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# Overexpression of Heme Oxygenase-1 Protects Dopaminergic Neurons against 1-Methyl-4-Phenylpyridinium-Induced Neurotoxicity

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## ABSTRACT

Heme oxygenase-1 (HO-1) is up-regulated in response to oxidative stress and catalyzes the degradation of pro-oxidant heme to carbon monoxide (CO), iron, and bilirubin. Intense HO-1 immunostaining in the Parkinsonian brain is demonstrated, indicating that HO-1 may be involved in the pathogenesis of Parkinsonism. We here locally injected adenovirus containing human *HO-1* gene (Ad-HO-1) into rat substantia nigra concomitantly with 1-methyl-4-phenylpyridinium ($MPP^+$). Seven days after injection of $MPP^+$ and Ad-HO-1, the brain was isolated for immunostaining and for measurement of dopamine content and inflammatory cytokines. It was found that overexpression of HO-1 significantly increased the survival rate of dopaminergic neurons; reduced the production of tumor necrosis factor $\alpha$ (TNF-$\alpha$) and interleukin-1$\beta$ (IL-1$\beta$) in substantia nigra; antagonized the reduction of striatal dopamine content induced by $MPP^+$; and also up-regulated brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) expression in substantia nigra. Apomorphine-induced rotation after $MPP^+$ treatment was also inhibited by Ad-HO-1. On the other hand, inhibition of HO enzymatic activity by zinc protoporphyrin-IX facilitated the $MPP^+$-induced rotatory behavior and enhanced the reduction of dopamine content. HO-1 overexpression also protected dopaminergic neurons against $MPP^+$-induced neurotoxicity in midbrain neuron-glia cocultures. Overexpression of HO-1 increased the expression of BDNF and GDNF in astrocytes and BDNF in neurons. Our results indicate that HO-1 induction exerts neuroprotection both in vitro and in vivo. Pharmacological or genetic approaches targeting HO-1 may represent a promising and novel therapeutic strategy in treating Parkinsonism.



Heme oxygenase (HO) is an enzyme that degrades intracellular heme to free iron, carbon monoxide (CO), and biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase, and free iron is sequestered by ferritin (Tenhunen et al., 1969). HO can be inhibited by synthetic enzyme inhibitors, zinc or tin protoporphyrin-IX (ZnPPIX or SnPPIX) (Drummond and Kappas, 1981). HO-1, a 32-kDa cellular stress response protein (also known as Hsp32) can be rapidly induced under oxidative challenge and other noxious stimuli in the brain or other tissues (Le et al., 1999). In normal brain, the level of HO-1 is rather low (Schipper et al., 1995; Baranano et al., 2002). On the other hand, HO-2 is constitutively expressed and does not respond to environmental stress (Schipper, 2000; Kim et al., 2004). Under stress conditions, neuronal and non-neuronal cells in the brain increase the synthesis of HO-1, indicating that HO-1 plays an important role in response to stress (Schipper, 2000; Ryter et al., 2006). The end products from HO enzymatic activity, CO and bilirubin, exert many biological functions (Ryter et al., 2006). CO is an endogenous gaseous molecule that activates soluble guanylate cyclase at a lower rate than nitric oxide and plays significant role in antiapoptosis, anti-inflammation, antiproliferation, neurotransmission, and vasodilatory actions (Otterbein et al., 2003). On the other hand, bilirubin

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**ABBREVIATIONS:** HO, heme oxygenase; PPIX, protoporphyrin-IX; ROS, reactive oxygen species; PD, Parkinson's disease; $MPP^+$, 1-methyl-4-phenylpyridinium; Ad, adenovirus; TNF, tumor necrosis factor; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BDNF, brain-derived neurotrophic factor ; GDNF, glia cell line-derived neurotrophic factor; TH, tyrosine hydroxylase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; HPLC, high-performance liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; moi, multiplicity of infection; PCR, polymerase chain reaction; CNS, central nervous system; $[Ru(CO)_3Cl_2]_2$, tricarbonyldichlororuthenium (II) dimer; GFAP, glial fibrillary acidic protein.

acts as reactive oxygen species (ROS) scavenger and exerts strong anti-inflammatory function (Baranano et al., 2002).

Parkinson's disease (PD) is a common movement disorder of uncertain etiology and is pathogenically characterized by the progressive degeneration of dopaminergic neurons in substantia nigra and the subsequent loss of their projecting nerve fibers in the striatum (Hornykiewicz and Kish, 1987). Several mechanisms have been proposed to explain the pathogenesis of PD, including the production of ROS, which is generated by dopamine auto-oxidation or mitochondrial energy metabolism in midbrain, $\alpha$-synuclein deposition, or mitochondrial dysfunction (Olanow, 1990; Valente et al., 2004). The synthetic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropryidine or 1-methyl-4-phenylpyridinium ($MPP^+$) has been widely used for experimental PD animal models and also in cell cultures to cause the death of dopaminergic neurons. Once $MPP^+$ enters the dopaminergic neurons, it inhibits mitochondrial complex I activity (Storch et al., 2004). Schipper et al. (1998) demonstrated that HO-1 expression in substantia nigra but not in other brain regions of PD specimens is 4-fold higher than age-matched control subjects, suggesting that the substantia nigra is bearing oxidative stress. The HO-1 up-regulation may thus play some roles in neuropathology of Parkinsonism. Smith et al. (1993) found that the metalloporphyrin inducers of HO, cobalt and tin protoporphyrinIX, not only increase HO-1 induction but also increase the expression of metallothioneins and ferritin. We here up-regulated HO-1 expression specifically via a replication-defective adenoviral (Ad) vector in $MPP^+$-induced rat model of PD to investigate the neuroprotective action of HO-1. The effects of HO-1 on both in vivo animal models and in vitro cell cultures were explored and discussed.

## Materials and Methods

**Preparation of Recombinant Adenovirus.** Replication-defective empty Ad and recombinant Ad carrying human *HO-1* gene (Ad-HO-1) were kindly provided by Dr. Lee-Young Chau (Academia Sinica, Taiwan). Virus was prepared as described previously (Juan et al., 2001). In brief, virus was amplified in QBI-293 cells and the number of plaque-forming units (PFU) cultured in 21 days was used for determining the titer of Ad and Ad-HO-1 stocks.

**Intrasubstantia Nigra Injection.** Wistar rats weighing approximately 350 g were used. Rats received unilateral injections of $MPP^+$ (8 $\mu$g; Sigma, St. Louis, MO) with or without Ad-HO-1 ($5 \times 10^7$ PFU in 1 $\mu$l of buffer containing 10 mM Tris, pH 8.0, 2 mM $MgCl_2$, and 4% sucrose) or ZnPPIX (3 pg; Sigma, St. Louis, MO) into the substantia nigra. Stereotaxic coordinates for substantia nigra pars compacta were lateral, +2.0 mm; anteroposterior (from the bregma point), −5.3 mm; and dorsoventral, +7.8 mm (Sindhu et al., 2006). The solution was injected into the substantia nigra with a 10-$\mu$l Hamilton syringe coupled to a motorized injector (Stoelting, Wood Dale, IL) at a rate of 0.2 $\mu$l/min, and the needle was left in situ for at least 5 min after injection. The rats were sacrificed at different time intervals after injection as indicated under *Results*.

**Apomorphine-Induced Rotation and Cytokine Measurement.** Seven days after $MPP^+$ administration, the effect of apomorphine (5 mg/kg, i.p.; Sigma, St. Louis, MO) on motor asymmetry was examined for 30 min. Rotation to the lesioned side was measured. The net rotation asymmetry score was expressed as full body turns per minute. The rats were sacrificed after the behavioral test, and the substantia nigra was isolated. Total protein extraction from substantia nigra tissue of rat was used to measure the levels of TNF-$\alpha$ and IL-1$\beta$ by using an ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

**Immunohistochemistry.** Brain tissue sections (30 $\mu$m thickness) were pretreated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity before incubation of primary antibody. The sections were then incubated with 10% bovine serum albumin and 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 1 h, and then incubated overnight at 4°C with the following primary antibody: TH (1:3000; Calbiochem Inc., San. Diego, CA), HO-1 (1:600; StressGen Biotechnologies, Victoria, BC, Canada), BDNF or GDNF (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) in PBS. The sections were subsequently incubated for 1 h with the biotinylated secondary antibody and then for 30 min with avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories. Burlingame, CA). Finally, the labeling was revealed by treatment with 0.01% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine (Sigma). Tissue sections were then washed in PBS and mounted on slides.

For double-immunolabeling studies, the brain sections were stained with primary antibodies against BDNF, GDNF, GFAP, CD11b, or tyrosine hydroxylase (TH) and then with Alexa Fluor 488- or Alexa Fluor 543-conjugated goat anti-rabbit or anti-mouse as the secondary antibody (1:600; Invitrogen, Carlsbad, CA). The confocal images were obtained at excitation wavelengths of 488 and 543 nm, respectively (model SP2 TCS; Leica, Heidelberg, Germany).

**Quantification of Nigral $TH^+$ Neuron.** Immunohistochemistry of coronal sections of substantia nigra was performed as described above. $TH^+$ neurons were counted every tenth section (30 $\mu$m for each section) in both sides of substantia nigra pars compacta using Zeiss Axioplan light microscopy in a blind manner as described previously (Lo Bianco et al., 2002). Data were expressed as the total number of $TH^+$ neurons throughout the six representative sections.

**Western Blotting.** Substantia nigra and striatal tissues dissected from rats were prepared as described previously (Roberts and Kapur, 1977). The protein expression levels of HO-1, HO-2, BDNF, and GDNF were determined by Western blotting. Samples obtained from cells or tissues were homogenized in radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride, 25 $\mu$M leupeptin, and 1 $\mu$g/ml aprotinin. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). Protein at 30∼50 $\mu$g was separated by SDS-PAGE using a 10∼15% resolving gel under reducing conditions and electrotransferred onto Immobilon-P membrane (Millipore, Billerica, MA). After being blocked with 5% nonfat milk in 0.5% Tween 20 in 20 mM Tris and 137 mM NaCl for 1 h at room temperature, the membranes were incubated overnight at 4°C with rabbit anti-HO-1 (1:5000), anti-HO-2 (1:5000; StressGen Biotechnologies, Victoria, Canada), anti-BDNF (1:3000), or anti-GDNF (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies diluted in 0.5% Tween 20 in 20 mM Tris and 137 mM NaCl. The membranes were probed with a mouse anti-$\alpha$-tubulin antibody as a standard (1:10,000; Santa Cruz Biotechnology). The blots were then incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:20,000; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Protein bands were detected using the ECL Western Blotting Substrate (Pierce, Rockford, IL) and were estimated using the Image Analysis Program Labwork 4.5 (UVP, Inc., Upland, CA).

**Measurement of Dopamine Content by High-Performance Liquid Chromatography (HPLC).** The striatum was dissected from the cerebral hemisphere, frozen in liquid nitrogen, and stored at −80°C until used. Each striatum was weighed and homogenized in 0.1 N perchloric acid and then placed on ice for 1 h. The homogenate were then centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was analyzed for dopamine, DOPAC, and HVA contents with Waters HPLC system and electrochemical detector (Waters, Milford, MA). Standards of dopamine, DOPAC, and HVA (grade HPLC; Sigma) were used for comparison.

**Midbrain Culture.** Neuron-glia cocultures from the midbrain of E14 rats were prepared as described previously with some modification (Chen et al., 2006). In brief, the ventral portion of the midbrain was removed in sterile, ice-cold $CaCl_2$-, $MgCl_2$-, and $MgSO_4$-free

Hanks' balanced salt solution (Invitrogen, Carlsbad, CA). The tissues were cleaned, minced, and mechanically dissociated by passage through a flame-polished Pasteur pipette. Dissociated cells were seeded in DMEM (Invitrogen) with 10% fetal bovine serum (FBS; Biological Industries, Grand Island, NY), 4500 mg/liter glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were seeded at $3.5 \times 10^5$/well on poly-D-lysine-coated 48-well plates. The cultures were kept in a humidified chamber at 37°C in a 5% $CO_2$ atmosphere. Twenty-four hours after plating, the cells were changed to minimal essential medium (Invitrogen) with 2% FBS, 2% horse serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The day 4 cultures were preinfected with Ad-HO-1/Ad (10 moi) or pretreated with anti-BDNF/GDNF antibody (1 $\mu$g/ml IgG for each; Santa Cruz Biotechnology, Santa Cruz, CA) for 24 h (+24 h) and 5 nM $MPP^+$ was then added for another 48 h. Bilirubin or $[Ru(CO)_3Cl_2]_2$ (Sigma, St. Louis, MO) was coadministered with 5 nM $MPP^+$ for 48 h. Dopaminergic neurons were characterized by staining with a rabbit anti-tyrosine hydroxylase (anti-TH) antibody (1:10,000; Calbiochem Inc., San. Diego, CA). The cultures were then incubated for 1 h with the biotinylated secondary antibody and then for 30 min with avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories. Burlingame, CA). Finally, the labeling was revealed by treatment with 0.01% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine (Sigma). The total number of TH-immunopositive cells in each well was counted under inverted microscope at 200× magnification (Leica, Heidelberg, Germany).

**Cell Cultures of Cortical Neuron and Astrocyte.** Cortical neuron-enriched and glia-enriched cultures were obtained from cerebral cortex of E17 Wistar rats. The tissues were cleaned free of meningeal tissue, minced, and mechanically dissociated by passage through a flame-polished Pasteur pipette. Dissociated cells were seeded in DMEM with 10% FBS, 4500 mg/liter glucose, 100 U/ml penicillin and 0.1 mg/ml streptomycin. For cortical neuron-enriched cultures, the cells were seeded at $1.2 \times 10^6$ cells/well on poly-D-lysine-coated six-well plates. Twenty-four hours after plating, the cells were changed to DMEM with 2% B27, 4500 mg/liter glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The neuron-enriched cultures were maintained in a humidified chamber at 37°C in a 5% $CO_2$ atmosphere for 7 to 10 days, the medium was changed every 3 days before use. For glia-enriched cultures, the cells were seeded at $1 \times 10^7$ in a 75-$cm^2$ flask. The cultures were kept in a humidified chamber at 37°C in a 5% $CO_2$ atmosphere for 7 to 14 days, the medium (DMEM with 10% FBS, 4500 mg/liter glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) was changed every 4 days. On the last day, flasks were placed on a shaker platform and shaken at 220 rpm for 6 h at 37°C to remove the oligodendrocytes and microglia in the cultures. The glia-enriched cultures were then grown to confluence before use.

**Total RNA Extraction, Reverse Transcription-PCR, and Real-Time Quantitative PCR.** Total RNA was extracted from substantia nigra with the TRIzol kit (MDBio Inc., Taipei, Taiwan). Single-strand cDNA was synthesized using SSIII reverse transcription reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All cDNA samples were stored at −20°C. The primer sequences for each PCR reaction were shown in Table 1. Real-time quantitative PCR was performed to monitor the expression of GDNF and glyceraldehyde-3-phosphate dehydrogenase. The TaqMan technology was used, and the results were analyzed in a 7900 HT sequence detector system (Applied Biosystems, Foster City, CA). Primers and the TaqMan probe were purchased from Applied Biosystems. The amplification reaction mixture (25 $\mu$l) contained a 2.5-$\mu$l cDNA sample, TaqMan Universal PCR Master Mix, and TaqMan primer/probe premix. The thermal cycling conditions included 10 min at 95°C, proceeding with 40 cycles of 95°C for 15 s and 60°C for 1 min. The GDNF mRNA levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA and expressed relative to control using the $\Delta\Delta C_t$ method.

**Statistics.** Results are expressed as mean ± S.E.M. Results were analyzed with one-way analysis of variance and Neuman-Keuls post hoc test to determine statistical significance between specific groups. Difference was considered significant when $p < 0.05$.

## Results

**HO-1 Induction Reduces $MPP^+$-Induced Behavior Asymmetry.** Unilateral lesion using $MPP^+$ provides greater advantage as it produces behavioral abnormality and $TH^+$ neurons of two sides can be compared in the same rats. Apomorphine was injected into the rat (5 mg/kg i.p.) on the day 7 after local administration of $MPP^+$ (8 $\mu$g) in substantia nigra. The rats elicited significantly ipsilateral circling behavior in $MPP^+$ and $MPP^+$ + Ad groups upon apomorphine test. The ipsilateral circling behavior after the administration of apomorphine is consistent with those reported by others (Sindhu et al., 2006). The rotation frequency was 3.8 ± 1.3 turns/min in the $MPP^+$ group ($n = 6$; Fig. 1A) and 4.0 ± 1.4 turns/min in the $MPP^+$ + Ad group ($n = 7$) during the 30-min observation period. The rotation behavior of $MPP^+$ and $MPP^+$ + Ad groups was statistically significant compared with control (0.5 ± 0.2 turns/min, $n = 7$; Fig. 1A). HO-1 induction by the simultaneous administration of $MPP^+$ with Ad-HO-1 ($5 \times 10^7$ PFU) significantly reduced $MPP^+$-induced rotation rate (0.6 ± 0.4 turns/min, $n = 11$; Fig. 1A).

**HO-1 Induction Inhibits $MPP^+$-Induced Dopaminergic Neuronal Death.** Seven days after injection of $MPP^+$ in the substantia nigra of rats, the immunohistochemical staining of TH, a marker of dopaminergic neuron, showed a lose of $TH^+$ neurons in substantia nigra at $MPP^+$- and $MPP^+$ + Ad-lesioned side compared with control (Fig. 1, B and C). HO-1 induction reduced $TH^+$ neuronal death in substantia nigra on the $MPP^+$-lesioned side (Fig. 1, B and C). The total number of $TH^+$ neurons in substantia nigra was decreased approximately 34% on the ipsilateral side after $MPP^+$ treatment (397.0 ± 10.9 on the ipsilateral side and 621.4 ± 15.8 on the contralateral side, $n = 5$; Fig. 1B). HO-1 induction markedly increased the number of surviving $TH^+$ neurons to 571.8 ± 26.1 (617.0 ± 4.6 on the contralateral side; $n = 6$; Fig. 1B). The immunostaining in striatum also showed a great loss of $TH^+$ nerve terminals in $MPP^+$- or $MPP^+$ + Ad-treated groups (Fig. 1C, bottom). However, the $TH^+$ nerve terminals on the ipsilateral side of striatum were significantly rescued by Ad-HO-1 (Fig. 1C, bottom). To test whether HO-1 exerts neuroprotective effect in response to higher dose of $MPP^+$, we increased the dose of $MPP^+$ up to 10 $\mu$g and found that HO-1 induction ($5 \times 10^7$ PFU) still had neuropro-

TABLE 1
PCR primer sequences

| Gene | Primer Sequence |
|---|---|
| HO-1 | |
| Forward | 5′-ACTTT CAGAA GGGTC AGGTG TCC-3′ |
| Reverse | 5′-TTGAG CAGGA AGGCG GTCTT AG-3′ |
| BDNF | |
| Forward | 5′-GACTC TGGAG AGCGT GAAT-3′ |
| Reverse | 5′-CCACT CGCTA ATACT GTCAC-3′ |
| GDNF | |
| Forward | 5′-CAGCC CAGAG AATTC CAGAG-3′ |
| Reverse | 5′-TTTTG TCATA CATTG TCTCG GC-3′ |
| GAPDH | |
| Forward | 5′-GCCAT CAACG CCCCT TCATT GAC-3′ |
| Reverse | 5′-ACGGA AGGCC ATGCC AGTGA GCTT-3′ |

tection in reducing behavior asymmetry and increasing $TH^+$ neuronal survival in substantia nigra [rotation (turns/min): 0.5 ± 0.2, 5.6 ± 0.8, and 2.0 ± 0.8 for control ($n$ = 7), $MPP^+$ ($n$ = 7), and $MPP^+$ + Ad-HO-1 ($n$ = 8), respectively; $TH^+$ neuron (ratio of ipsilateral/contralateral): 0.97 ± 0.06, 0.50 ± 0.06, and 0.76 ± 0.03 for control ($n$ = 4), $MPP^+$ ($n$ = 7), and $MPP^+$ + Ad-HO-1 ($n$ = 7), respectively]. These results indicate that HO-1 induction inhibited $MPP^+$-induced dopaminergic neuronal death in substantia nigra and also the nerve terminal's degeneration in striatum.

Seven days after injection of $MPP^+$ into substantia nigra concomitantly with Ad or Ad-HO-1, the striatum was dissected and the amounts of dopamine, DOPAC, and HVA were determined by HPLC. The $MPP^+$- or $MPP^+$ + Ad-treated groups caused a marked reduction of dopamine and DOPAC but not HVA content on the ipsilateral side of striatum compared with the unlesioned contralateral side on day 7 ($n$ = 7 for each group; Fig. 2). HO-1 induction antagonized the $MPP^+$-induced reduction of striatal dopamine and DOPAC contents in the lesioned side of striatum ($n$ = 8; Fig. 2). These results further confirm the neuroprotective effect of HO-1 on dopaminergic neurons.

**HO-1 Induction Inhibits $MPP^+$-Induced TNF-$\alpha$ and IL-1$\beta$ Release.** Seven days after injection of $MPP^+$ into the substantia nigra, with or without Ad-HO-1 ($5 \times 10^7$ PFU), the substantia nigra was isolated and the contents of TNF-$\alpha$ and IL-1$\beta$ were determined by ELISA. In $MPP^+$-treated rats, TNF-$\alpha$ and IL-1$\beta$ release in the lesioned-side was significantly elevated compared with the unlesioned contralateral side ($n$ = 8; Fig. 3). The two inflammatory cytokines produced from the $MPP^+$-lesioned side were reduced approximately 50% after HO-1 induction ($n$ = 8; Fig. 3). These results suggest that HO-1 induction reduced the production of inflammatory cytokines accompanied with $MPP^+$-induced dopaminergic neuronal injury.

**Increase of both mRNA and Protein Levels of BDNF and GDNF by HO-1 Induction in Rat Substantia Nigra.** To investigate the possible neuroprotective action after HO-1 induction, we measured the mRNA and protein expression of two neurotrophic factors, BDNF and GDNF. BDNF and GDNF have been reported to play an important role in the survival of dopaminergic neurons (Lin et al., 1993; Kirschner et al., 1996). Twenty-four hours after a single unilateral injection of Ad-HO-1 ($5 \times 10^7$ PFU) in substantia nigra, HO-1 mRNA increased markedly on the ipsilateral side ($n$ = 3; Fig. 4A). Enhancement of BDNF and GDNF mRNA expression accompanied HO-1 induction ($n$ = 3; Fig. 4A). Real-time quantitative PCR also revealed that GDNF mRNA increased approximately 15-fold in the ipsilateral HO-1 induction side ($n$ = 7; Fig. 4B). We then performed immunostaining to examine the cellular type that expressed HO-1, BDNF, and GDNF 48 h after Ad-HO-1 administration. Ad-HO-1 infection was found to occur in both neuron and glia near the injection site in substantia nigra (data not shown). BDNF and GDNF

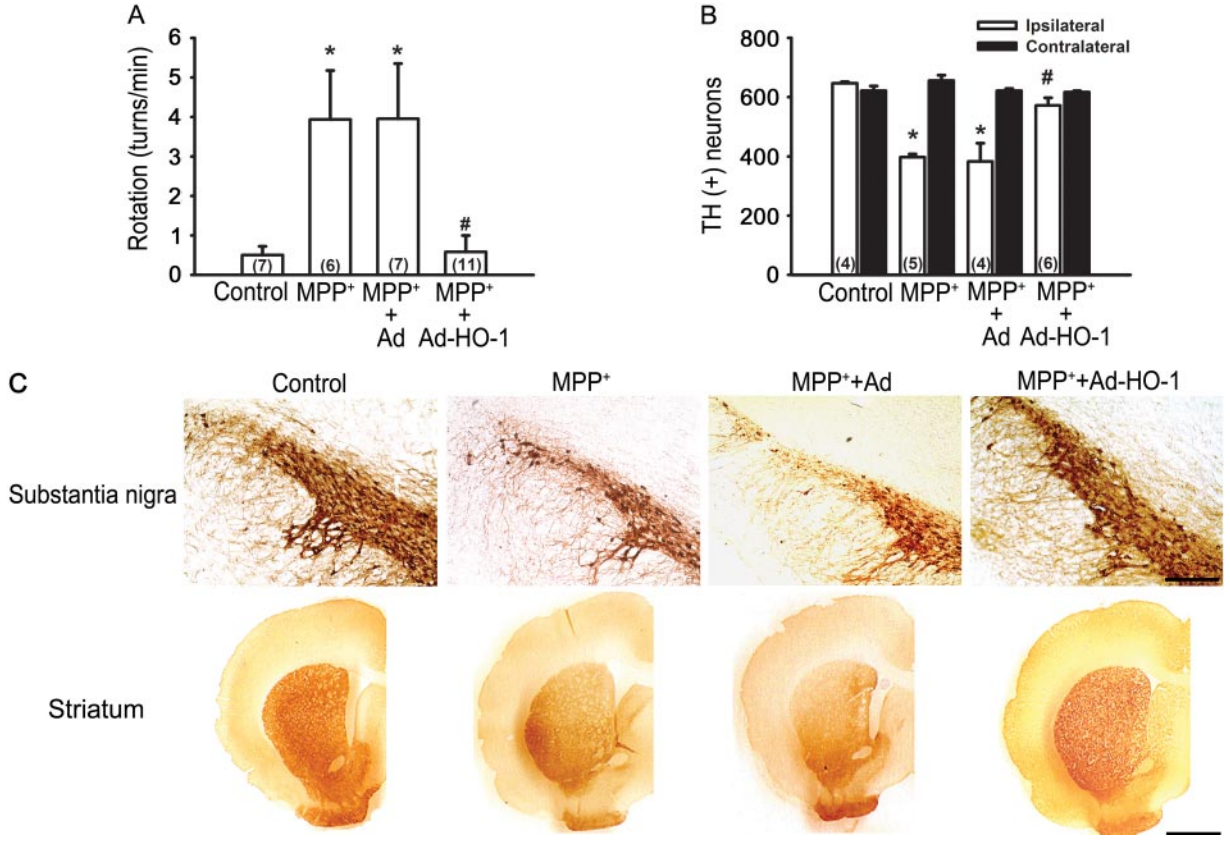


**Fig. 1.** HO-1 induction reduces $MPP^+$-induced behavior asymmetry, dopaminergic neuronal death in substantia nigra and terminal degeneration in striatum. A, seven days after local administration of $MPP^+$ (8 $\mu$g) into substantia nigra of rat, apomorphine was injected (5 mg/kg, i.p.) to induce rotation behavior. The rats were sacrificed after the behavior test and immunostaining was performed. B, the $TH^+$ neuron in substantia nigra was evaluated. C, representative graphs of the $TH^+$ neuron on the ipsilateral sides of substantia nigra (upper panels) and the $TH^+$ nerve terminals in striatum (bottom). Data are presented as mean ± S.E.M. (*, $p < 0.05$ compared with control; #, $p < 0.05$ compared with $MPP^+$ treatment alone). Scale bars: top, 200 $\mu$m; bottom, 2 mm.

immunopositive cells markedly increased in Ad-HO-1 injection side compared with contralateral Ad injection side (Fig. 4C). To further examine the cell type that expressed BDNF or GDNF in response to HO-1 induction, immunofluorescent double staining was performed. Figure 4D shows that BDNF mainly colocalized with $TH^+$ neuron and GDNF colocalized with $GFAP^+$ glia in substantia nigra (Fig. 4D). Therefore, exogenous administration of HO-1 up-regulated BDNF and GDNF expression in both mRNA and protein levels. We then further examined whether $MPP^+$ treatment increases the expression of endogenous HO-1. Rats receiving single injections of $MPP^+$ (8 $\mu$g) into the substantia nigra showed a large number of HO-1 immunopositive cells in substantia nigra on day 7 on the $MPP^+$-lesioned side compared with the unlesioned contralateral side (Fig. 5A). Western blotting also revealed that $MPP^+$ treatment increased endogenous HO-1 protein level without affecting the HO-2 level in substantia nigra (Fig. 5B). The double staining in Fig. 5C further demonstrates that $MPP^+$-induced the expression of HO-1 colocalized with $TH^+$ neuron and $GFAP^+$ glia, suggesting that $MPP^+$ treatment up-regulated endogenous HO-1 expression both in dopaminergic neuron and astroglia.

**ZnPPIX Treatment Enhances $MPP^+$-Induced Dopaminergic Neuronal Death.** Because $MPP^+$ treatment enhanced the endogenous HO-1 induction and HO-2 is constitutively expressed in the cells, we further examined the role of heme oxygenase (HO) in $MPP^+$-induced neurotoxicity. We locally administered HO inhibitor ZnPPIX (3 pg) concomitantly with $MPP^+$ (8 $\mu$g) in substantia nigra. The rotation behavior was examined earlier than in previous experiments. Apomorphine (5 mg/kg i.p.) was given on day 3. It was found that ZnPPIX markedly increased the rotation behavior induced by $MPP^+$ ($0.5 \pm 0.2$, $2.4 \pm 0.9$, and $6.3 \pm 1.3$ turns/min, respectively, for control, $MPP^+$ alone, and $MPP^+$ + ZnPPIX, respectively, $n = 5\sim7$; Fig. 6A). $MPP^+$ and ZnPPIX treatment alone did not induce significant rotation. On day 3, the TH immunostaining in striatum also demonstrated that cotreatment of $MPP^+$ with ZnPPIX significantly potentiated the loss of dopaminergic nerve terminals (Fig. 6B). HPLC analysis also showed the similar death-potentiating effect of ZnPPIX (Fig. 6, C–E). These results indicate that endogenous HO-1 induction by $MPP^+$ treatment and also constitutive expression of HO-2 may exert inhibition against $MPP^+$-induced dopaminergic neurotoxicity.

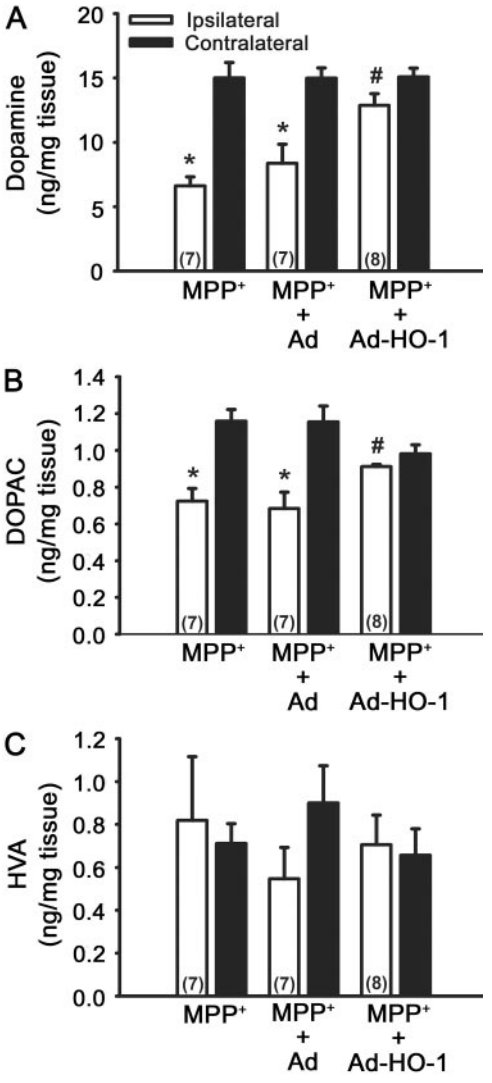


**Fig. 2.** HO-1 induction inhibits $MPP^+$-induced decrease of dopamine and DOPAC contents in striatum. $MPP^+$ (8 $\mu$g) was locally injected into substantia nigra of rat concomitantly with Ad or Ad-HO-1. The striatum was isolated 7 days later for HPLC analysis of dopamine (A) and its metabolites DOPAC (B) and HVA (C). Note that Ad-HO-1 antagonized the reduction of dopamine and DOPAC induced by $MPP^+$ treatment. Data are given as mean ± S.E.M. (*, $p < 0.05$ compared with contralateral side; #, $p < 0.05$ compared with $MPP^+$ treatment alone).

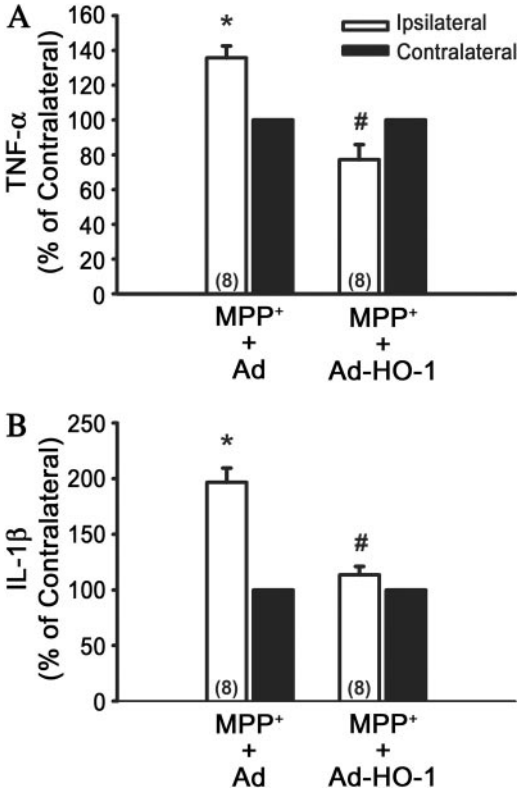


**Fig. 3.** Reduction of $MPP^+$-induced TNF-$\alpha$ and IL-1$\beta$ release by HO-1 induction. $MPP^+$ (8 $\mu$g) was locally injected into substantia nigra of rat concomitantly with or without Ad-HO-1. The substantia nigra was isolated 7 days later for the measurement of TNF-$\alpha$ (A) and IL-1$\beta$ (B) using ELISA kit. Note that both cytokines increased after $MPP^+$ treatment and that Ad-HO-1 administration antagonized this potentiating effect. Data are given as mean ± S.E.M. ($n = 8$) (*, $p < 0.05$ compared with contralateral side; #, $p < 0.05$ compared with $MPP^+$ + Ad-treated group).

**Neuroprotection by HO-1 in Neuron-Glia Cocultures of Rat Midbrain.** HO-2 is constitutively and abundantly expressed in normal mammalian CNS, and HO-1 is induced by stress (Schipper, 2004). We further examined the neuroprotective action of HO-1 in midbrain neuron-glia cocultures. There was a time- and dose-dependent expression of HO-1 after Ad-HO-1 administration in the cell (Fig. 7, A and B). The HO-2 protein level was not altered by Ad-HO-1 infection. The quantitative analysis revealed that treatment of day 4 midbrain neuron-glia cocultures with 5 nM $MPP^+$ for 48 h caused the loss of $TH^+$ neurons by 58.5 ± 2.7% (Fig. 7C). In the presence of Ad-HO-1 but not Ad (preinfection, 24 h), the toxic effect of $MPP^+$ was significantly reduced, the survival of $TH^+$ neurons was 92.8 ± 5.3% in the 3 moi Ad-HO-1-infected group (Fig. 7C), suggesting that HO-1 induction exerts protective action in dopaminergic neuron against $MPP^+$-induced neurotoxicity. We further examined the neuroprotective ability of HO-1 induction by treatment of Ad-HO-1 after $MPP^+$ preincubation. It was found that infection of Ad-HO-1 after addition of $MPP^+$ for 6~12 h (+6~+12 h) exerted less neuroprotective effect (Fig. 7D). Furthermore, the effect of downstream products of HO enzyme, bilirubin, and CO on $MPP^+$-induced dopaminergic neuronal toxicity was also evaluated. Western blotting showed that treatment of midbrain cultures with bilirubin or the CO-releasing molecule $[Ru(CO)_3Cl_2]_2$ did not affect HO-1 expression (Fig. 7B). It was found that

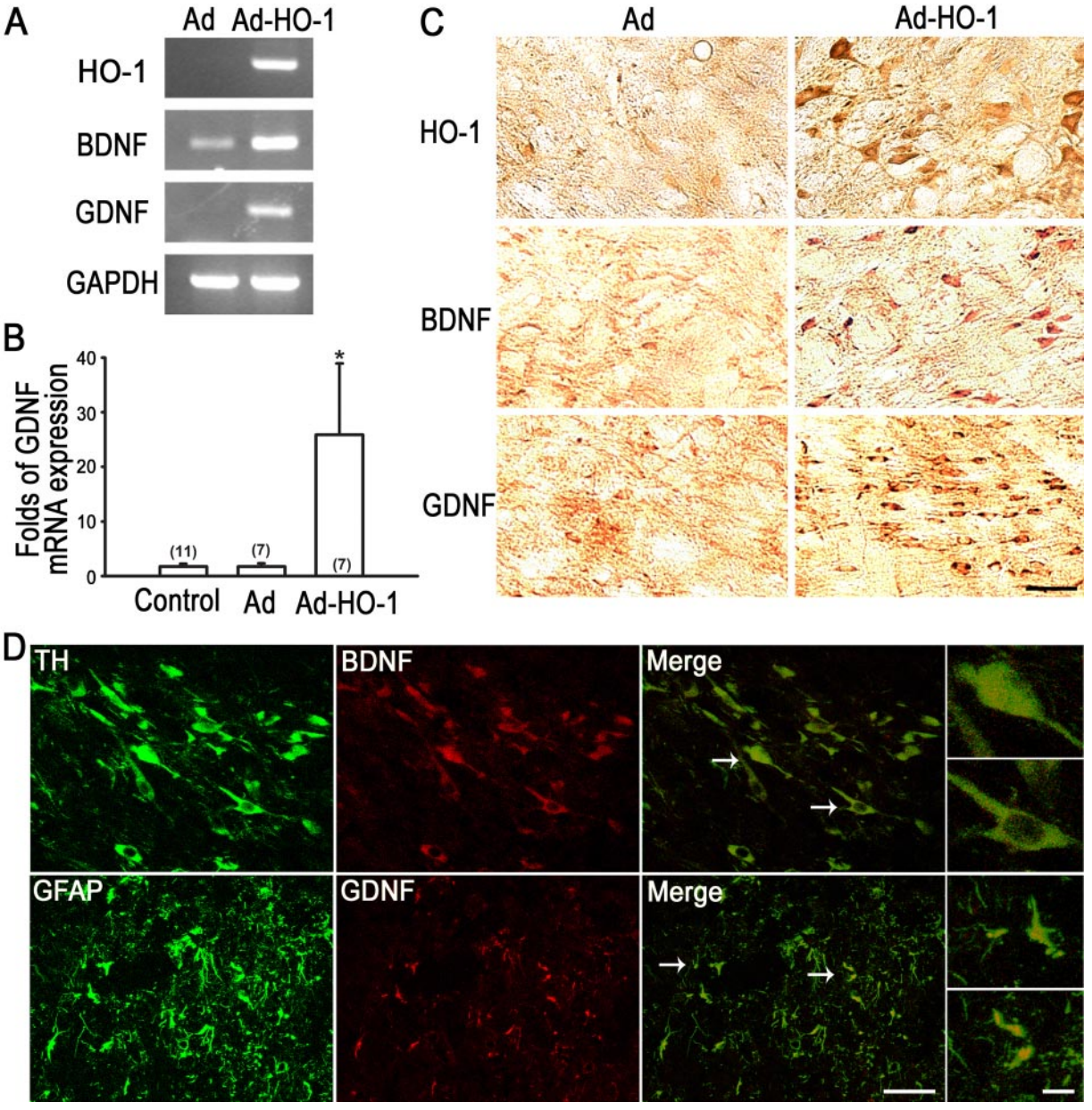


**Fig. 4.** Increase of mRNA and protein levels of BDNF and GDNF by HO-1 induction in rat substantia nigra. A, Ad-HO-1 ($5 \times 10^7$ PFU) was locally administered into substantia nigra, which was isolated 24 h later for the mRNA analysis of HO-1, BDNF, and GDNF. B, HO-1 induction increased GDNF mRNA expression using real-time quantitative PCR. C, immunohistochemical staining of HO-1, BDNF, and GDNF was performed 48 h after Ad-HO-1 administration in substantia nigra. D, immunofluorescent double staining shows that BDNF colocalized with $TH^+$ neuron and GDNF colocalized with $GFAP^+$ astroglia. The colocalized cells were indicated by arrows and enlarged at right. Note that HO-1 induction increased the expression of BDNF in $TH^+$ neurons and GDNF in astroglia. Data are presented as mean ± S.E.M. *, $p < 0.05$ compared with control. Scale bars: C and D, 50 $\mu$m; D, right, 10 $\mu$m.

bilirubin concentration-dependently increased the survival rate of $TH^+$ neuron (65.0 ± 3.3% and 84.2 ± 14.4% for 10 and 30 $\mu$M, respectively; Fig. 7E). However, the CO donor $[Ru(CO)_3Cl_2]_2$ at 30 and 100 $\mu$M did not affect $MPP^+$-induced neuronal death (Fig. 7E). When the CO donor was administered twice a day, the neuronal loss was unchanged. The photomicrographs show that $MPP^+$ treatment for 48 h caused a decrease in $TH^+$ neurons and damage of dopaminergic neurite. Treatment of Ad-HO-1 rescued these neurotoxic effects (Fig. 7F). Bilirubin also exerted a similar dopaminergic neuroprotection. These results indicate that HO-1 and the main downstream product bilirubin increased the

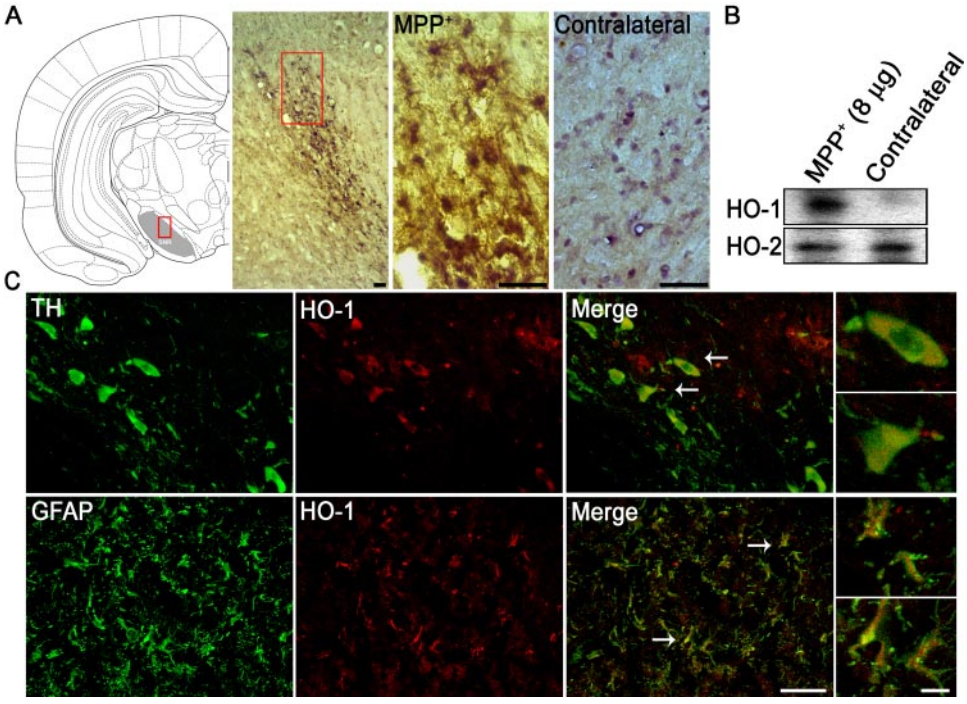


**Fig. 5.** Increase of endogenous HO-1 expression by $MPP^+$ treatment in rat substantia nigra. $MPP^+$ (8 $\mu$g) was locally injected into substantia nigra, and the rats were sacrificed 7 days later. A, immunohistochemical staining shows the intense HO-1 expression in the lesioned side compared with the contralateral side. Hematoxylin was used as counter-staining. The square area was sequentially enlarged on the right. B, Western blotting shows that $MPP^+$ treatment increased HO-1 but not HO-2 expression. C, immunofluorescent staining shows that endogenous HO-1 expression after $MPP^+$ treatment occurred both in $TH^+$ neuron and $GFAP^+$ glia. The colocalization as indicated by arrow was enlarged on the right. Scale bars: A and C, 50 $\mu$m; C, right, 10 $\mu$m.

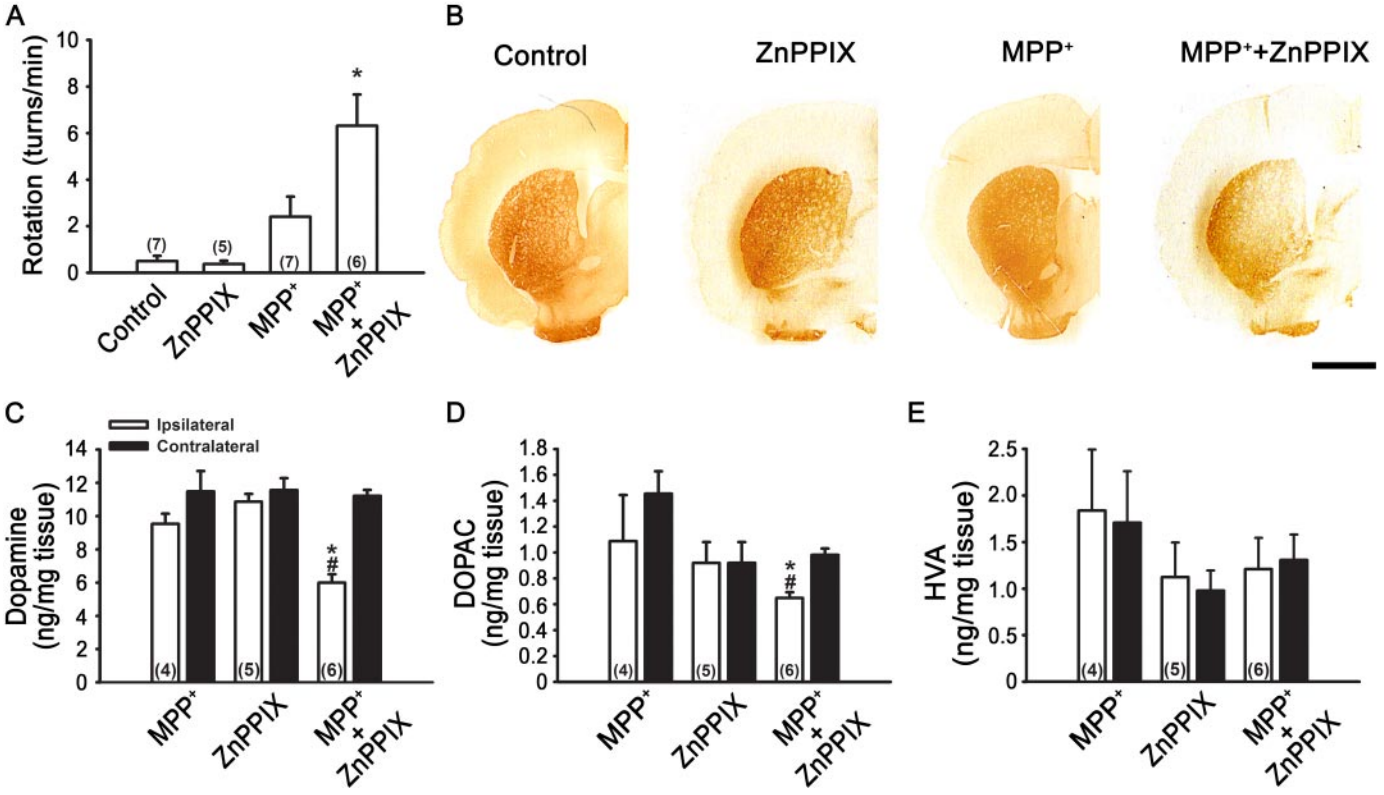


**Fig. 6.** ZnPPIX cotreatment enhances $MPP^+$-induced neurotoxicity. A, $MPP^+$ (8 $\mu$g) was locally injected into substantia nigra with or without ZnPPIX (3 pg). The behavior test was performed on day 3, and the rats were scarified after the test. B, the striatum was used for immunostaining of tyrosine hydroxylase. C–E, HPLC analysis of dopamine and its metabolites. Data are given as mean ± S.E.M. *, $p < 0.05$ compared with contralateral side; #, $p < 0.05$ compared with $MPP^+$ treatment alone (ipsilateral side). Scale bar, 2 mm.

survival rate and also maintained the morphology of dopaminergic neurons against $MPP^+$-induced neurotoxicity in rat midbrain neuron-glia cocultures.

**Increase of Neurotrophic Factor Expression by HO-1 in Rat Glia-Enriched and Cortical Neuron-Enriched Cultures.** Neurotrophic factors are very important for neu-

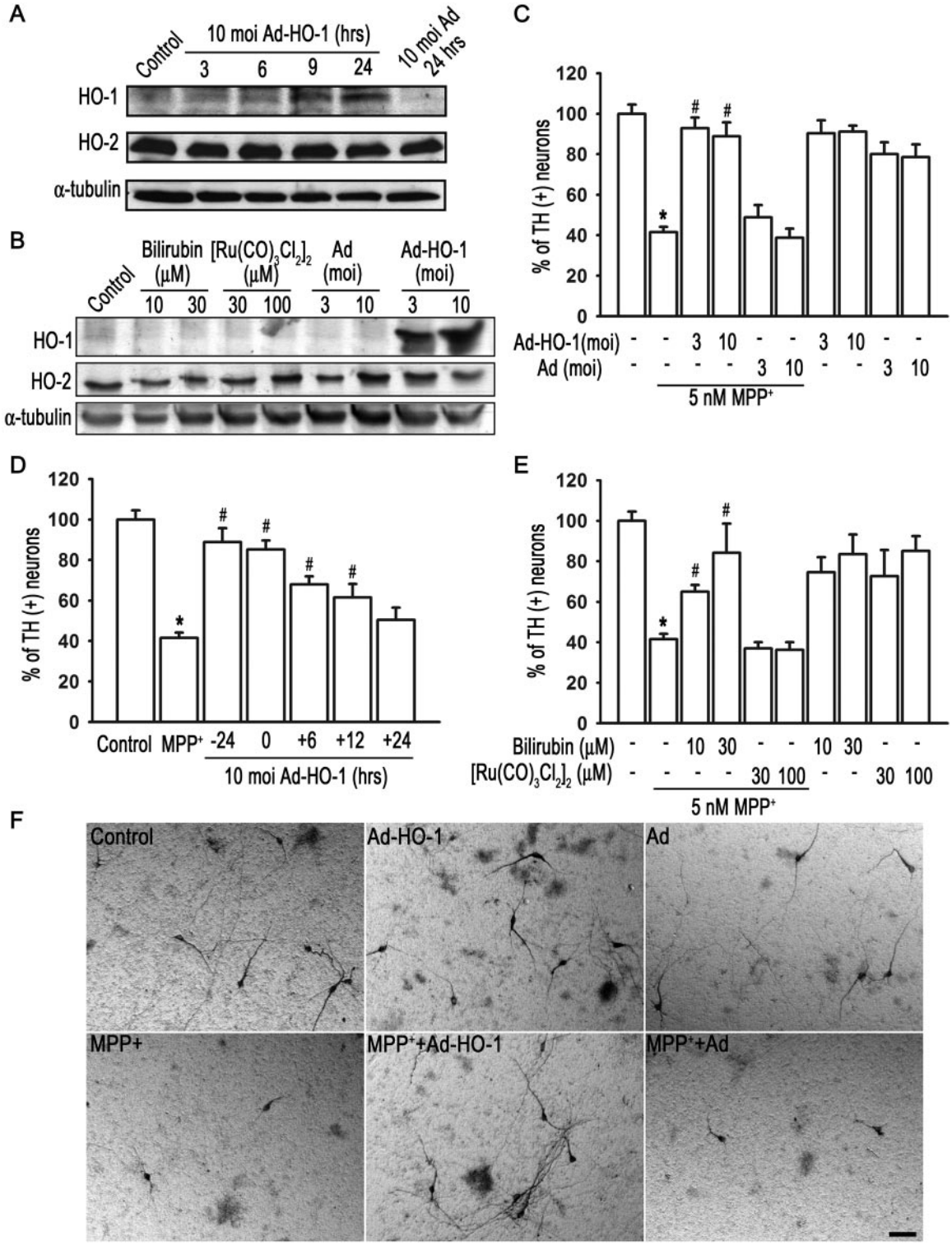


**Fig. 7.** Neuroprotection by HO-1 and bilirubin in rat midbrain neuron-glia cocultures. Midbrain neuron-glia cocultures were obtained from E14 rats. A, time-dependent expression of HO-1 protein after infection with 10 moi Ad-HO-1. B, dose-dependent expression of HO-1 after administration of Ad-HO-1 for 24 h. C–E, quantitative analysis of survival of $TH^+$ neuron in midbrain cultures in response to the treatment of 5 nM $MPP^+$ for 48 h. C, concomitant treatment of Ad-HO-1 with $MPP^+$ inhibited $MPP^+$-induced dopaminergic neurotoxicity. D, pretreatment of Ad-HO-1 for 24 h (−24) and coadministration (0) of Ad-HO-1 with $MPP^+$ exerted more profound neuroprotection. However, administration of Ad-HO-1 6 (+6)–12 h (+12) after $MPP^+$ treatment had less neuroprotective action. E, concomitant administration of $MPP^+$ with bilirubin but not $[Ru(CO)_3Cl_2]_2$ inhibited $MPP^+$-induced neuronal death. F, representative photography shows the neuroprotection of Ad-HO-1 against $MPP^+$-induced neurotoxicity. Data are presented as mean ± S.E.M. ($n = 3 \sim 4$). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with $MPP^+$ treatment alone. Scale bar, 50 $\mu$m.

ronal survival, and the in vivo study showed that HO-1 induction in the substantia nigra of rat increased BDNF/GDNF expression, which colocalized with dopaminergic neuron/glia. We further examined the effect of HO-1 induction on the BDNF and GDNF expression in primary cell cultures. RT-PCR showed that HO-1 induction in astrocytes increased BDNF and GNDF mRNA expression 24 h after infection with Ad-HO-1 (10 moi; Fig. 8A). Immunoblotting indicated that HO-1 induction increased the protein expression of BDNF pro-form (pro-BDNF; 28 kDa), mature BDNF (BDNF; 14 kDa), and mature GDNF (GDNF; 15 kDa) 24 h after infection of Ad-HO-1 (Fig. 8B). $MPP^+$ treatment increased endogenous HO-1 induction as well as BDNF and GDNF expression in glia-enriched cultures (Fig. 8C). Furthermore, cotreatment of $MPP^+$ with HO inhibitor ZnPPIX antagonized the potentiating action on neurotropic factor expression (Fig. 8C). ZnPPIX

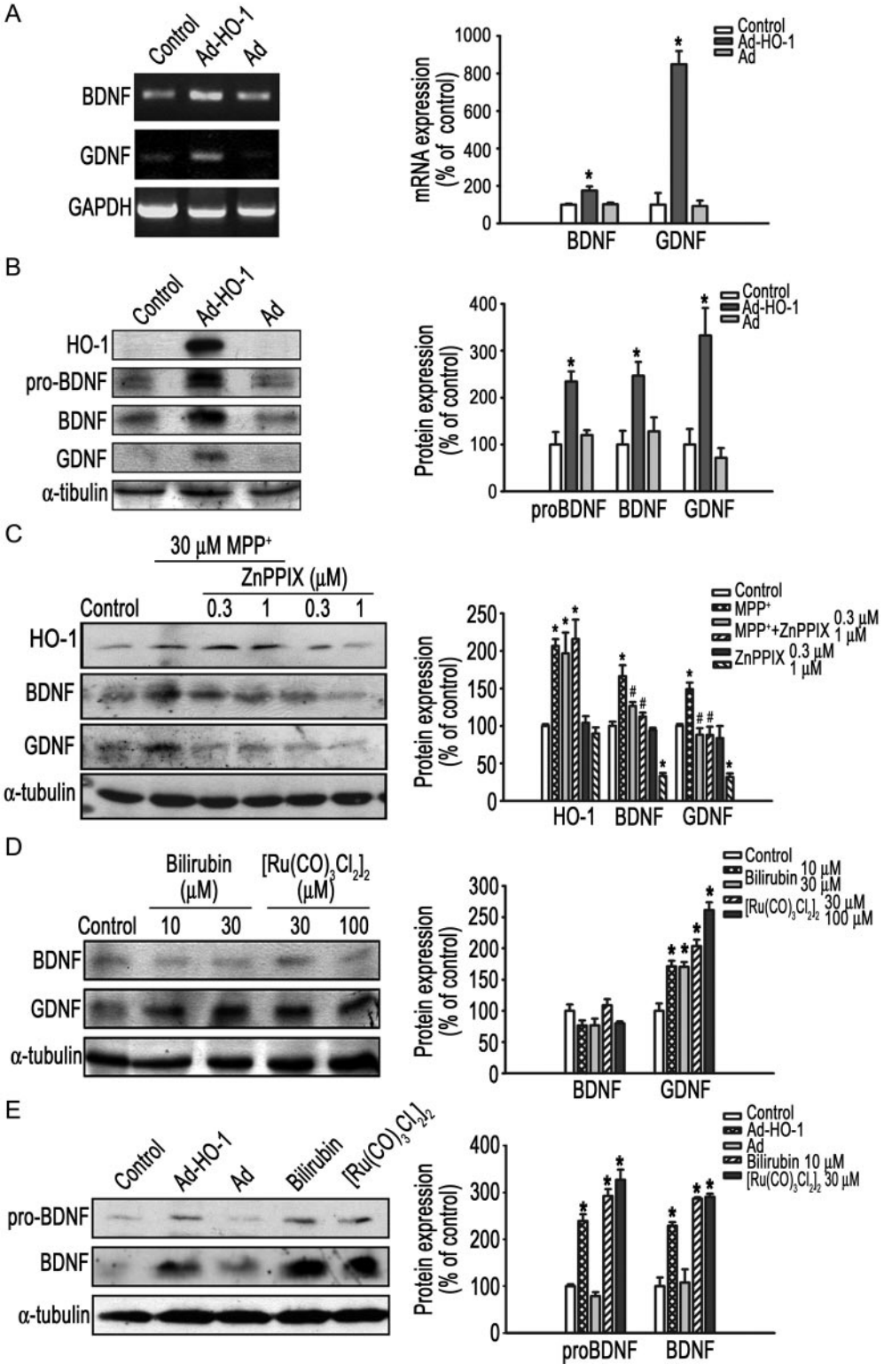


**Fig. 8.** Increase of BDNF and GDNF expression by HO-1 in glia-enriched and cortical neuron-enriched cultures. A, glia-enriched cultures were infected with 10 moi Ad-HO-1 or Ad for 24 h, the RT-PCR shows that BDNF and GDNF mRNA expression was increased by HO-1 induction. B, protein expression of BDNF pro-form (pro-BDNF; 28 kDa), mature BDNF (BDNF; 14 kDa) and mature GDNF (GDNF; 15 kDa) increased after 24 h infection with Ad-HO-1 in glia-enriched cultures. C, endogenous HO-1 induction by $MPP^+$ treatment (30 $\mu$M) for 24 h increased GDNF and BDNF expression in glia-enriched cultures. Note that concomitant administration of $MPP^+$ with ZnPPIX antagonized the potentiating action. D, treatment of bilirubin or $[Ru(CO)_3Cl_2]_2$ for 24 h increased GDNF protein expression in glia-enriched cultures. E, increase of pro-BDNF and BDNF protein expression in cortical neuron-enriched cultures by the 24-h treatment of Ad-HO-1 (10 moi), Ad (10 moi), bilirubin (10 $\mu$M), or $[Ru(CO)_3Cl_2]_2$ (30 $\mu$M). The summarized results were shown in the right panels. Data are given as mean ± S.E.M. ($n = 3\sim4$). *, $p < 0.05$ compared with control; #, $p < 0.05$ compared with $MPP^+$ treatment alone.

treatment alone at a higher concentration (1 $\mu$M) also reduced the basal level of BDNF and GDNF expression in astrocytes, which may be due to the inhibition of HO-2 enzyme activity (Fig. 8C). Treatment of glia-enriched cultures with bilirubin or $[Ru(CO)_3Cl_2]_2$ also increased GDNF but not BDNF expression (Fig. 8D). Because midbrain culture contains both neuron and glia, we therefore used cortical neuron-enriched cultures to investigate whether the BDNF expression was affected by HO-1 induction, bilirubin, or $[Ru(CO)_3Cl_2]_2$ treatment. Cortical neuron-enriched cultures were treated with Ad-HO-1 (10 moi), Ad (10 moi), bilirubin (10 $\mu$M), or $[Ru(CO)_3Cl_2]_2$ (30 $\mu$M) for 24 h; the Western blot shows that pro-BDNF and BDNF expression increased after HO-1 induction and bilirubin or $[Ru(CO)_3Cl_2]_2$ treatment (Fig. 8E). The quantitative data were all shown in the right panels. These results further confirm our in vivo studies that BDNF in neurons and GDNF in astrocytes were up-regulated by HO-1 induction. We further used BDNF and GDNF antibody to neutralize endogenously released BDNF/GDNF in neuron-glia cocultures of midbrain to determine the effect of endogenous release of BDNF and GDNF on the survival of dopaminergic neurons. The cultures treated with BDNF or GDNF antibody (1 $\mu$g/ml) but not control IgG for 72 h had a significant reduction in $TH^+$ neurons compared with control (50.1 $\pm$ 6.6%, 50.2 $\pm$ 5.3% and 93.3 $\pm$ 5.1% for BDNF antibody, GDNF antibody, and control IgG, respectively; $n = 3$), suggesting that endogenous release of BDNF/GDNF plays an important role in the survival of dopaminergic neurons.

## Discussion

Neurons in CNS are particularly vulnerable to oxidative stress, and HO-1 has been shown to act as a protectant during neuronal injury, because HO-1 is induced by oxidative challenge or kainic acid-induced excitotoxic injury (Chen et al., 2000; Huang et al., 2005). Astrocytes of *HO-1*(−/−) mice also show more vulnerable to heme-mediated oxidative damage (Chen-Roetling et al., 2005). However, adenoviral transfer of HO-1 protects astrocyte from heme-mediated injury (Teng et al., 2004). HO-1 significantly increases especially in the hippocampi of patients with Alzheimer's disease and in the substantia nigra of patients with Parkinson's disease (Schipper et al., 1995, 1998). Munoz et al. (2005) reported that HO-1 induced in glia may act as one useful marker in early stages of striatal damage. These observations provide support for the hypothesis that HO-1 induction is a key element for anti-oxidant defense in the brain and that HO-1 plays an important role during brain injury and CNS degenerative diseases.

Recent studies have demonstrated that adenovirus-mediated gene transfer of HO-1 in animal models can inhibit hypoxia-induced lung injury, reperfusion-induced injury of transplanted liver, and the development of atherosclerosis (Amersi et al., 1999; Otterbein et al., 1999; Juan et al., 2001). In this study, we induced HO-1 expression by using a replication-defective adenovirus vector encoding human HO-1 in substantia nigra of rats or midbrain neuron-glia cocultures to investigate whether exogenous induction of HO-1 is useful in treating Parkinsonism. HO-1 induction in substantia nigra by adenovirus at a dose of $5 \times 10^7$ PFU was found to have a strong neuroprotective effect in a rat Parkinsonism model via 1) reducing $MPP^+$-induced behavioral impairment in response to apomorphine test, 2) increasing the survival of dopaminergic neurons in substantia nigra, 3) increasing the dopamine and DOPAC contents in striatum, and 4) reducing inflammatory cytokine production in substantia nigra. In in vitro study in E14 midbrain neuron-glia cocultures, HO-1 significantly reduced the death of $TH^+$ neurons and maintained neurite morphology after $MPP^+$ treatment. Pretreatment of Ad-HO-1 for 24 h or concomitant administration with $MPP^+$ exerted more profound neuroprotection than post-treatment. On the other hand, $MPP^+$ treatment increased expression of endogenous HO-1 but not HO-2 both in dopaminergic neurons and in glia cells of the substantia nigra. Cotreatment of $MPP^+$ with a very low dose of the HO inhibitor ZnPPIX (3 pg) was found to significantly exert the following effects: 1) enhancement of $MPP^+$-induced behavioral impairment upon apomorphine test, 2) potentiation of the reduction of dopamine and DOPAC contents in striatum, and 3) increase of the dopaminergic nerve terminal's degeneration in striatum compared with $MPP^+$ treatment alone on day 3. Because ZnPPIX inhibits both HO-1 and HO-2 and the downstream products of HO-1 and HO-2 are the same (bilirubin and CO), the enhancement of $MPP^+$-induced dopaminergic neurotoxicity by ZnPPIX may result from the inhibition of these two types of HO.

In vivo animal experiments show that HO-1 induction in substantia nigra up-regulated BDNF expression in dopaminergic neuron and GDNF expression in glia. Furthermore, BDNF and GDNF were up-regulated by both endogenous and exogenous HO-1 induction in primary glia-enriched cultures. Bilirubin and $[Ru(CO)_3Cl_2]_2$ could also increase GDNF expression in the primary cultures. In addition, proBDNF and BDNF were up-regulated by Ad-HO-1, bilirubin, and $[Ru(CO)_3Cl_2]_2$ treatment in cortical neuron-enriched culture. It has been shown that each mole of $[Ru(CO)_3Cl_2]_2$ releases approximately 0.7 mol of CO gas (Motterlini et al., 2002). We also found that 30 $\mu$M $[Ru(CO)_3Cl_2]_2$ increased GDNF expression in primary glia-enriched culture through the soluble guanylate cyclase/protein kinase G-dependent pathway (S.-Y. Hung, H.-C. Liou, and W.-M. Fu, unpublished data). Therefore, we cannot exclude the involvement of CO in dopaminergic neuroprotection in vivo. Binding of endogenously released BDNF/GDNF with respective antibody resulted in neuronal death, indicating that the neuroprotective action of HO-1 may result not only from the antioxidant ability but also from the modulation of BDNF and GDNF expression.

Bilirubin at nanomolar concentrations is a potent antioxidant and neuroprotectant, which may be due to its ROS scavenging ability (Dore et al., 1999). We examined the neuroprotective role of the downstream products of HO-1, bilirubin, and CO. The midbrain neuron-glia cells were cultured in minimal essential medium with 2% FBS and 2% horse serum, which contained 16.6 $\mu$M albumin. On the other hand, glia-enriched cells were cultured in DMEM with 10% FBS that contained 41.5 $\mu$M albumin. Because bilirubin can bind with albumin, the free form of 10 $\mu$M bilirubin is approximately 211 and 99 nM, respectively, in these two kind of media according to the calculation reported by Weisiger et al. (2001). Free bilirubin at such a low concentration was found to be able to increase dopaminergic neuron survival against $MPP^+$-induced neurotoxicity in midbrain coculture. In addition, bilirubin also enhanced GDNF expression in astrocyte and BDNF expression in neurons. Although $Fe^{2+}$ is the

downstream product of HO-1, Juan et al. (2001) demonstrate that infection with Ad-HO-1 effectively reduces the hemin-induced iron overload in rat aortic smooth muscle cells and also the iron deposition in aortic lesions of young apoE-deficient mice, suggesting that overexpression of HO-1 will not increase iron deposition.

BDNF, a 28-kDa protein dimer, has a more widespread distribution than nerve growth factor in brain (Connor and Dragunow, 1998). BDNF has been reported to increase the survival of dopaminergic neurons (Kirschner et al., 1996). GDNF promotes the morphological differentiation and survival of dopaminergic neurons through a heterodimetric receptor complex consisting of a transmembrane receptor tyrosine kinase (Ret), and a ligand binding component GDNF-family receptor $\alpha$1; GDNF is a glycosylated and disulfide-bonded homodimer and belongs to the TGF-$\beta$ superfamily (Lin et al., 1993; Treanor et al., 1996). The established treatment for Parkinson's disease is oral 3,4-dihydroxy-L-phenylalanine and stereotaxic surgery, such as deep brain stimulation (Lang and Lozano, 1998a). It is necessary to develop new therapeutic strategy to treat Parkinsonism. BDNF and GDNF protect dopaminergic neurons from neuronal injury and also attenuate the behavioral deficits in Parkinson's disease (Gill et al., 2003; Love et al., 2005; Patel et al., 2005). Here we found that HO-1 induction increased both BDNF and GDNF expression, which may have the potential to treat Parkinsonism. Furthermore, HO-1 has additional strong antioxidant ability. To our knowledge, this is the first report to show the modulation of GDNF expression by HO-1. The detailed molecular mechanism and regulatory pathway regarding how HO-1, bilirubin, or CO modulates BDNF or GDNF expression needs further investigation.

In summary, the administration of exogenous HO-1 in the $MPP^+$-treated Parkinsonian rat model is beneficial in reducing dopaminergic neuronal degeneration, attenuating behavior asymmetry induced by $MPP^+$, and up-regulation of BDNF and GDNF expression both in vitro and in vivo. Because HO-1 expression is a sensitive marker in response to cellular oxidative stress. Immunohistochemical staining from postmortem brain specimens derived from subjects with Parkinson's disease also shows that HO-1 is strongly expressed both in dopaminergic neurons that contain Lewy bodies and in GFAP immunopositive astrocytes in substantia nigra (Schipper et al., 1998; Munoz et al., 2005). Furthermore, inhibition of endogenous HO-1/HO-2 activity by ZnPPIX after $MPP^+$ treatment enhanced neurotoxicity. Our data indicate that endogenous HO-1 expression in response to oxidative stress plays an important role in neuroprotection, and exogenous HO-1 induction may have possible therapeutic value to treat brain disorders, such as Parkinsonism, neurotrophic factor deprivation, or other inflammation-induced neurodegenerative diseases.

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