Neuroprotective Effects of Bak Foong Pill in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-Induced Parkinson’s Disease Model Mice

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Neuroprotective effects of estrogen and estrogen-like chemicals on neurodegenerative diseases, especially Parkinson’s disease, have been well established. In the present study, we compared the effects of Bak Foong Pill (BFP), a well-known gynaecological tonic in China, and 17β-estradiol, on dopamine transporter (DAT) and tyrosine hydroxylase (TH) gene expression patterns in ovariectomized, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson’s disease (PD) model mice, using multiplex reverse transcription-polymerase chain reaction (RT-PCR). MPTP, a specific dopaminergic neurotoxin, significantly decreased DAT and TH mRNA levels in the striatum, midbrain and cerebellum, but not the cortex, of C57BL/6 mice. However, MPTP-challenge with BFP pretreatment demonstrated reduced neurotoxicity, with DAT and TH mRNA levels either not affected by MPTP or affected to a significantly lesser extent in the midbrain and striatum as compared to the MPTP treated controls. 17β-estradiol treatment prevented MPTP-induced reduction of DAT expression in striatum and midbrain, but failed to alter TH expression. These results suggest that BFP is able to protect dopaminergic neurons against MPTP-induced neuronal damage in a mechanism that is different from the protective effect of estrogen.

Key words Bak Foong Pill; estrogen; Parkinson’s disease; tyrosine hydroxylase; dopamine transporter

Parkinson’s disease (PD) is a chronic neurodegenerative disorder characterized by the loss of dopaminergic neurons of substantia nigra pars compacta in the ventral midbrain.1) The loss of dopaminergic neurons leads to the reduction of dopamine release into the striatum, and is responsible for the clinical features of PD including bradykinesia, resting tremor, rigidity, and difficulty in initiating movements.2) Pathological patterns of PD are usually examined by inducing Parkinson’s-like symptoms in rodents by injection of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and is widely used for study of PD.3) MPTP is converted to MPP+, which structurally resembles dopamine, by brain monoamine oxidase B (MAO-B) and can be actively transported into the dopaminergic nerve terminals of the striatum by dopamine transporter (DAT).4) It is important to note that DAT is found only in dopaminergic neurons and may be the best and only unique marker for dopaminergic neurons.5) It has been shown that administration of MPP+ alone decreases dopamine transporter protein expression by Western blot.6) In addition, MPTP intoxication has been reported to be associated with reduced immunoreactivity of tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis,7) in the dopaminergic neurons of substantia nigra via the dopamine uptake system. This is considered to lead to energy failure and subsequently the inhibition of protein expression.8) The reduction of TH-positive neurons is also considered to be a good marker for dopaminergic cell loss. Current therapy for PD is essentially symptomatic, with the use of levodopa, the direct precursor of dopamine, remaining the primary treatment choice since its first use over 30 years ago.9)–11) However, long-term therapy with levodopa is associated with significant side effects.12) Neuroprotective therapy to rescue dopamine neurons, which could alter and prevent the progression of PD,13) has thus been proposed as an alternative to symptomatic treatment. It has been shown that neuroprotection can be observed in the PD models with iron chelators, radical scavenger antioxidants, MAO-B inhibitors, nitric oxide synthase inhibitors, calcium channel antagonists, glutamate antagonist trophic factors.12) Even nicotine and low exposure to cigarette smoke may have a neuroprotective effect on the dopaminergic nigrostriatal system.14)

In addition, epidemiological data suggest that steroid hormone 17β-estradiol plays an important role in protecting the brain from neurodegenerative processes, including Parkinson’s disease. Estrogen, but not androgen, has been reported to prevent MPTP-induced dopamine depletion,15) decrease of DAT mRNA and DAT specific binding.16) Phytoestrogens6,17) and Chinese medicines,18) as well as acupuncturing without the use of any drug19) have also been demonstrated to have neuroprotective effects in PD. As a well-known gynaecological tonic in China, Bak Foong Pill (BFP, China registration number Z980035, also known as Bai Feng Wan) has long been recognized to have estrogen-like activities, such as its ability to increase the expression of cystic fibrosis transmembrane-conductance regulator,20) reduce blood pressure, increase vasorelaxation, reduce serum triglyceride, and even its antiplatelet activity21,22) and stimulating effects on dopamine release of the brain.23) To explore possible neuroprotective effect of BFP and related molecular mechanism(s), we examined the expression of DAT and TH in several brain regions of MPTP intoxicated mice with and without pretreatment of BFP, and compared their expression in PD mice with 17β-estradiol pretreatment.

MATERIALS AND METHODS

Reagents BFP was obtained from Eu Yan Sang (Hong Kong) Ltd, with the ingredients described previously.22) 17β-Estradiol and MPTP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade and were from commercial sources.

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Animals Adult female C57/BL/6 mice (10—12 weeks of age, 20—25 g), bred under specified pathogen free (SPF) conditions at the Laboratory Animal Services Centre of the Chinese University of Hong Kong, were maintained at 20—24 °C, 50—60% relative humidity, light/dark cycle of 12:12, and with food and water available ad libitum. Ovariectomy was performed on all animals, as described previously, to remove the influence of endogenous estrogen. Following a two-weeks post-surgical recovery period, the mice were divided randomly into groups and treated with BFP (3 g/kg, p.o.), 17β-estradiol (50 μg/kg, i.p.) or vehicle (10 ml/kg H2O, p.o. or 0.2 ml saline, i.p.) for 2 weeks. On the 14th day, the mice were treated with MPTP (15 mg/kg×4 times with a 2 h interval) or vehicle, followed by a further 5 d of BFP, estrogen or vehicle treatment. On the final day of treatment, the mice were sacrificed and the striatum, midbrain, cortex and cerebellum were harvested according to the mouse brain atlas, rapidly frozen in liquid nitrogen, and stored at −70 °C until used for RT-PCR analysis.

During the experiments, all the procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications No. 85—23, revised 1985) and were performed according to institutional animal experimentation ethical guidelines.

Total RNA Extraction Total RNA was extracted from the brain tissues of mice using TRIzol (Invitrogen, Life Technologies). The concentration of RNA was estimated by spectrophotometer at 260 nm wavelength. Reverse transcription was carried out with SuperScript Pre-amplification System according to manufacturers instructions. Briefly, 1 ml TRIzol was added to every 500 mg tissues and homogenized, followed by addition of 200 μl chloroform and centrifugation at 14000 × g at 4 °C for 10 min. The aqueous phase was then transferred to a centrifuge tube containing 500 μl isopropanol and incubated at −20 °C for 10 min. The resulting mixture was then centrifuged at 14000 × g at 4 °C for 10 min and the visible RNA pellet washed with 1 ml 75% ethanol and re-suspended in sufficient diethyl pyrocarbonate-treated H2O (DEPC-H2O).

Reverse Transcription (RT)-Complement DNA (cDNA) Preparation RNA samples were reverse transcribed by incubation with a reverse transcription mixture containing the following constituents: oligo(dT)12-18 primer, 10×PCR buffer, 25 mM MgCl2, 0.1 mM DTT, 10 mM 2’ deoxynucleotide 5’ triphosphates (dNTPs), 30 U of RNase inhibitor (Amer sham) and 200 U of Superscript II RT (Life Technologies). Synthesis occurred for 50 min at 42 °C, followed by treatment at 70 °C for 15 min to inactivate the RT enzyme.

Multiplex Polymerase Chain Reaction (PCR) The cDNA in the RT product was amplified using Taq DNA polymerase. The reaction conditions in a total volume of 25 μl PCR solution were as follow: 15 μg/μl RT mixture, 10×PCR buffer, 10 mM dNTPs, 15 pmol of each primer pair and 1 U of Taq DNA polymerase. The specific oligo nucleotide primers for DAT, TH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are indicated in Table 1. The cDNA was amplified under the following reaction conditions: 94 °C for 1 min, 60 °C for 45 s, 72 °C for 45 s. The cyclic process was performed 28—30 times for DAT, TH and GAPDH, respectively. A 10 μl aliquot of each PCR product was visualized by luminescence following electrophoresis through a 2% agarose gel containing 0.5 μg/ml ethidium bromide. PCR products were illuminated with Alphaimager 2200 documentation & analysis system and analysed by computerized densitometric scanning of the images. PCR products were sequenced to make sure they were the target mRNA.

Statistical Analysis Values shown are mean ± S.E.M. Data analyses were performed using one-way analysis of variance (ANOVA), followed by a post-hoc Tukey. p<0.05 was considered statistical significant.

RESULTS To examine the effects of BFP on DAT and TH gene expression in the brain of MPTP-intoxicated mice, multiplex RT-PCR was carried out. The results showed that DAT and TH mRNA were prominently expressed in the striatum and midbrain of mice, while detectable levels of TH but not DAT mRNA could be found in the cerebellum. However, neither could be reliably detected in the cortex. MPTP significantly decreased DAT mRNA expression in the striatum (91.5%) and midbrain (83.9%), but not the cerebellum and cortex, of the C57BL/6 mice (Fig. 1). However, MPTP-challenge following BFP treatment demonstrated reduced neurotoxicity, with DAT mRNA levels either not affected by MPTP or affected to a significantly lesser extent in the midbrain and striatum, when compared to vehicle treated animals. BFP itself did not affect DAT gene expression in the brain regions examined (data not shown). Similar MPTP-induced decreases in TH mRNA levels were also observed in the striatum, midbrain and cerebellum with a reduction of 70.8%, 61.3% and 48.4%, respectively (Fig. 2). Treatment with BFP significantly inhibited the MPTP-induced reduction in TH mRNA with almost no neurotoxicity found in the striatum and cerebellum. The TH mRNA levels in the cortex were so low that no significant differences could be detected between control and treatment groups.

Similar experiments were also conducted where 17β-estradiol was used instead of BFP for pretreatment. It was shown that 17β-estradiol prevented the reduction of DAT expression in striatum and midbrain of the MPTP-challenged mice (Fig. 3) to an extent similar to that produced by BFP. In contrast to

<table>
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<th>Genes</th>
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<th>Nucleotides position</th>
<th>RT-PCR product</th>
<th>Gene reference</th>
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<td>TH</td>
<td>Sense: AAA ATC CAC CAC TTA GAG ACC</td>
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<td>Sense: GAC CAC AGT CCA TGC CAT CAC</td>
<td>No. 565—904</td>
<td>340 bp</td>
<td>XM 132897</td>
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Table 1. RT-PCR Primers for Selected Genes
BFP, however, 17β-estradiol, failed to inhibit MPTP-induced decreases in TH expression in all brain regions examined (Fig. 4).

DISCUSSION

PD is a chronic neurodegenerative disorder characterized by the loss of dopaminergic neurons in the nigro-striatal system. In the present study, we detected DAT and TH gene expression not only in the nigro-striatal system, but also in the cerebellum and cortex with much reduced expression levels compared to the nigro-striatal system. It is well known that considerable numbers of dopaminergic neurons are located in the midbrain. Brain regions of striatum, cortex and cerebellum receive dopaminergic innervation from the midbrain and ventral tegmental area, but are thought to be devoid of dopamine-synthesizing cells. However, it has been shown that TH mRNA in the striatum may be transported axonally from the midbrain. The presently observed high levels of DAT and TH expression in the midbrain are consistent with the abundance of dopaminergic neurons in these regions, while detectable expression levels found in other regions suggest considerable dopaminergic innervation from the midbrain or ventral tegmental area.

Gonadal steroid hormones, especially estrogen, play an essential role in maintaining the integrity of the nigral dopamine system. It has been shown that estrogen deprivation for 30 d results in an apparently permanent loss of >30% of the total number of substantia nigra dopamine cells. However, estrogen treatment has been shown to in-
crease dopamine release in the female mice but not the male,\textsuperscript{28} whereas neurotoxin induced greater lesions in males than females as evidenced by greater striatal dopamine depletions.\textsuperscript{29} Indeed, even changes in estrogen levels during estrous cycle can also influence the susceptibility of these neurons to neurotoxins.\textsuperscript{30} Furthermore, estrogen plays an important role during differentiation of midbrain dopaminergic neurons, due to the presence of estrogen receptors and the transient expression of the estrogen-forming enzyme aromatase within the dopaminergic cell groups.\textsuperscript{31}

It has long been suggested that BFP has estrogen-like activities. Our previous studies have shown that BFP promotes dopamine release in the rat brain,\textsuperscript{23} which suggests that BFP may act on dopaminergic neurons and modulate dopamine function. The present results also demonstrate a regulatory effect of BFP on gene expression of dopamine synthesis enzyme in the brain of MPTP-induced PD mice. BFP treatment prior to and continued through the MPTP treatment, showed reduced neurotoxic effects than that of vehicle treated animals, suggesting that BFP may either protect dopaminergic neurons from neurotoxic damage of MPTP, or promote the synthesis of dopamine in the residual neurons. The preserved DAT gene expression in the midbrain of PD mice with BFP pretreatment indicates significant neuroprotective effect of BFP on dopamine functions since DAT is the main factor for determining dopamine neurotransmission and in maintaining dopamine homeostasis in the central nervous system. Noteworthy is the apparent reversal of DAT gene expression in the midbrain rather than in the striatum of BFP-treated model mice, suggesting that BFP exerts its neuroprotective

Fig. 3. Effect of Estradiol on DAT Expression in Different Brain Regions of MPTP-Treated Mice

Upper panel: representative RT-PCR results. Lower panel: DAT-to-GAPDH mRNA ratio in the striatum, midbrain, cortex and cerebellum of the control, MPTP-treated and estradiol-MPTP-treated mice. Mice were injected with 17β-estradiol (50 μg/kg, i.p.) or vehicle for two weeks before MPTP (15 mg/kg×4) administration. Figures are mean±S.E.M. n=5—7. * p<0.05, *** p<0.001 versus the control; † p<0.05, †† p<0.01 versus the MPTP group (ANOVA).

Fig. 4. Effect of Estradiol on TH Expression in Different Brain Regions of MPTP-Treated Mice

Upper panel: representative RT-PCR results. Lower panel: TH-to-GAPDH mRNA ratio in the striatum, midbrain, cortex and cerebellum of the control, MPTP-treated and estradiol-MPTP-treated mice. Mice were injected with 17β-estradiol (50 μg/kg, i.p.) or vehicle for two weeks before MPTP (15 mg/kg×4) administration. Figures are mean±S.E.M. n=5—6. *** p<0.001 versus the control.
effects in the neuronal bodies rather than the terminals. However, the difference between midbrain and striatum might also because of the sensitivity.

The present study has also demonstrated differential effects of BFP and estrogen on DAT and TH expression in different brain regions. Most notably, BFP could reverse both MPTP-induced reduction in DAT and TH expression, while 17β-estradiol only prevented MPTP-induced decrease in DAT but not TH mRNA in the striatum, midbrain and cerebellum. As many steroids may act as direct transcription modulators and thus affect protein synthesis and/or protein integration in the cytoplasmic membrane, estrogen may exert neuroprotective effects on dopaminergic neurons by activating transcription of DAT and, consequently, the synthesis of new DAT to compensate their loss on dopaminergic terminals. It has been shown, however, the potential neuroprotective effect of estrogen may occur only under special circumstances. For example, long-term estrogen replacement treatment of ovariectomized rats did not protect substantia nigra neurons from an insult with 6-hydroxydopamine and low endogenous levels of estrogen may provide neuroprotection. It has also been noted that neuroprotective effect of estrogen against MPP+ insult was not found at a concentration at which the hormone is specific for dopaminergic cells in primary culture. That the present study did not detect significant effect of estrogen on TH expression suggest that at least the estrogen dose used and duration of administration were not appropriate to produce significant neuroprotective effect on TH expression. It is interesting to note that TH gene expression measured in rat locus ceruleus was not regulated by chronic estrogen administration. Therefore, the ability of BFP to reverse MPTP-induced reduction in TH expression suggests that it has neuroprotective effects, as well as the underlying mechanism(s), different from that of estrogen. Although BFP contains phytoestrogens such as ginseng which may act similarly to estrogen, which has been shown to prevent neuronal degeneration caused by increased oxidative stress and apoptosis; other component herbs of BFP may act through different mechanisms, the details of which remain to be elucidated.

Consistent with our research, TH and DAT gene expression and protein patterns of dopaminergic neurons have also been described by the other researchers. The intensity of TH- and DAT-immunoreactive dopaminergic cell bodies and terminals was also decreased in the substantia nigra and striatum of mice after MPTP treatment. The present study demonstrated the neuroprotective effects of BFP on dopaminergic neurons, which may be beneficial for sufferers of Parkinson’s disease, a disease that manifests with damaged dopaminergic tissue and impaired dopaminergic functions. Neuroprotective effects of BFP expressed on TH expression, which was not seen with estrogen, warrants further investigation.

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