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CATALPOL ATTENUATES MPTP INDUCED NEURONAL DEGENERATION OF NIGRAL-STRIATAL DOPAMINERGIC PATHWAY IN MICE THROUGH ELEVATING GLIAL CELL DERIVED NEUROTROPHIC FACTOR IN STRIATUM

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Abstract—The protective effect of an iridoid catalpol extracted and purified from the traditional Chinese medicinal herb *Rehmannia glutinosa* on the neuronal degeneration of nigral-striatal dopaminergic pathway was studied in a chronic 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP)/probenecid C57BL/6 mouse model and in 1-methyl-4-phenylpyridimium (MPP^+) intoxicated cultured mesencephalic neurons. Rotarod performance revealed that the locomotor ability of mice was significantly impaired after completion of model production and maintained thereafter for at least 4 weeks. Catalpol orally administered for 8 weeks (starting from the second week of model production) dose dependently improved the locomotor ability. HPLC revealed that catalpol significantly elevated striatal dopamine levels without changing the metabolite/dopamine ratios. Nor did it bind to dopamine receptors. Therefore it is unlikely that catalpol resembles any of the known compounds for treating Parkinsonism. Instead, catalpol dose dependently raised the tyrosine hydroxylase (TH) neuron number in substantia nigra pars compacta (SNpc), the striatal dopamine transporter (DAT) density and the striatal glial cell derived neurotrophic factor (GDNF) protein level. Linear regression revealed that both the TH neuron number and DAT density were positively correlated to the GDNF level. In the cultured mesencephalic neurons, MPP^+ decreased the dopaminergic neuron number and shortened the neurite length, whereas catalpol showed protective effect dose dependently. Furthermore, the expression of GDNF mRNA was up-regulated by catalpol to a peak nearly double of normal control in neurons intoxicated with MPP^+ for 24 h but not in normal neurons. The GDNF receptor tyrosine kinase RET inhibitor 4-amino-5-(4-methyphenyl)-7-(t-butyl)-pyrazolo-[3,4-d]pyrimidine (PP1) abolished the protective effect of catalpol either partially (TH positive neuron number) or completely (neurite length). Taken together, catalpol improves locomotor ability by attenuating the neuronal degeneration of nigral-striatal dopaminergic pathway, and this attenuation is at least partially through elevating the striatal GDNF expression.

Key words: MPP^+ intoxication, dopamine transporter (DAT), tyrosine hydroxylase (TH), MPTP chronic Parkinsonism model, rotarod performance, glial cell derived neurotrophic factor (GDNF).

Parkinson's disease (PD) is a chronic neurodegenerative disease, the main pathological changes of which involve selective degeneration of dopaminergic neurons, reduced expression of tyrosine hydroxylase (TH) of substantia nigra pars compacta (SNpc) and decreased density of dopamine transporter (DAT) and dopamine (DA) content in the striatum (Kish et al., 1988; Nutt et al., 2004; Nagatsu and Sawada, 2007). The effects of current drugs are mostly palliative, and may gradually decrease after prolonged use or cause side effects such as dyskinesia (Olanow and Tatton, 1999; Savit et al., 2006). New therapeutic approaches aimed at delaying or reversing the neurodegenerative process have attracted the attention of many investigators in recent years (Silverdale et al., 2003; Savit et al., 2006; Peterson and Nutt, 2008).

Many studies showed that neurotrophic factors such as the brain derived neurotrophic factor (BDNF) and the glial cell derived neurotrophic factor (GDNF) have powerful neuroprotective effects (Murer et al., 2001; Dawson and Dawson, 2002; Cass et al., 2006; Evans and Barker, 2008). Recently, it has been hypothesized that neurotrophic factors, most notably GDNF, might have the ability to restore function of dopaminergic neurons (Hong et al., 2008; Yang et al., 2009). However, these factors cannot reach their target areas in brain by systematic administration. Therefore, an important new research field involves the search for small molecules that can enter the brain tissue and then trigger the endogenous neuroprotective mechanisms.

The iridoid catalpol (Fig. 1) is an active component in some important medicinal herbs such as *Rehmannia glutinosa* (Oshio and Inouye, 1981), that are frequently used for treating neurodegenerative diseases in traditional Chinese medicine, but the underlying mechanisms are mostly unclear. Preliminary data suggest that it can improve the memory of animal models by raising the choline acetyltransferase activity in brain (Wang et al., 2006) and can ameliorate beta amyloid-induced degeneration of cholin-

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Abbreviations: AUC, area under the curve; DA, dopamine; DAT, dopamine transporter; DOPAC, dihydroxy-phenyl acetic acid; FP-CIT, 2β-carbomethoxy-3β-(4-iodophenyl)-N-(3-fluoropropyl) nortropane; GDNF, glial cell derived neurotrophic factor; HVA, homovanillic acid; MPP^+, 1-methyl-4-phenylpyridimium; MPTP, 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine; PD, Parkinson's disease; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

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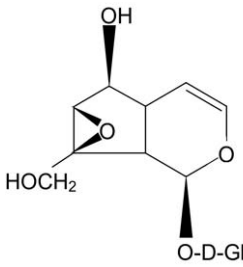

Fig. 1. Chemical structure of catalpol.

ergic neurons by elevating BDNF (Wang et al., 2009), yet it is neither a cholinesterase inhibitor nor a muscarinic receptor (M receptor) agonist (Li et al., 2004; Liu et al., 2007a,b). It has also reported that catalpol could elevate the activity of mitochondria complex 1 in the 1-methyl-4-phenylpyridimium (MPP^+) intoxicated cultured mesencephalic neurons (Tian et al., 2007). However, several important questions have not yet been addressed. Can catalpol improve locomotor ability in animal models of Parkinsonism? Can catalpol attenuate the neurodegenerative changes of dopaminergic pathway such as DAT and DA in striatum? Can catalpol really attenuate the loss of TH positive cells in SNpc if a realible method is used to count the cells (for example, the unbiased stereological counting)? Is the improvement of locomotor ability closely related to the neuroprotection effect? Furthermore, what happens to be most interested to us is whether the neuroprotective effect is related to GDNF which has been said to be the most potent neurotrophic factor for Parkinsonism?

In an attempt to answer these questions, we first carried out *in vivo* experiments to study the effect of catalpol on the locomotor deficit. For this purpose, the chronic 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) mouse model (Petroske et al., 2001; Meredith et al., 2008; Schintu et al., 2009) was chosen since the locomotor deficit in Parkinsonism is a chronic progressive process and according to the experience of traditional Chinese medicine, the effect of catalpol is also a chronic progressive process. When the test of locomotor ability was completed, dopamine and its principal metabolites in striatum were measured to examine whether catalpol is different from the known drugs currently in clinical use for treating Parkinsonism. Subsequently, another set of *in vivo* experiments using the chronic MPTP model was carried out to study the effect of catalpol on the neurodegenerative changes of dopaminergic neurons, involving the transporter density in striatum, the TH positive neuron number in SNpc, and the striatal GDNF content. Finally, *in vitro* experiments using the cultured mesencephalic neurons intoxicated with MPP^+ were carried out to study the molecular mechanism of the neuroprotection of catalpol, especially the role played by GDNF synthesis.

EXPERIMENTAL PROCEDURES

Production of an animal model for *in vivo* experiments

Male C57BL/6 mice (21.2±2.7 g, 10 weeks old), purchased from Shanghai SIPPR-BK Laboratory Animal Company, were housed five per cage with room temperature and relative humidity set at 22±2 °C and at 55%±15% respectively, and lit by artificial light for 12 h each day. Sterilized drinking water and standard chow diet were supplied *ad libitum*. The animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996, and approved by the Animal Ethics Committee of Shanghai Jiaotong University School of Medicine.

The chronic model was produced as described (Petroske et al., 2001) with slight modifications. In short, 10 doses of probenecid (Sigma, St. Louis, MO, USA) plus MPTP (Sigma, St. Louis, MO, USA) were given to each mouse over 5 weeks (two doses per week). For each dose, an i.p. injection of 250 mg/kg of probenecid in DMSO was given 0.5 h prior to the s.c. injection of 15 mg/kg of MPTP in saline. The normal control group was injected simultaneously with solvents.

Drug administration for *in vivo* experiments

Experiment 1. Three groups of mice (seven in each group), the normal control, the model control, and model treated with catalpol (50 mg/kg dissolved in saline) for 8 weeks once daily through a gastric tube starting from the third dose of model production. Catalpol was extracted and purified in our laboratory from fresh roots of the traditional Chinese medicinal herb *Rehmannia glutinosa* and identified with MS and NMR. Its purity was >97% as determined by HPLC. The locomotor ability was tested in the last 3 days of drug administration and the animals were sacrificed for HPLC assay of striatal DA and its metabolites (dihydroxy-phenyl acetic acid (DOPAC) and homovanillic acid (HVA)).

Experiment 2. Six groups of mice (eight in each group) were involved: the normal control, the normal control treated with catalpol (50 mg/kg), the model control, and models treated with catalpol at doses of 5, 15 and 50 mg/kg per day. Catalpol or vehicle was administrated by the same way as in experiment 1. On the last 3 days of drug/vehicle administration, locomotor ability was tested. On the next day (4 weeks after the last injection of MPTP), five mice were taken from each group, sacrificed, and their brains were rapidly taken and kept at −70 °C until assayed for DAT and GDNF. The other three mice of each group were prefixed with 4% paraformaldehyde for immunohistochemical examination of TH-positive cells in SNpc.

Primary culture of mesencephalic cells and treatment of the cultured neurons

Sprague–Dawley (SD) pregnant rats were acquired from Shanghai SIPPR-BK Laboratory Animal Company and the rat mesencephalic neurons were cultured as described previously (Zhang et al., 2008). In brief, freshly dissected ventral mesencephalons from brains of rat embryos (E14–15 days) were dispersed by trypsin digestion and trituration. After centrifugation, cells were resuspended in DMEM/F12 containing 10% fetal calf serum and 5% horse serum, seeded on poly-D-lysine-coated 96-well plates at 1×10^5 cells/cm^2 and cultured at 37 °C in 5% CO_2. The medium was changed to DMEM/F12 plus 2% B27 on the next day and was then renewed every 3–4 days. In order to study the effect of catalpol against MPP^+ (Sigma, St. Louis, MO, USA) induced toxicity, the culture medium was replaced with fresh medium containing various concentrations of catalpol (10^{-6} M to 10^{-4} M) or vehicle on the 6th day. Twenty four hours later, MPP^+ (10^{-5} M) was added to the medium and the cells were further cultured for 48 h. For studying the effect of catalpol (10^{-5} M) on the expression of GDNF mRNA, cells were harvested at 0 h, 6 h, 24 h, 48 h after the administration of MPP^+(10^{-5} M). For blocking the GDNF receptor tyrosine kinase RET, 4-amino-5-(4-methyphenyl)-7-(t-butyl)-pyrazolo-[3,4-d]pyrimidine (PP1) (Biomol, Alexis Corp., UK) was added to the medium 10 min before administration of catalpol