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Paeoniflorin attenuates neuroinflammation and dopaminergic neurodegeneration in the MPTP model of Parkinson's disease by activation of adenosine A_1 receptor

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1 This study examined whether Paeoniflorin (PF), the major active components of Chinese herb *Paeoniae alba* Radix, has neuroprotective effect in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (PD).

2 Subcutaneous administration of PF (2.5 and 5 mg kg^{-1}) for 11 days could protect tyrosine hydroxylase (TH)-positive substantia nigra neurons and striatal nerve fibers from death and bradykinesia induced by four-dose injection of MPTP (20 mg kg^{-1}) on day 8.

3 When given at 1 h after the last dose of MPTP, and then administered once a day for the following 3 days, PF (2.5 and 5 mg kg^{-1}) also significantly attenuated the dopaminergic neurodegeneration in a dose-dependent manner. Post-treatment with PF (5 mg kg^{-1}) significantly attenuated MPTP-induced proinflammatory gene upregulation and microglial and astrocytic activation.

4 Pretreatment with 0.3 mg kg^{-1} 8-cyclopentyl-1,3-dipropylxanthine, an adenosine A_1 receptor (A_1AR) antagonist, 15 min before each dose of PF, reversed the neuroprotective and antineuroinflammatory effects of PF.

5 In conclusion, this study demonstrated that PF could reduce the MPTP-induced toxicity by inhibition of neuroinflammation by activation of the A_1AR, and suggested that PF might be a valuable neuroprotective agent for the treatment of PD.
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Abbreviations: A_1AR, adenosine A_1 receptor; AD, Alzheimer's disease; CNS, central nervous system; DMSO, dimethylsulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GFAP, glial fibrillary acidic protein; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; MPP^+, 1-methyl-4-phenyl-pyridiniumion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NECA, *N*-ethylcarboxamidoadenosine; PD, Parkinson's disease; PF, paeoniflorin; RFC, relative fold change; ROS, reactive oxygen species; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TNF-α, tumor necrosis factor-alpha; VM, ventral midbrain

Introduction

Parkinson's disease (PD) is characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Even after numerous studies, the cause of dopaminergic cell degeneration in SNc of PD patients still has not been identified with certainty. Recently, increasing evidence from human and animal studies has suggested that neuroinflammation is an important contributor to the neuronal loss in PD (McGeer *et al*., 1988; Hunot & Hirsch, 2003). The hallmark of brain inflammation is the activation of glia, particularly microglia. Microglia, the resident immune cells in the brain, are sensitive to even minor disturbances in central nervous system (CNS) homeostasis and become readily activated during most neuropathological conditions, such as Alzheimer's disease (AD), multiple sclerosis, AIDS dementia, trauma, stroke and PD (Kreutzberg, 1996; Liu & Hong, 2003). Activated microglia are thought to contribute to neuronal damage, particularly in neurodegenerative diseases, *via* the release of proinflammatory and neurotoxic factors. These factors include proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL-1β), reactive nitrogen species, reactive oxygen species (ROS), eicosanoids and excitatory amino acids (Merrill & Benveniste, 1996; Liu & Hong, 2003). Moreover, microglia-derived neurotoxic factors might act in concert to trigger or exacerbate the neurodegeneration. For example, TNF-α induces the expression of inducible nitric oxide synthase (iNOS) in glia, thus amplifying NO-mediated neuronal damage (Merrill & Benveniste, 1996). Additionally, in the mature brain, the higher density of resting microglia in the SNc compared with other brain regions (Lawson *et al*., 1996; Kim *et al*., 2000) might be one of the reasons why dopamine-containing neurons are extremely vulnerable to oxidative stress in PD. Recently, growing experimental evidence demonstrated that inhibition of the inflammatory response could, in part, prevent degeneration of nigrostriatal dopamine-containing neurons in several animal models of PD, suggesting that inhibition of inflammation might become a promising therapeutic intervention for

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PD (Mogi *et al*., 1998; Liberatore *et al*., 1999; Gao *et al*., 2003; Teismann *et al*., 2003; Ferger *et al*., 2004; Furuya *et al*., 2004; Zhou *et al*., 2005).

Paeoniae alba Radix (red peony root; Chishao), the dried root of *Paeonia lactiflora* Pallas or *Paeonia veitchii* Lynch, is one of the Chinese traditional crude drugs. It has been widely used as a component of traditional Chinese prescriptions to regulate the amenorrhea, to treat traumatic injuries, epistaxis, inflammation, boils and sores, and to relieve pain in the chest and costal regions. Paeoniflorin (PF), a characteristic main principal bioactive component of *P. alba* Radix (PF, Liu *et al*., 2005), has been reported to exhibit many pharmacological effects such as anti-inflammatory and antiallergic effects (Yamahara *et al*., 1982), antihyperglycemic effects (Hsu *et al*., 1997), analgesic effects (Sugishita *et al*., 1984), neuromuscular blocking effects (Dezaki *et al*., 1996), cognition-enhancing effects (Takeda *et al*., 1995) and inhibitory effects on steroid protein binding (Tamaya *et al*., 1986). Moreover, recent studies indicated that PF might exert their activities by activation of adenosine A_1 receptors (A_1AR) (Lai *et al*., 1998; Cheng *et al*., 1999; Yang *et al*., 2001; Tang *et al*., 2003; Liu *et al*., 2005).

A_1AR has been found to be involved in nigrostriatal dopaminergic neurodegeneration. The administration of adenosine A_1AR agonists improved the impairment of dopaminergic system caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Lau & Mouradian, 1993) or methamaphetamine (DelleDonne & Sonsalla, 1994; Gol'embiowska & Żylewska, 1998), while the antagonists for this receptor subtype enhanced the damage of dopaminergic system caused by mitochondrial inhibitor malonate (Alfinito *et al*., 2003). Adenosine was also found to regulate the suppression of inflammation (Bouma *et al*., 1994; Cronstein, 1994; Hasko *et al*., 1996; Le Moine *et al*., 1996; Sajjadi *et al*., 1996; Schwaninger *et al*., 1997; Tsutsui *et al*., 2004). In the CNS, A_1AR is highly expressed on microglia/macrophages and neurons (Johnston *et al*., 2001). It has been found that activation and upregulation of A_1AR could attenuate neuroinflammation and demyelination in the mice model of multiple sclerosis (Tsutsui *et al*., 2004). These data indicated that the activation of A_1AR might have anti-inflammatory and neuroprotective effects on doparminergic neurons. Recently, we demonstrated that PF has neuroprotective effects in cerebral ischemic rat, and the neuroprotective effects were mediated by A_1AR (Liu *et al*., 2005). In the present studies, we investigated whether PF could prevent the neuroinflammation and dopaminergic neurodegeneration in a MPTP mouse model of PD. Moreover, A_1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was used to investigate whether A_1AR was involved in the protective effects of PF.

Methods

Chemicals and animals

PF was extracted from the dried and powdered roots of *P. alba,* one species in *Paeony*. The purity of PF is above 98% (Liu *et al*., 2005). DPCPX and MPTP were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). MPTP was dissolved in saline, and DPCPX was dissolved in saline with 5% dimethyl-sulfoxide (DMSO).

Male C57BL/6 mice, weighing 18–22 g, were used in the present studies. The animals had free access to solid food and water *ad libitum* under standard conditions of temperature, humidity and light. The study was performed in compliance with National Institutes of Health (NIH) guidelines and was approved by Animal Care and Use Committee, Shanghai Institute of Materia Medica, Chinese Academy of Science.

Experimental protocols

Two experimental paradigms of MPTP delivery have been used as described by Iwashita *et al*. (2004) with minor modifications. One is the four-dose paradigm of MPTP intoxication to induce severe cell injury, and the other is the two-dose paradigm to induce milder cell injury. To determine the effect of pre-MPTP treatment with PF, the four-dose paradigm (severe model) was used in the present studies. PF in saline was administered at 2.5 or 5 mg kg^{-1} s.c. for 11 days, on day 8, the animals received 4×20 mg kg^{-1} MPTP at 2 h intervals for the severe model. To determine the effect of post-MPTP treatment with PF, two-dose paradigm of MPTP (mild model) was used. The animals were injected with 2×20 mg kg^{-1} MPTP at 2 h intervals. Daily treatment with 2.5 or 5 mg kg^{-1} of PF was started at 1 h after the second injection of MPTP, and continued to day 3 after MPTP injection. Furthermore, to determine whether A_1AR was involved in the protective effects of PF, DPCPX, the selective A_1AR antagonist (0.3 mg kg^{-1}, i.p.) was given to MPTP-treated animals at 15 min before each PF administration in the post-MPTP treatment. In all the experiments, each group contained eight mice.

Behavioral observations

The pole test was used to measure bradykinesia, a typical symptom of parkinsonism (Matsuura *et al*., 1997; Araki *et al*., 2001; Kato *et al*., 2004). In the present studies, pole test was conducted 4 days after MPTP treatments with each experimental paradigm. The mice were placed head upward near the top of a vertical rough-surfaced pole (diameter 8 mm, height 55 cm). The time taken to turn completely downward (time to turn; T-turn) and the time until all four feet of the mouse reached the floor (locomotion activity time; T-LA) were recorded with the cutoff limit of 30 s. The test was performed five times for each mouse.

Immunohistochemistry

After anesthetization with pentobarbital, the mice were perfused by the intracardiac route with PBS, followed by 4% paraformaldehyde in PBS. The mice were then decapitated, and the brains were removed and immersed for 48 h in 4% paraformaldehyde for fixation. Midbrain and striatum coronal sections (25 μm thick) were then prepared with a cryostat. For all immunostaining, the sections were first rinsed with PBS containing 0.1% Triton-X (PBS-T), and then were immersed in a solution of 0.5% H_2O_2 for 30 min. After incubated overnight with primary antibody in PBS-T containing 10% normal serum at 4°C, the sections were washed three times in PBS-T and incubated with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, U.S.A.) in PBS-T for 2 h at room temperature. Then, sections were incubated in

avidin–biotin peroxidase complex (Vector Laboratories) for 1 h. A final incubation in DAB was performed for visualization. All the sections were then washed in PBS, mounted on amino propyltriethoxy silan-coated slides, dried, dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped. Following primary antibodies were used in this experiment: mouse monoclonal anti-tyrosine hydroxylase (TH, dilution 1 : 1000, Sigma Chemical Co., St Louis, MO, U.S.A.) for dopaminergic neurons, mouse monoclonal anti-glial fibrillary acidic protein (GFAP, dilution 1 : 1000; Chemicon International, Temecula, CA, U.S.A.) for astrocytes, mouse monoclonal anti-cd11b (1 : 200; Serotec, Raleigh, NC, U.K.) for activated microglia. For the microglial activation assay, the mice were killed 24 h after the last dose of MPTP. Whereas for astrocytic activation examination, mice were killed 96 h after the last MPTP injection.

Assessment of neuronal loss

Loss of neurons in the SNc of mice was determined by serial section analysis of the total number of TH-positive neurons at day 4 after MPTP treatment as described previously (Conti *et al*., 2005). Briefly, total numbers of TH-immunoreactive and Nissl-stained SNc neurons were counted by using stereology as previously described (Coggeshall, 1992; Gundersen, 1992; Volpe *et al*., 1998; Sugama *et al*., 2003). Non-neuronal cells were excluded by counting clearly defined nucleus, cytoplasm and prominent nucleolus by Nissl-staining. This procedure was carried out on four sections at a periodicity of 100 μm in the SNc. Average neuron density was obtained by summing the number of neuron profiles divided by the calculated volume. The total number of neurons was calculated as the product of the neuron density and the volume of SNc as described previously (Coggeshall, 1992; Gundersen, 1992; DeGiorgio *et al*., 1998; Volpe *et al*., 1998; Sugama *et al*., 2003). With this procedure, the number of cells counted is not affected by the volume of the SNc or the size of the neurons.

To determine the gray density of the TH-immunoreactive staining in the striatum, a square frame of 700×700 μm was placed in the dorsal part of the striatum. A second square frame of 200×200 μm was placed in the region of the corpus callosum to measure background values. To control for variations in background illumination, the average of the background density readings from the corpus callosum was subtracted from the average of density readings of the striatum for each section. TH optical density was given in arbitrary units (a.u.).

Real-time reverse transcription PCR

Total RNA was extracted from the ventral midbrain (VM) of mice with TRIzol reagent (Invitrogen, Gaithersburg, MD, U.S.A.) 24 h after the last dose of MPTP. The concentration and purity of the RNA preparations were determined by measuring the absorbance at 260 and 280 nm in a spectrophotometer. First-strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.). The cDNA template was then amplified by PCR using Ex-*Taq* (TaKaRa, Kyoto, Japan). The nucleotide sequences of the primers were based on published cDNA sequences (Table 1). Semiquantitative analysis was performed by monitoring in real-time the increase of fluorescence of the SYBR-green dye (Molecular Probes, Carlsbad, CA, U.S.A.) on DNA Engine Opticon 2 thermal cycler (MJ Research, Waltham, MS, U.S.A.). Real-time fluorescence measurements were performed, and a threshold cycle value for each gene of interest was determined, as reported previously (Power *et al*., 2003). All data were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level and expressed as mRNA relative fold change (RFC).

Table 1 The specific primers for interested genes

Oligonucleotides designed for RT–PCR	*Sequence*
IL-1β forward	5′-CTGTGTCTTTCCCGTGGACC-3′
IL-1β reverse	5′-CAGCTCATATGGGTCCGACA-3′
iNOS forward	5′-TCACTGGGACAGCACAGAAT-3′
iNOS reverse	5′-TGTGTCTGCAGATGTGCTGA-3′
TNF-α forward	5′-GCGGTGCCTATGTCTCAGCC-3′
TNF-α reverse	5′-TGAGGAGCACGTAGTCGGGG-3′
GAPDH forward	5′-GGTTGTCTCCTGCGACTTCA-3′
GAPDH reverse	5′-TGGTCCAGGGTTTTTACTCC-3′

N-*methyl-4-phenylpyridinium ion measurements*

1-methyl-4-phenyl-pyridiniumion (MPP^+) measurements were performed as described previously (Iwashita *et al*., 2004). Briefly, PF 5 mg kg^{-1} was injected subcutaneously (s.c.) 7 days before or 1 h after a single dose of MPTP (20 mg kg^{-1} i.p.). At 2 h after MPTP dosing, striatal concentrations of MPP^+ was measured by HPLC as described previously (Crocker *et al*., 2003).

Statistical analysis

Data were presented as the mean $\pm$ s.e.m. Statistical differences were determined by Paired student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* comparison. For all cases, significance of differences were accepted at $P<0.05$.

Results

Effects of pre- and post-treatment with PF on MPTP-induced dopaminergic neurodegeneration and motor deficit

To ascertain whether PF exerts the neuroprotective effects in the MPTP mouse model, PF was first evaluated on the four-dose paradigm of MPTP delivery (severe model). PF was administered 11 days to C57BL/6 mice (2.5 or 5 mg kg^{-1} s.c.), and MPTP was injected to mice on day 8. In this model, pretreatment of PF significantly and dose dependently ameliorated the loss of the nigrostriatal dopaminergic neurons in the SNc (Figure 1) and terminals in the striatum (Figure 2). The MPTP injection resulted in significant motor deficits in the pole test. The time of T-turn of the pole test was increased by 4.6-fold, the time T-LA was increased by 1.9-fold (all with significance at $P<0.01$ compared with normal control). Treatment with PF significantly reduced the motor abnormalities in the pole tests in a dose-dependent manner (Figure 3).

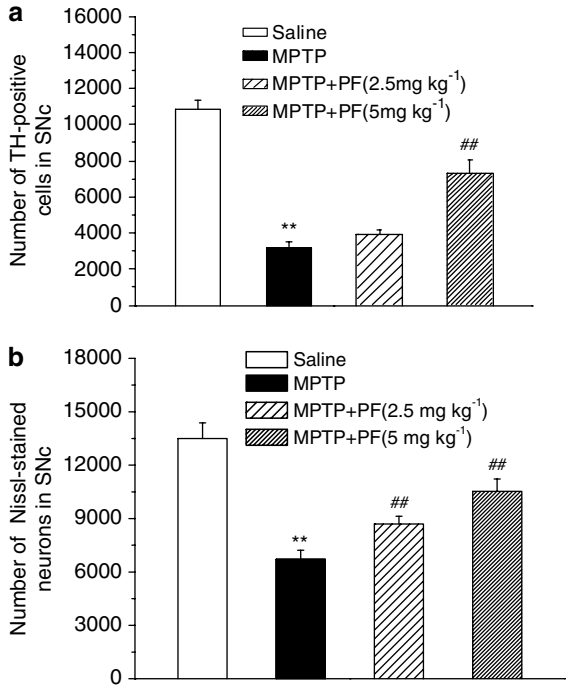

Figure 1 Effects of PF on MPTP-induced dopaminergic neuron loss in the SNc with pretreatment. Quantitative analysis of the TH-positive cells (a) and Nissl-stained neurons (b) in the SNc of mice. Each column and vertical bar represents the mean ± s.e.m. of results from eight mice. $^{**}P<0.01$ compared with saline-treated mice, $^{\#\#}P<0.01$ compared with MPTP-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison).

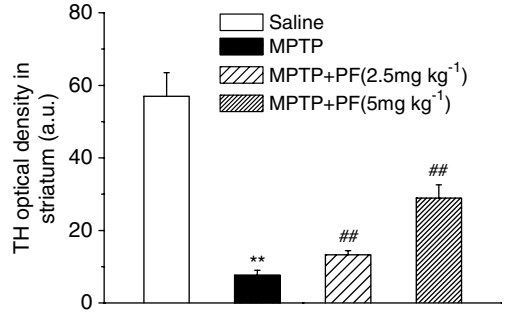

Figure 2 Effects of PF on MPTP-induced dopaminergic fiber loss in the striatum with pretreatment. Each column and vertical bar represents the mean ± s.e.m. of results from eight mice. $^{**}P<0.01$ compared with saline-treated mice, $^{\#\#}P<0.01$ compared with MPTP-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison).

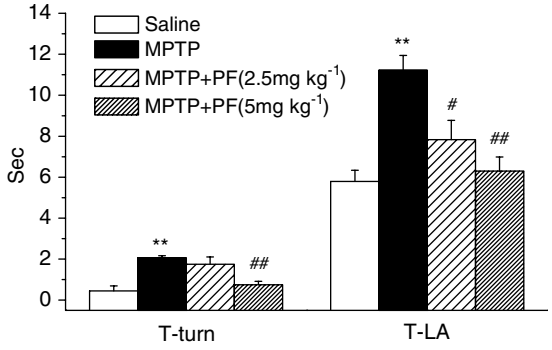

Figure 3 Effects of PF on MPTP-induced motor deficit in mice with pretreatment. Each column and vertical bar represents the mean ± s.e.m. of results from eight mice. $^{**}P<0.01$ compared with saline-treated mice, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with MPTP-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison).

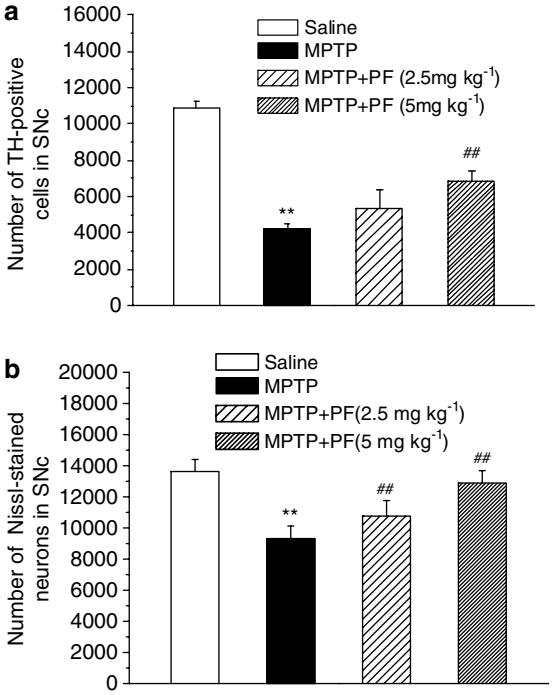

Figure 4 Effects of PF on MPTP-induced dopaminergic neuron loss in the SNc with post-treatment. Quantitative analysis of the TH-positive cells (a) and Nissl-stained neurons (b) in the SNc of mice. Each column and vertical bar represents the mean ± s.e.m. of results from eight mice. $^{**}P<0.01$ compared with saline-treated mice, $^{\#\#}P<0.01$ compared with MPTP-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison).

To determine whether the neuroprotective effect of PF would also be obtained with delayed treatment, post-treatment of PF after MPTP injections was conducted in C57BL/6 mice. On the two-dose paradigm of MPTP delivery (mild model), MPTP was injected twice at a 2 h interval. Administration of MPTP with this regimen resulted in mild-to-moderate reduction of SNc dopaminergic neuron survive (approximately 39% of normal level; Figure 4) and striatal dopaminergic terminals level (approximately 23% of normal level; Figure 5). When PF (2.5 or 5 mg kg^{-1}) was subcutaneously administered 1 h after second MPTP injections and administered once a day for the following 3 days, post-treatment of PF also significantly attenuated the motor abnormalities in the pole tests (Figure 6), neuronal loss assessed by the quantification of TH-positive cells and Nissl-stained neurons in the SNc (Figure 4) and TH-positive terminals in the striatum (Figure 5). The loss of TH-positive neurons in the SNc was significant but relatively mild compared with TH positive in the striatum. Thus, the depletion of TH-positive terminals in the striatum was coincident with cell death in the SNc.

Effect of PF on glial activation and upregulation of proinflammatory molecules

As PF has been reported to have an anti-inflammatory effect, we determined whether PF treatment could attenuated the glial-mediated inflammatory response in the MPTP mouse model of PD. Potent microglial activation in SNc and striatum was observed at 24 h after the last dose of MPTP (Figure 7b and e), while the astrocytic activation was at observed 96 h

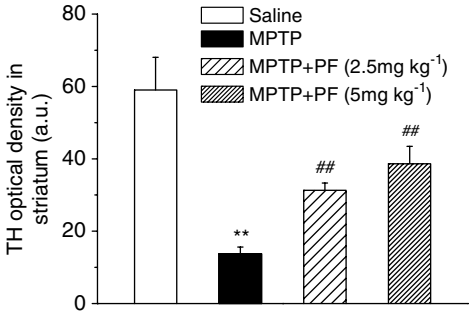

Figure 5 Effects of PF on MPTP-induced dopaminergic fiber loss in the striatum with post-treatment. Each column and vertical bar represents the mean ± s.e.m. of results from eight mice. $^{**}P<0.01$ compared with saline-treated mice, $^{\#\#}P<0.05$ compared with MPTP-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison).

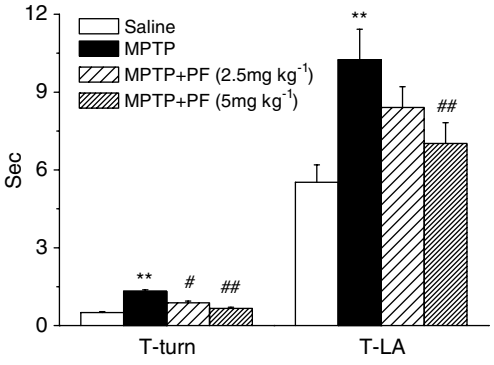

Figure 6 Effects of PF on MPTP-induced motor deficit in mice with post-treatment. Each column and vertical bar represents the mean ± s.e.m. of results from eight mice. $^{**}P<0.01$ compared with saline-treated mice, $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with MPTP-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison).

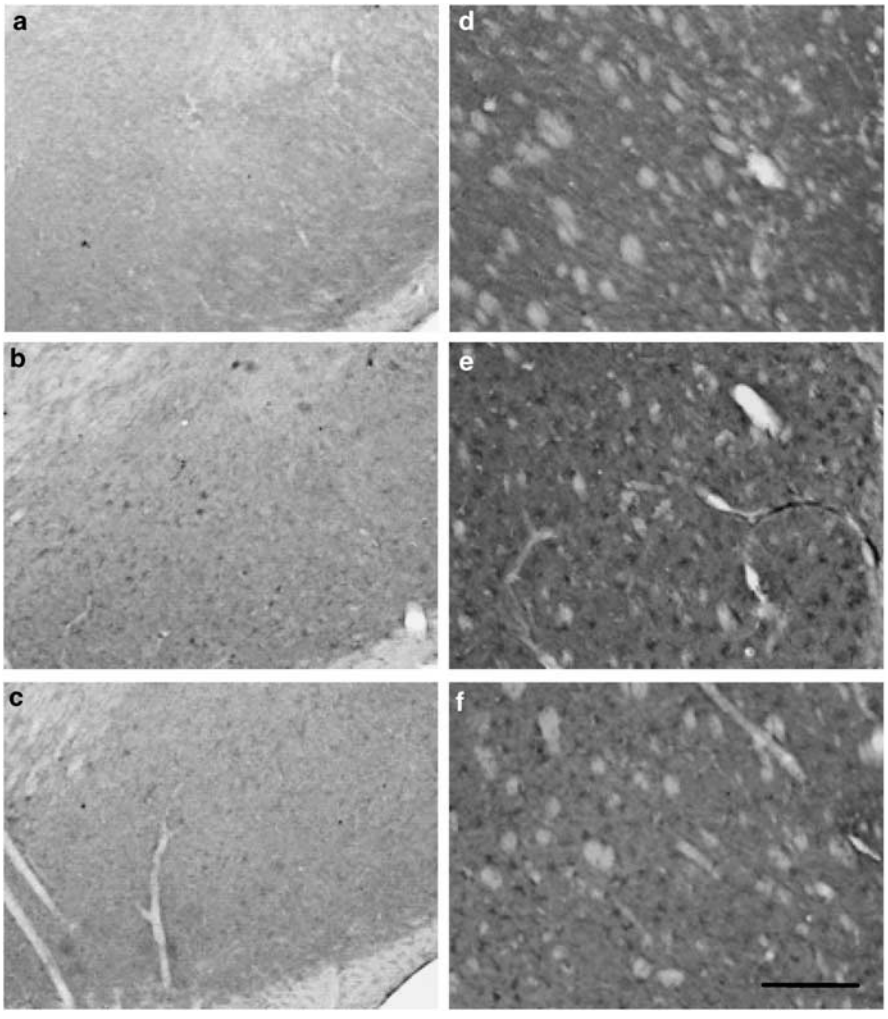

Figure 7 Effects of PF on MPTP-induced microglial activation in the SNc and striatum. Cd11b immunohistochemistry in the SNc (a–c) and striatum (d–f) of mice. (a, d) saline-treated mice, (b, e) MPTP-treated mice, (c, f) MPTP plus PF-treated mice. Microglial activation after MPTP were attenuated by PF treatment in both the SNc and striatum, $n=8$, scale bar, 100 μm.

(Figure 8b and e). In terms of morphological characteristics, both the cd11b and GFAP-positive cells appeared more compact, rounded, and with obvious cellular thickening, indicative of an activated state (Soltys *et al*., 2001). Post-MPTP treatment of PF (5 mg kg^{-1}) significantly attenuated the microglial and astrocytic activation (Figures 7 and 8).

It has been reported that the selective expression and release of inflammatory mediators, such as cytokines and NO, from activated glial cells contribute to the pathogenesis of PD (Mogi *et al*., 1994a, b; Przedborski & Jackson-Lewis, 1998). Given the effect of PF on MPTP-induced glial activation, we examined whether the production of known proinflammatory molecules was also inhibited by PF. The RFC in mRNA expression of IL-1β of MPTP-treated mice showed a significant 5.3-fold and 1.4-fold increase in the SNc and striatum, respectively, compared with that of saline-treated mice. Similarly, treatment of MPTP significantly augmented the expression of TNF-α and iNOS compared with saline group. The upregulation of these proinflammatory molecules was reduced by post-MPTP treatment of PF (5 mg kg^{-1}) (Table 2). These data suggested that the anti-inflammatory property of PF might be contributed to its neuroprotective effect.

Effect of DPCPX on the neuroprotective and anti-inflammatory effects of PF

To determine whether A_1AR is involved in the neuroprotective effects of PF, DPCPX (0.3 mg kg^{-1}), a selective A_1AR antagonist, was injected i.p. at 15 min before PF administration in the MPTP mild model. DPCPX alone did not cause or enhance the dopaminergic neurodegeneration and neuroinflammation in saline or MPTP-treated mice (Figures 9 and 10). However, the protective effect of PF (5 mg kg^{-1}) was abolished by pretreatment with the selective A_1AR antagonist DPCPX (0.3 mg kg^{-1}) (Figures 9 and 10). Notably, the anti-gliosis effect of PF was reversed (Figures 11 and 12). The mRNA expression of proinflammatory molecules was also elevated by DPCPX administration compared with PF plus MPTP treatment (Table 2).

Effect of PF on MPTP metabolism

To confirm that the neuroprotective effect of PF is not caused by reducing metabolism of MPTP to MPP^+, MPP^+ level in the brain was measured after PF treatment. At 2 h after the

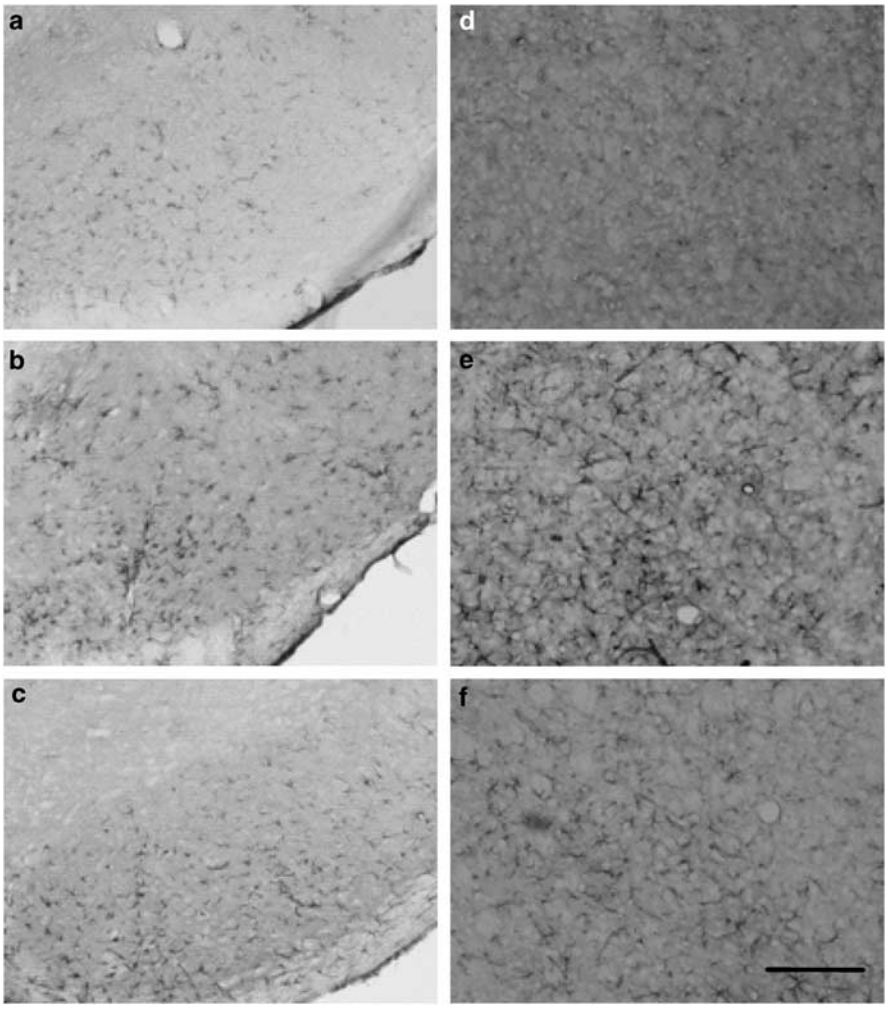

Figure 8 Effects of PF on MPTP-induced astrocytic activation in the SNc and striatum. GFAP immunohistochemistry in the SNc (a–c) and striatum (d–f) of mice, (a, d) saline-treated mice, (b, e) MPTP-treated mice, (c, f) MPTP plus PF-treated mice. Astrocytic activation after MPTP were attenuated by PF treatment in both the SNc and striatum, $n = 8$, scale bar, 100 μm.

Table 2 Effects of PF on the mRNA upregulation of proinflammatory molecules induced by MPTP

	Saline	*MPTP*	*PF* (5 mg kg^{-1}) + *MPTP*	*DPCPX* (0.3 mg kg^{-1}) + *PF* (5 mg kg^{-1}) + *MPTP*
VM				
iNOS	1.00±0.17	2.98±0.50**	1.02±0.17$^{\#\#}$	2.23±0.37$^{\$}$
TNF-α	1.00±0.17	2.69±0.45**	1.12±0.19$^{\#\#}$	1.74±0.29
IL-1β	1.00±0.09	5.26±0.84**	2.12±0.03$^{\#\#}$	3.51±0.14$^{\$\$}$
Striatum				
iNOS	1.00±0.03	1.95±0.32*	1.17±0.27	1.68±0.07
TNF-α	1.00±0.08	3.17±0.26**	1.30±0.02$^{\#\#}$	2.63±0.03$^{\$\$}$
IL-1β	1.00±0.18	1.42±0.06*	1.06±0.17	1.24±0.04

All data were normalized to the GADPH mRNA level and expressed as RFC±s.e.m. ($n=8$). *$P<0.05$, **$P<0.01$ compared with saline-treated mice; $^{\#\#}P<0.01$ compared with MPTP-treated mice; $^{\$}P<0.05$, $^{\$\$}P<0.01$ compared with PF (5 mg kg^{-1}) + MPTP-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison).

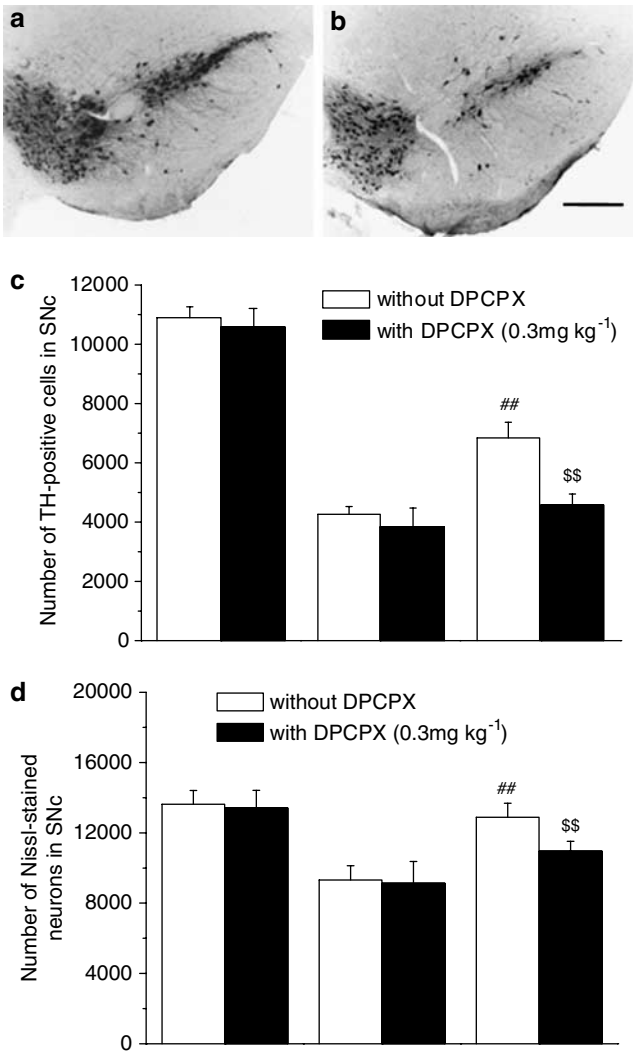

Figure 9 Effects of DPCPX on the dopaminergic neuron-protective effects of PF in the SNc. TH immunohistochemistry in the SNc (a, b), quantitative analysis of the TH-positive cells (c) and Nissl-stained neurons (d) in the SNc of mice. (a) MPTP plus PF-treated mice, (b) MPTP plus PF-treated mice that pretreated with DPCPX before PF administration. The protective effect of PF (5 mg kg^{-1}) on dopaminergic neurons in the SNc was abolished by pretreatment with the selective A_1AR antagonist DPCPX (0.3 mg kg^{-1}). Each column and vertical bar represents the mean±s.e.m. of results from eight mice. $^{\#\#}P<0.01$ compared with MPTP-treated mice, $^{\$\$}P<0.01$ compared with MPTP plus PF-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison), scale bar, 200 μm.

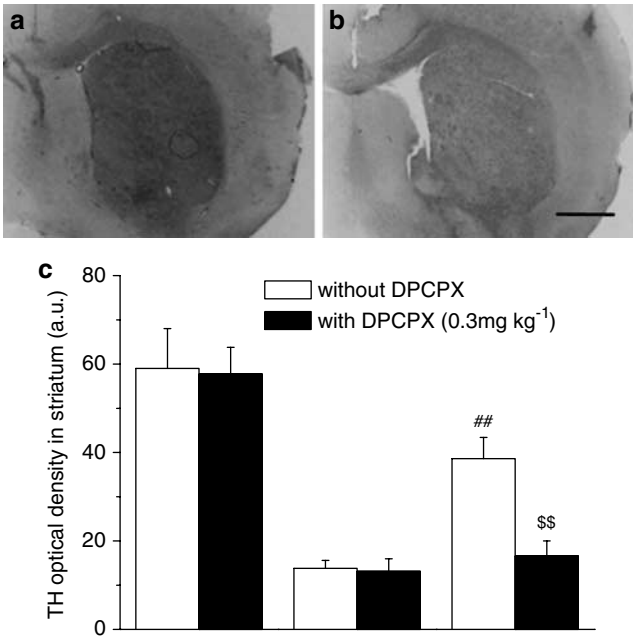

Figure 10 Effects of DPCPX on the dopaminergic fiber-protective effects of PF in the striatum. TH immunohistochemistry in the striatum (a, b), quantitative analysis of the TH optical density in the striatum of mice (c). (a) MPTP plus PF-treated mice, (b) MPTP plus PF-treated mice that pretreated with DPCPX before PF administration. The protective effect of PF (5 mg kg^{-1}) on dopaminergic fibers in the striatum was abolished by pretreatment with the selective A_1AR antagonist DPCPX (0.3 mg kg^{-1}). Each column and vertical bar represents the mean±s.e.m. of results from eight mice. $^{\#\#}P<0.01$ compared with MPTP-treated mice, $^{\$\$}P<0.05$ compared with MPTP plus PF-treated mice (one-way ANOVA followed by Dunnett's *post hoc* comparison), scale bar, 500 μm.

MPTP treatment, MPP^+ levels in the striatal were 2.21±0.25 μg g^{-1} (pretreated with 5 mg kg^{-1} PF for 7 days) and 2.38±0.11 μg g^{-1} (PF was injected 1 h after MPTP) in PF-treated mice, or 2.23±0.17 μg g^{-1} in vehicle treated mice, respectively. Thus, PF treatment had no effect on the concentration of MPP^+ in the brain of MPTP-treated C57BL/6 mice.

Discussion

In the present studies, two experimental paradigms of MPTP delivery (Iwashita *et al.*, 2004) were used to determine the

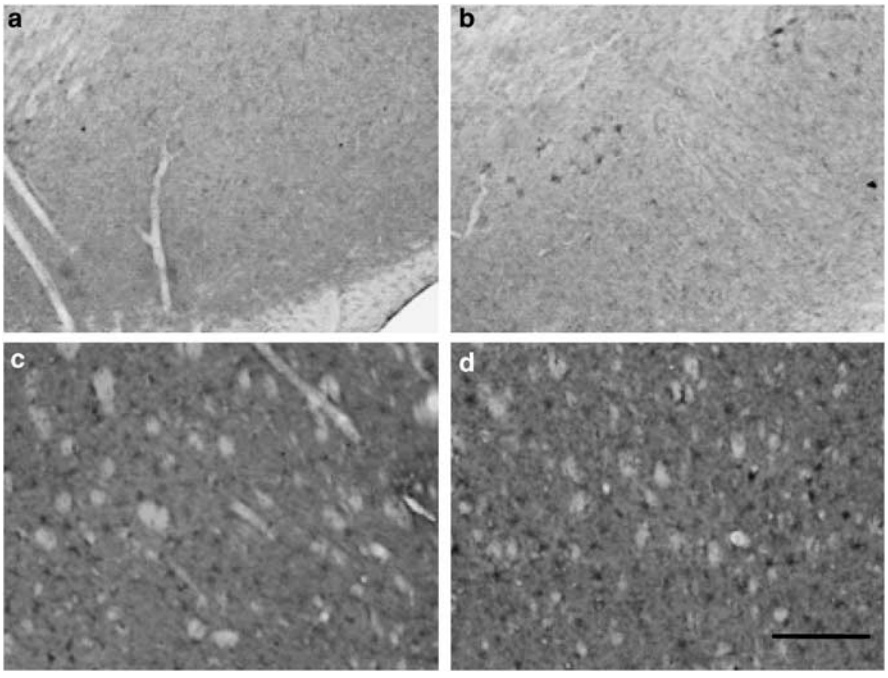

Figure 11 Effect of DPCPX on the microglia-modulating effects of PF. Cd11b immunohistochemistry in the SNc (a, b) and striatum (c, d), (a, c) MPTP plus PF-treated mice, (b, d) MPTP plus PF-treated mice that pretreated with DPCPX before PF administration. The microglia-modulating effects of PF (5 mg kg^{-1}) were reversed by pretreatment with DPCPX (0.3 mg kg^{-1}), $n = 8$, scale bar, 100 μm.

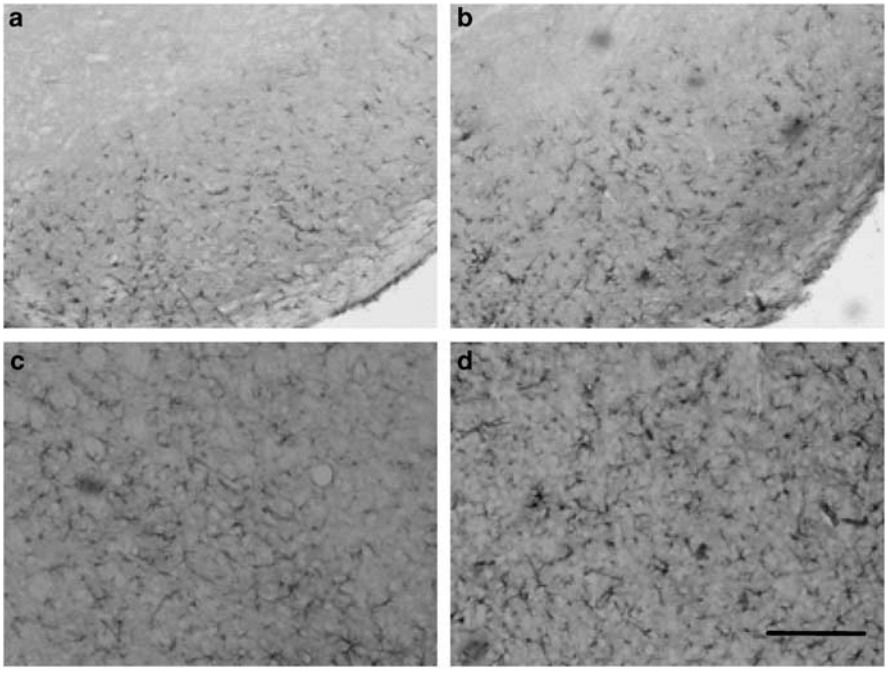

Figure 12 Effect of DPCPX on the astrocyte-modulating effects of PF. GFAP immunohistochemistry in the SNc (a, b) and striatum (c, d) of mice. (a, c) MPTP plus PF-treated mice, (b, d) MPTP plus PF-treated mice that pretreated with DPCPX before PF administration. The astrocyte-modulating effects of PF (5 mg kg^{-1}) were reversed by pretreatment with DPCPX (0.3 mg kg^{-1}), $n = 8$, scale bar, 100 μm.

neuroprotective properties of PF. Both the severe and mild MPTP treatment caused a selective dopaminergic neurodegeneration in SNc and striatum (Figures 1, 2, 4 and 5), which result in the motor deficit in the pole test in mice (Figures 3 and 6). Our results were similar to those reported by Liberatore *et al*. (1999), Benner *et al*. (2004), Kurosaki *et al*. (2004) and Conti *et al*. (2005). In the present studies, we demonstrated that 11 days' treatment with PF attenuated the

dopaminergic neurotoxicity and bradykinesia induced by four-dose injection of MPTP on day 8 in mice (Figures 1–3). Since two-dose paradigm of MPTP has been proved to be an excellent PD model for drug evaluation with delayed treatment (Iwashita *et al*., 2004), this paradigm was also used to determine the effects of post-MPTP treatment with PF in the present studies. We demonstrated that the neuroprotective effects were also obtained with post-MPTP treatment of PF (Figures 4–6). In addition, the HPLC-UV assay indicated that PF administration 7 days before or 1 h after MPTP had no effect on the striatal MPP^+ content in mice, suggesting that the neuroprotection afforded by PF was not attributable to impairment of MPTP metabolism. Thus, the key finding of the present studies was that PF, a monoterpene glucoside isolated from the Chinese herbal *Paeony* radix, could potently protected dopaminergic neurons against MPTP-induced degeneration and motor deficit in mice (Figures 1–6).

It is known that inflammation has an important role in the pathogenesis of PD (McGeer *et al*., 1988; Hunot & Hirsch, 2003). The hallmark of brain inflammation is the activation of glia, particularly microglia. Activation of microglia is thought to contribute to neuronal damage by the release of proinflammatory and neurotoxic factors. These factors include proinflammatory cytokines such as TNF-α and IL-1β, reactive nitrogen species, ROS, eicosanoids and excitatory amino acids (Mogi *et al*., 1994a, b; Merrill & Benveniste, 1996; Przedborski & Jackson-Lewis, 1998; Liu & Hong, 2003). In the MPTP mouse model of PD, reactive gliosis has been characterized with both reactive astrogliosis and microgliosis observed in the SNc and striatum (Francis *et al*., 1995; Kohutnicka *et al*., 1998; Kurkowska-Jastrzebska *et al*, 1999; Kay & Blum, 2000). It has been reported that the expression of cd11b, the marker of activated microglia, reached maximum at 24–48 h after acute MPTP injection (Kohutnicka *et al*., 1998; Ferger *et al*., 2004; Furuya *et al*., 2004). While GFAP, the marker of astrocytes, has been reported peaked at 4–7 days after MPTP injection (Schneider & Denaro, 1988; Kohutnicka *et al*., 1998; Kato *et al*., 2004). Therefore, in the present studies, mice were killed at either 24 h or 96 h after the last dose of MPTP, respectively, in order to examine the activation of either microglia or astrocytes. We demonstrated that MPTP treatment induced a marked increase in microglial and astrocytic activation within the SNc and striatum, and that the reactive gliosis was reduced in the PF ($5\,mg\,kg^{-1}$)-treated animals (Figures 7 and 8). Upregulation of the proinflammatory molecules such as IL-1β, TNF-α and iNOS has been reported in MPTP-treated mice (Grunblatt *et al*., 2000; Wu *et al*., 2002; Ferger *et al*., 2004; Furuya *et al*., 2004; Hebert *et al*., 2005; Marchetti *et al*., 2005; Shen *et al*., 2005). The inhibition of these upregulation has been clarified to be neuroprotective (Mogi *et al*., 1998; Liberatore *et al*., 1999; Ferger *et al*., 2004). Consistent with these reports, we demonstrated that the mRNA levels of IL-1β, TNF-α, and iNOS were significantly upregulated in VM and striatum by MPTP treatment in the present studies. Moreover, the upregulation of these proinflammatory molecules was significantly reversed by PF ($5\,mg\,kg^{-1}$) (Table 2). These results strongly suggested that the neuroprotective effect of PF observed in the present investigation is most probably derived from its anti-inflammatory property.

Several lines of evidence indicate that adenosine may be an endogenous neuroprotective agent in the CNS (Rudolphi *et al*., 1992; Ongini & Schubert, 1998; Von Lubitz, 1999; De Mendonça *et al*., 2000). A_1AR agonists were shown to attenuate dopaminergic neurodegeneration in different models of PD (Lau & Mouradian, 1993; DelleDonne & Sonsalla, 1994; Gol'embiowska & Żylewska, 1998), while blockade of A_1AR has been found to enhance the dopaminergic system damage (Alfinito *et al*., 2003). To investigate whether A_1AR was involved in the neuroprotection of PF, DPCPX, a selective A_1AR antagonist, was used in the present studies. Since it has been reported that doses higher than $0.5\,mg\,kg^{-1}$ DPCPX tended exacerbate MPTP toxicity (Chen *et al*., 2001), the lower but effective dose of $0.3\,mg\,kg^{-1}$ DPCPX (Zarrindast *et al*., 1999) was chosen here. We demonstrated that DPCPX alone did not cause dopaminergic neurodegeneration in saline-treated mice or enhance the neurotoxicities in MPTP-treated mice. However, pretreatment with DPCPX reversed the neuroprotective effects of PF (Figures 9 and 10), indicating that A_1AR might play an important role in the PF-induced neuroprotection. In addition, our previous studies reported that PF was able to displace the binding of [^{3}H] *N*-ethylcarboxamidoadenosine (NECA), the A_1AR agonist, in competitive binding assays (Liu *et al*., 2005).

Recent studies have demonstrated that activation of adenosine receptors on immune cells suppressed the production of proinflammatory mediators, including TNF-α (Bouma *et al*., 1994; Hasko *et al*., 1996; Sajjadi *et al*., 1996) and matrix metalloproteinases (Boyle *et al*., 1996). Adenosine receptor agonists appear to influence other macrophage properties, such as phagocytosis and chemotaxis, thereby reducing leukocyte accumulation at sites of inflammation (Cronstein, 1994; Olah & Stiles, 1995). In the CNS, the A_1AR is highly expressed on microglia/macrophages and neurons (Johnston *et al*., 2001). However, A_1AR has not been detected in dopaminergic neurons in SNc (Alexander & Reddington, 1989), indicated that the activation of A_1AR might not have direct effects on dopaminergic neurons. It has been reported that activation or upregulation of A_1AR which located in glial cells could attenuate neuroinflammation and demyelination in the mice model of multiple sclerosis, another disease of the CNS characterized by neuroinflammation (Tsutsui *et al*., 2004). In the present studies, we demonstrated that treatment with DPCPX ($0.3\,mg\,kg^{-1}$) alone did not cause reactive gliosis or upregulation of proinflammatory molecules in saline-treated mice. Treatment with DPCPX ($0.3\,mg\,kg^{-1}$) alone did not potentiate the neuroinflammation in MPTP-treated mice either (data not shown). However, pretreatment with DPCPX reversed the anti-inflammatory effects of PF (Figures 11 and 12; Table 2). The results suggested that A_1AR might be involved in the anti-inflammatory effects of PF.

None of the classical A_1AR agonists was used as the positive control in the present studies. One reason is that the classical A_1AR agonists caused severe cardiovascular side effects (Daval *et al*., 1991; Collis & Hourani, 1993; Van Schaick *et al*., 1997). Another and more important reason is that the mechanism of PF was different from that of the classical A_1AR receptor agonists (Liu *et al*., 2005).

In conclusion, our results demonstrated that PF, a characteristic monoterpene glucoside isolated from the root of *P. alba*, had a potent neuroprotective effect on dopaminergic neurons in the MPTP mouse model of PD. The

neuroprotective effects of PF might be mediated though its modulation of neuroinflammation by activation of the A_1AR. Our results suggested that PF might represent a promising candidate for the prevention and treatment of PD.

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