increases in the expression of iNOS, TNF-\(\alpha\), MIP-1\(\alpha\), IL-6, IL-1\(\beta\), and gp91 mRNA as measured by real-time PCR (Fig. 5A). Because it is known that LPS up-regulates MHC class I, MHC class II, and costimulatory molecules CD40 and CD80 in microglia (37), we also examined whether morphine can regulate these surface markers expression by flow cytometry. Fig. 5B shows that 24 h after LPS stimulation, significant increases of MHC class I expression were detected. Pretreatment with either \(l\)- or \(d\)-morphine (10\(^{-14}\) M) significantly inhibited LPS-induced MHC class I expression. In addition, both isomers slightly reduced CD40 expression, but the reduction did not reach a statistically significant level. On the other hand, morphine treatment with either enantiomer did not show significant effects on LPS-induced MHC class II and CD80 expression.
PHOX plays an important role in morphine-mediated protection against LPS-induced neuron degeneration

The results mentioned above indicate that morphine significantly reduced both LPS- and MPP+/H11001−/H11001-induced production of superoxide. To further investigate the role of ROS in morphine-elicited neuroprotection, we used neuron-glia cells from mice deficient in the catalytic subunit of PHOX, which is the key enzyme required for the production of ROS. Neuron-glia cultures were prepared from PHOX−/−/− knockout and PHOX+/+ wild-type mice and, as shown in Fig. 6A, LPS treatment of neuron-glia cultures prepared from PHOX−/−/− mice substantially reduced [3H]DA uptake. Similarly, both l- and d-morphine at 10−14 M significantly attenuated the decrease in [3H]DA uptake (Fig. 6A). In contrast, although LPS treatment also showed a significant albeit smaller reduction in [3H]DA uptake capacity in PHOX−/− mice, both l- and d-morphines failed to show any protective effect on DA neurons from these mice. We also observed that LPS-induced TNF-α production in PHOX−/− mice is significantly less than that seen in PHOX+/+ mice (Fig. 6B) and that both enantiomers of morphine were able to significantly reduce TNF-α production in PHOX+/+ mice. In contrast, neither l- nor d-morphines were capable of significantly reducing TNF-α production in PHOX−/− mice (Fig. 6B). These results demonstrate that LPS can induce some degree of neurotoxicity in PHOX−/− cell cultures, presumably through the production of other toxic mediators such as TNF-α, IL-6, and NO. Therefore, our data support the idea that inhibition of ROS production and subsequent TNF-α production is associated with the neuroprotective effect of morphine at submicromolar concentrations.

Morphine inhibits LPS-induced translocation of cytosolic subunit of PHOX p47phox to the cell membrane

It has been reported that activation of PHOX requires phosphorylation and subsequent translocation of the cytosolic component p47phox, together with other PHOX cytoplasmic subunits to the cell membrane, where they complex with cytochrome b558 to assemble into an active enzyme (38). Because morphine significantly inhibits superoxide production induced by either LPS or MPP+, we sought to determine whether morphine inhibits PHOX activation by preventing the translocation of p47phox from the cytosol to the membrane after LPS stimulation. Using confocal microscopy, we observed that LPS initiated the translocation of cytosolic

FIGURE 8. Western blot analysis of MAPK phosphorylation. Enriched microglia were treated with LPS (10 ng/ml) in the presence or absence of morphine (10−14 M) for 5 or 30 min, cells were then harvested, and the levels of phosphorylated MAPK (ERK1/2, p38, and JNK1/2) relative to total MAPK were determined by Western blot using specific Abs against phosphorylated or total MAPK, respectively. Representative Western blots for ERK1/2 (A), p38, and JNK (B) phosphorylation are shown from three independent experiments.
translocation. In cells treated with vehicle alone (Fig. 7A), the toxicity. Various concentrations of anti-TNF-α were added to the neuron-glia cultures concurrently with 10 ng/ml LPS. Eight days after LPS stimulation, [3H]DA uptake assays were performed to measure neurotoxicity. Results were expressed as a percent of the vehicle-treated control cultures and were the means ± SE from three independent experiments in triplicate. *p < 0.05 compared with the LPS-treated cultures.

p47phox to the membrane (Fig. 7A, III and IV), and pretreatment of cells with either l-morphine (Fig. 7A, V and VI) or d-morphine (Fig. 7A, VII and VIII) at 10⁻¹¹ M significantly attenuated this translocation. In cells treated with vehicle alone (Fig. 7A, I and II) or 10⁻¹⁰ M morphine (data not shown) in the absence of LPS stimulation, p47phox remained localized primarily in the cytosol. Consistent with the result of the confocal microscopy study, Western blot assay clearly showed an increase in the immunoreactivity of p47phox in the membrane fraction of HAPI cells 10 min after LPS treatment, and this increase in the immunoreactivity was significantly blocked in the presence of morphine (Fig. 7B). Therefore, one mechanism by which morphine inhibits superoxide production in microglial cells is through the inhibition of p47phox translocation to the cell membrane after LPS stimulation.

Morphine suppresses LPS-induced ERK1/2 phosphorylation

It has been shown that translocation of p47phox to the cell membrane in activated neutrophils requires activation of the MAPK pathway, resulting in the phosphorylation and subsequent translocation of p47phox to the membrane (39–41). Thus, we investigated whether the inhibitory effect of morphine on the activation of PHOX enzyme by LPS is mediated through the ERK1/2, p38, or JNK pathways. Microglia cells were pretreated with subpicomolar concentration of morphine followed by stimulation with LPS for 5–30 min. ERK1/2, p38, and JNK pathways. Microglia cells were pretreated with subpicomolar concentration of morphine followed by stimulation with LPS for 5–30 min. ERK1/2, p38, and JNK were all phosphorylated in microglia after LPS stimulation, and the phosphorylation of p44 ERK (ERK1) but not p42 ERK (ERK2) was significantly reduced by pretreatment with 10⁻¹⁰ M of either l- or d-morphine (Fig. 8A) at 5 min after LPS treatment. Pretreatment with either l- or d-morphine significantly reduced both ERK1 and ERK2 phosphorylation 30 min after LPS treatment (Fig. 8A). Interestingly, d-morphine showed a stronger inhibitory effect on ERK1/2 phosphorylation compared with l-morphine at this time point. In contrast, neither l- nor d-morphine inhibited LPS-induced phosphorylation of p38 and JNK at the time points studied (Fig. 8B). These results suggest that morphine-mediated inhibition of p47phox phosphorylation and translocation occurs in an ERK (most likely ERK1)-dependent manner in microglia.

TNF-α and IL-6 partially contribute to LPS-induced neurotoxicity

The above-mentioned results indicate that PHOX plays an important role in LPS-induced neurotoxicity. However, there is a highly significant PHOX-dependent neurotoxicity after LPS stimulation in neuron-glia cultures (Fig. 6A). Thus, we further studied the role of other proinflammatory factors in LPS-induced neuronal death. Various concentrations of anti-TNF-α or IL-6 Abs were added to the neuron-glia cultures to neutralize these two proinflammatory cytokines, respectively. Our results (Fig. 9) indicated that both anti-TNF-α Ab (10 μg/ml) and anti-IL6 Ab (3 and 10 μg/ml) significantly reduced LPS-induced neurotoxicity. Therefore, TNF-α and IL-6 partially contribute to LPS-induced neuronal damage.

Discussion

This study demonstrates that both isomers of morphine, at subpicomolar concentrations, exhibit potent anti-inflammatory and neuroprotective effects and operate through a novel mechanism that is independent of conventional opioid receptors. Using the well-established LPS- and MPP⁺-mediated PD in vitro models, we found that: 1) morphine shows strong efficacy as a neuroprotective agent; 2) both enantiomers are equally potent in neuroprotection at femtomolar concentrations; 3) morphine is neuroprotective in both the LPS and MPP⁺ models of neurotoxicity, and that this protection is mediated through the inhibition of microglia overactivation rather than via a direct effect on neurons or astrocytes; 4) mechanistically, one novel and important finding from this paper is that PHOX is the site of action mediating the anti-inflammatory effect of morphine; 5) finally, we have elucidated the signaling pathway by which morphine affects PHOX activation by showing that morphine reduced the LPS-induced PHOX cytosolic subunit p47phox translocation to the cell membrane by inhibiting ERK phosphorylation. Our studies strongly suggest that anti-inflammatory activity underlying the neuroprotective effect of morphine. This notion was supported by the finding that both l- and d-morphine showed potent anti-inflammatory effects in the subpicomolar doses, which is much lower than the nanomolar concentrations required for the activation of mu-opioid receptor (42).

Although the analgesic effect of l-morphine is believed to be mediated through the mu-opioid receptor, it is not clear if the anti-inflammatory functions of morphine are mu-opioid receptor dependent. Our previous studies have shown that other morphinan compounds, such as dextromethorphan (43), 3-hydroxydextromethorphan (44), as well as morphine antagonists naloxone and naltrexone, are also highly neuroprotective both in vivo and in vitro (20, 21). The results from this study strongly suggest that the anti-inflammatory effects of morphine are mu-opioid receptor independent given that d-morphine, which does not bind to the mu-opioid receptor, was equally and in some cases more effective than l-morphine in protecting DA neurons against LPS or MPP⁺-induced neurotoxicity. More recently, Tseng et al. (19) showed that both l- and d-morphines also possess potent antianalgesic effects on mouse spinal cord responses at extremely low concentrations. Although the nature and activity of this novel receptor have yet to be characterized, results from our studies and those of Tseng et al. suggest that the target of the activity of morphine is mediated through the alteration of glial cell activity rather than on neurons.
Interestingly, our results showed that morphine has protective effects in both the LPS and the MPP\textsuperscript{+} model of PD even though the target of these two agents is different. LPS leads to the direct activation of microglia, which results in death of DA neurons through the production of inflammatory mediators. Our previous study has demonstrated that removal of microglia but not astroglial cells from neuron-glia cultures prevents the neurotoxicity of LPS (28). However, more compelling data for the crucial role of microglia in neurotoxicity and the target of morphine action come from the use of MPP\textsuperscript{+} as the neurotoxic agent. Even though MPP\textsuperscript{+} directly targets DA neurons for toxicity, morphine also provides significant although more modest protective effects to these cultures. We demonstrate that this effect is due to the fact that a significant amount of the MPP\textsuperscript{+}-mediated neurotoxicity requires microglia cells, suggesting that MPP\textsuperscript{+} works both to directly kill a subset of DA neurons but also indirectly, by activating microglia, which contributes to additional neurotoxicity by producing toxic inflammatory mediators. Increasing evidence has shown that the microglia response to neuronal damage is a long-term self-propelling process (35, 45, 46), where a small percentage of dying or damaged neurons release proinflammatory signals that activate microglia through either the release of soluble factors or the loss of cell inhibition between neurons and microglia (47–50). The activated microglia then damage the remaining DA neurons, thus creating a continuous cycle of reactive microgliosis that mimics a chronic progressive neurodegeneration such as observed in PD. Reports from our laboratory and others have shown that MPP\textsuperscript{+} can cause reactive microgliosis and that oxidative stress is involved in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine/MPP\textsuperscript{+} induced neurotoxicity (35, 48, 51). On the basis of our current evidence, we propose that the anti-inflammatory effect of morphine is capable of inhibiting both LPS-induced microglial activation and MPP\textsuperscript{+}-induced reactive microgliosis, and its ultimate result is to suppress the inflammation that mediates chronic neurodegeneration in PD.

We further propose that morphine inhibits microglia-induced neurotoxicity by inhibiting the activation of PHOX. Support for this conclusion comes from the following sets of observations: 1) our previous studies have shown that inhibiting microglia production of superoxide was critical in eliciting a neuroprotective effect against microglia-mediated inflammatory damage, indicating that superoxide is a key element associated with the degeneration of DA neurons (24); 2) morphine significantly inhibits superoxide production induced by LPS by preventing translocation of the cytosolic subunit p47\textsuperscript{phox} to the cellular membrane (Fig. 7), which is required for the activity of PHOX (52); 3) we found that subpicomolar concentrations of both l- and d-morphine could significantly reduce LPS-induced DA uptake reduction in wild-type mice but did not provide significant protection to cells derived from PHOX\textsuperscript{−/−} mice (Fig. 6A); 4) the production of intracellular ROS and TNF-\(\alpha\) are reduced in PHOX\textsuperscript{−/−} mice, suggesting that PHOX can indirectly regulate intracellular ROS concentration and TNF-\(\alpha\) production, presumably through the reduction of NF-\(\kappa\)B activity (28, 53). This reduction in NF-\(\kappa\)B activity would likely explain our results which found that morphine also reduces the transcription of proinflammatory genes such as iNOS, IL-1\(\beta\), IL-6, TNF-\(\alpha\), MIP-1\(\alpha\), gp91\textsuperscript{phox}, and MHC class I, all of which are regulated by NF-\(\kappa\)B (54–56).

Although the above-mentioned results show that PHOX plays a role in LPS-mediated neurotoxicity, there is a highly significant PHOX-independent neurotoxicity induced by LPS. We have previously reported that TNF-\(\alpha\) in conjunction with IFN-\(\gamma\) produced neurotoxicity in neuron-glia cultures (57) and that inhibition of iNOS activity and NO production prevents LPS-induced neurotoxicity, indicating that NO production is also involved in LPS-induced neurodegeneration (58). Moreover, we recently found that a systemic injection of TNF-\(\alpha\) can cause neuronal death (59) and the production of superoxide (L. Qin and J.-S. Hong, unpublished observations) in the brain. Furthermore, our studies indicated that after neutralization of endogenous TNF-\(\alpha\) or IL-6, LPS-induced neurotoxicity was significantly decreased (Fig. 9). These observations are consistent with the notion that LPS-elicited neuronal damage is resulted from the combined toxic effects from an array of pro-inflammatory factors, such as free radicals, cytokines, and prostaglandins released by microglia. Therefore, PHOX plays an important, but not exclusive role in mediating inflammation-related neurotoxicity.

While investigating the mechanism by which morphine inhibits PHOX activity, we found that both l- and d-morphines inhibited the phosphorylation of ERK 1/2 as early as 5 min after LPS stimulation. Several studies have already shown that inhibitors of the ERK and p38 pathways inhibit the respiratory burst in fMLP, a chemoattractant released from bacteria, activated neutrophils (39–41). In our previous study, we show that p47\textsuperscript{phox} translocation to the cell membrane occurred 10 min after LPS stimulation (28). In this study, we provide evidence that morphine down-regulates ERK phosphorylation within 5 min of LPS exposure, which correlated with the inhibition of superoxide production and p47\textsuperscript{phox} translocation. Interestingly, we observed that although morphine can inhibit ERK phosphorylation for at least 30 min after LPS treatment, morphine has no effect on the phosphorylation of either p38 or JNK. Moreover, morphine treatment can lower the basal levels of phospho-p38 and ERK, however, the mechanism for the lower expression of this basal level of phosphorylation is not known. Because we repeatedly do not see a significant effect of morphine on the level of the LPS-induced phospho-p38 signal, these results demonstrate that the major effect of morphine after LPS stimulation is to inhibit ERK but not p38 phosphorylation. Although it has been previously reported that protein kinase C is involved in the regulation of p47\textsuperscript{phox} phosphorylation and its translocation to the cellular membrane in monocytes (60, 61), Neither l- nor d-morphine exhibited any inhibitory effect on protein kinase C phosphorylation (data not shown). Taken together, our results suggest that morphine at 10\textsuperscript{−11} M inhibits LPS-induced ROS production in microglia by inhibiting p47\textsuperscript{phox} translocation, which is regulated by ERK signaling pathway.

Increasing evidence from both in vivo and in vitro studies suggest that morphinans possess important immunomodulatory potential (62). Morphinans are a group of compounds structurally similar to morphine, but lacking the E-ring found in the naturally occurring opioids, as well as the 6-OH and the 7,8-double bond. Our studies and those of others have shown that these compounds work at very low concentrations, to inhibit both cellular activation and microglial cell-mediated neurotoxicity. More importantly, the dextrorotatory form of this compound, which has no affinity or effect on the mi-opioid receptors and therefore does not cause addiction, is equally if not more effective than its l form in mediating anti-inflammatory effects on microglia (62). Given the effectiveness of these compounds at such low concentrations and the broad spectrum of activity in inhibiting inflammatory responses, these compounds appear to have significant potential in the treatment of chronic inflammatory disorders. In addition, PHOX is an ideal target for the development of anti-inflammatory drugs because of its central role in tissue destruction, through the production of ROS, as well as its role in amplifying the production of other proinflammatory factors, such as TNF-\(\alpha\), PGE\textsubscript{2}, and NO (2). In addition, the expression of PHOX is highly cell type specific, with high expression in cells of the monocytic lineage. However, since these proinflammatory neurotoxic factors also exhibit immunoregulatory

The Journal of Immunology
functions necessary for normal immune responses, the use of these compounds to regulate inflammatory stimuli must be carefully studied to determine their efficacy for long-term usage.

Acknowledgments
We thank Dr. Jie Liu at National Institute of Environmental Health Sciences for his help in performing the real-time PCR studies and thank Dr. Brian Mill at the same institution for his help in editing this paper.

Disclosures
The authors have no financial conflict of interest.

References


