Genetics and Molecular Research

Puerarin prevents inflammation and apoptosis in the neurocytes of a murine Parkinson's disease model

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Genet. Mol. Res. 15 (4): gmr.15047501
Received January 5, 2016
Accepted August 5, 2016
Published October 5, 2016
DOI http://dx.doi.org/10.4238/gmr.15047501

ABSTRACT. The aim of this study was to investigate Parkinson's disease (PD) using a murine model of PD. Specifically, we aimed to explore the mechanism by which puerarin prevents inflammation and apoptosis in neurocytes. Eighty healthy male C57/BL6 mice were randomly selected and divided into four groups (N = 20 each): control group; PD group; PD+puerarin group; and puerarin group. At the end of the treatment period, the animals' brains were removed after perfusion and decollation. The protein expression levels of tyrosine hydroxylase (TH) in the murine brains were assessed by immunohistochemistry and the protein expression levels of TH, glial fibrillary acidic protein (GFAP), inducible nitric oxide synthase (iNOS), cleaved Caspase-3, and Bax in the substantia nigra and corpus striatum of the animals were assessed

by western blotting. The spontaneous activity of the PD mice was found to be significantly higher after puerarin treatment and the distance traveled by mice in an open field assessment was 1700 cm further in puerarin-treated PD mice than in PD mice. Immunohistochemistry and western blotting analyses indicated that the expression of TH was significantly higher (2.63-fold) in puerarin-treated PD mice than in untreated PD mice and that the expression of GFAP in PD mice was significantly reduced (~45%) by puerarin treatment. These findings lead us to conclude that puerarin significantly alleviates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced injury in dopaminergic neurons. Puerarin mediates anti-apoptotic and anti-inflammatory activities and plays a neuroprotective role.

Key words: Puerarin; Animal models; Inflammation; Neuroprotection

INTRODUCTION

Parkinson's disease (PD), also known as shaking palsy, is the second most common chronic degenerative disease of the nervous system following Alzheimer's disease (Nussbaum and Ellis, 2003). The main symptoms of PD include tremor, muscle rigidity, hypokinesia, and postural balance disorders (Nussbaum and Ellis, 2003); while the main pathological characteristics of PD include selective degenerative loss of dopaminergic neurons of the substantia nigra pars compacta and the presence of acidophilous inclusion body in the residual neuron plasma (Lewybody) (Paumier et al., 2015). A decline in the tyrosine hydroxylase (TH) protein content and activity in the substantia nigra and corpus striatum is the leading cause of PD onset (Afonso-Oramas et al., 2014). The TH enzyme, the rate-limiting enzyme in the dopamine synthesis process, is considered a direct indicator of PD. Nowadays, most research on PD is focused on the pathogenesis of PD toward the identification of therapeutic targets, which can effectively block or delay PD-induced neuron death (Davie, 2008).

The mechanism by which selective death of dopaminergic neurons occurs remains unclear. Correlations and cross-talk between multiple pathways and processes including genetic variation, mitochondrial dysfunction, oxidative stress, cell apoptosis, inflammation, abnormal expression of proteins, and misfolding form a complex vicious circle finally leading to dopaminergic neuron loss (Qiao et al., 2014). The Bcl-2 superfamily proteins play important roles in apoptosis of dopaminergic neurons in PD (van der Heide and Smidt, 2013). The Bax protein can be inserted into the mitochondrial membrane to form a pro-apoptotic pathway, promote release of cytochrome C, trigger the cascade reaction of caspase-3, finally resulting in cell apoptosis (Youle and Strasser, 2008). The Bcl-2 family proteins play an important role in regulating cell apoptosis induced by MPP^+ (Xu et al., 2012). Glial fibrillary acidic protein (GFAP) is the main intermediate fibrous protein in mature intracerebral astrocytes. High levels of GFAP are specifically expressed in astrocytes, and GFAP is the marker protein for astrocyte activation (Kanski et al., 2014). Inducible nitric oxide synthase (iNOS) is the marker for inflammatory reactions and can be used to evaluate inflammatory reactions (Suschek et al., 2004).

Puerarin is a flavonoid glycoside extracted from *Pueraria thomsonii,* a leguminous plant known as Kudzu. Puerarin is similar to estradiol in structure (Yeung et al., 2006) and its main constituents are 8-D-glucopyranose-4'-7-dihydroxy isoflavone glycosides. Puerarin

has hydroxide radicals at positions 4 and 7 and these are closely associated with the activity of the estrogen-like hormone (Yeung et al., 2006). The 4-hydroxide radical can bind to the estrogen receptor to strengthen the role of estrogen in promoting the release of calcitonin by the thyroid (Michihara et al., 2012). Puerarin has multiple pharmacologic effects including reducing blood fat, providing oxidation resistance, inhibiting oxidative modification of low-density lipoprotein, eliminating reactive oxygen species, and regulating the activity of NOS (Liu et al., 2011). Puerarin furthermore has a protective effect of various vital organs including the heart by inhibiting apoptosis of neurocytes, vascular endothelial cells, myocardial cells, and vascular smooth muscle cells (Zhang et al., 2014a). The anti-apoptotic mechanism of puerarin is associated with apoptosis-regulating factors such as Bcl-2 family proteins, p53, Fas, as well as other effectors in the apoptosis pathway (Zhu et al., 2012). Little research, however, has been conducted on the role of puerarin in protecting dopaminergic neurons in the substantia nigra in the C57/BL6 murine model of PD.

Immunohistochemical methods were used to assess the TH content in the brain tissue of mice and western blotting was used to assess the expression levels of GFAP, iNOS, cleaved-caspase-3, and Bax in the substantia nigra and corpus striatum of the animals. A further aim of this study was to investigate the mechanism by which puerarin protects the dopaminergic neurons from apoptosis and inflammation.

MATERIAL AND METHODS

Materials

Healthy male C57/BL6 mice were obtained from the Animal laboratory of Inner Mongolia Medical University, China; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 99% puerarin were purchased from Sigma (USA) and Conba Pharmaceutical Co., Ltd., (Zhejiang, China), respectively.

Animal experiments

Eighty healthy male C57/BL6 mice were selected and divided into four groups of 20 each, where group A was the control group (vehicle: saline); group B was the PD group; group C was the PD + puerarin group; and group D was the puerarin group. The MPTP injection volume in groups B, and C was 10 mL/kg and the intraperitoneal injections of MPTP continued for 20 consecutive days. An equivalent volume of normal saline was administered to the mice in groups A and D. The mice were subjected to a CatWalk pathway walking (Nam et al., 2013) exercise 8 days before puerarin was administered intraperitoneally in the relevant groups for 20 consecutive days after the exercise. All experimental animals were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine perfused with 20 mL normal saline and 30 mL paraformaldehyde through the heart at 20 days after puerarin treatment. The brains of the animals were removed after perfusion and decollation.

Immunohistochemical assessment of TH protein levels in the brain tissue

Immunohistochemical analyses were performed using a previously published method with slight modifications (Viswanath et al., 2001). Briefly, the brain tissue was fixed,

dehydrated, infiltrated and embedded with paraffin, dried in an incubator, removed and sectioned, dewaxed, subjected to antigen retrieval, blocked with BSA (5% in PBS), and then incubated with the primary antibody (Leica Biosystems, Germany) at 4°C overnight before being incubated with the secondary antibody (Leica Biosystems, Germany) for 30 min at room temperature. The tissue sections were then subjected to color development, H&E double staining, and dehydration followed by additional treatment with ethyl alcohol and xylene. Thereafter, the sections were mounted with neutral gum and air dried.

Western blotting

The relative protein expression levels of TH, GFAP, iNOS, cleaved Caspase-3, and Bax in the substantia nigra and corpus striatum in each animal group were assessed by western blotting according to Viswanath et al. (2001). Total protein was extracted from the frozen tissue using whole cell lysis buffer and protein concentration in the lysates was determined using the bicinchoninic acid method. The denatured proteins were separated by SDS-PAGE (20 μg/well), transferred to PVDF membrane, blocked in 5% skim milk for 2 h, incubated with the murine primary antibody (Sigma) at 4°C overnight, and were incubated with goat anti-mouse secondary antibody (Sigma). The membrane was washed, subjected to ECL luminescence analysis, and scanned using a BioDoc-It 220 Gel-Imaging system (UVP, Upland, CA, USA). The bands were analyzed using Quantity One software (Zhang et al., 2014b).

Statistical analysis

The SPSS 16.0 (IBM, Seattle, WA, USA) statistical software package was used for data analysis and all data are reported as means ± SD. Comparisons between groups were carried out using one-way analysis of variance and the *t*-test was used to compare means between two samples. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Puerarin increases the excitability of the nervous system in MPTP PD mice

As shown in Figure 1, the spontaneous activity (Figure 1A) of mice injected with MPTP was significantly lower than that of the animals in the control group. The travel distance (Figure 1B) and the motion duration (Figure 1C) measured over 10 min for the C57/BL6 mice injected with MPTP were found to decreased by 2900 ± 35 cm and 70 ± 5 s, respectively, relative to the corresponding parameters measured for the mice in the PD group. The rest duration was 75 ± 5 s ($P < 0.05$) longer in the PD mice relative to the control mice (Figure 1D). The travel distance (Figure 1B) of the PD mice treated with puerarin was significantly higher (1700 ± 35 cm; $P < 0.05$) than that of the untreated PD mice; the motion duration (Figure 1C) of the puerarin-treated PD mice increased by 30 ± 5 s relative to the untreated PD mice; and the rest duration (Figure 1D) in puerarin-treated mice decreased by 70 ± 5 s ($P < 0.05$) relative to the rest duration in the untreated PD mice.

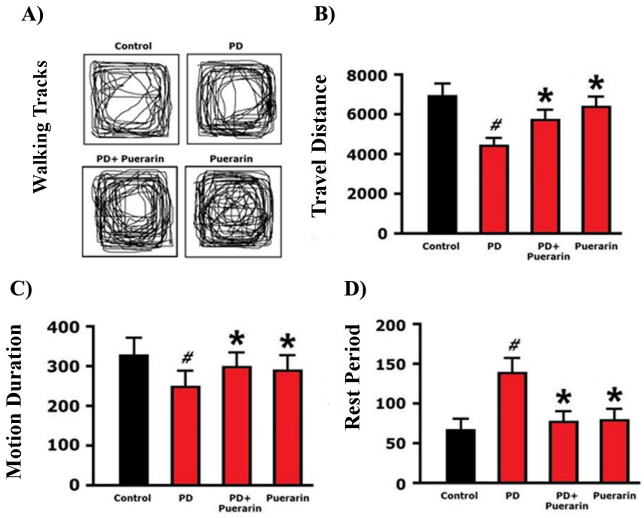

Figure 1. Spontaneous activity of PD mice treated with puerarin. **A.** Walking tracks of the mice in each group assessed in open field test; **B.** travel distances; **C.** motion durations of mice; **D.** rest periods after motion. *P < 0.05, PD group versus control group; #P < 0.05 puerarin-treated group versus the PD group.

Puerarin inhibits the MPTP-induced loss of TH-positive cells

Western blot analysis (Figure 2) demonstrated the protein expression level of TH in the substantia nigra of MPTP-treated mice to be 0.13 ± 0.02 of that in the substantia nigra of the control group (P < 0.05), while TH expression in puerarin-treated PD mice was >2.5 times higher than in untreated PD mice (P < 0.01). Treatment of mice with puerarin was therefore shown to have a significant protective effect against the toxicity of MPTP to TH-positive cells in the substantia nigra and corpus striatum and was therefore shown to protect dopaminergic neurons.

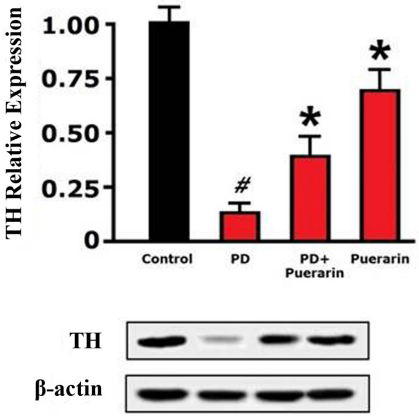

Figure 2. MPTP in mice leads to decreased TH protein expression in the substantia nigra compared with control animals. The protein expression of TH increased after treatment with puerarin. *P < 0.05, PD group versus control group; #P < 0.05, PD + puerarin group versus PD group.

Puerarin inhibits the inflammatory responses induced by MPTP

Western blot analysis (Figure 3) furthermore showed that the expression levels of GFAP were >2.5 times higher in MPTP-treated mice than in the control group animals ($P < 0.05$) and that GFAP expression in PD mice was reduced by ~45% by treatment with puerarin ($P < 0.05$; Figure 3A). The expression of iNOS (Figure 3B) in the midbrains of the mice was shown to be >6 times higher in PD mice than in control animals ($P < 0.05$), while puerarin treatment was shown to reduce iNOS expression in PD mice by ~64% ($P < 0.05$).

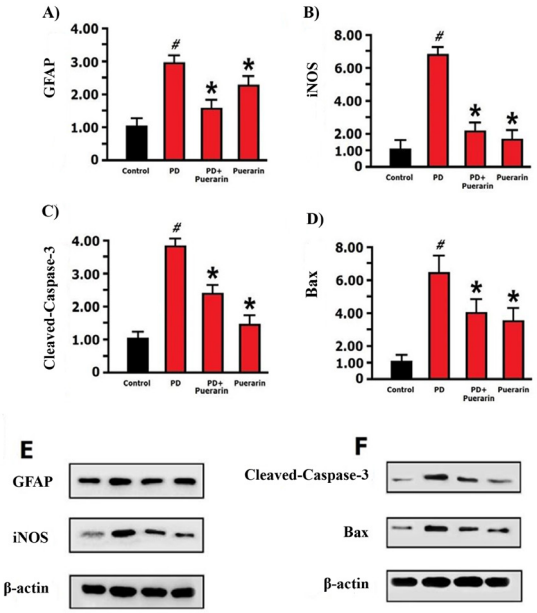

Figure 3. Relative protein expression levels of GFAP, iNOS, Bax, and cleaved Caspase-3 in the substantia nigra of mice. **A. B.** MPTP led to a significant increase in GFAP and iNOS expression compared with the control group; **C. D.** iNOS expression decreased significantly in PD mice following puerarin treatment. Cleaved Caspase-3 and Bax expression levels were higher in PD mice than in control mice and were significantly lower in puerarin-treated PD mice than in untreated PD mice; **E. F.** Western blot analysis of GFAP, iNOS, Bax, and cleaved Caspase-3 expression. *$P < 0.05$, PD group versus control group; #$P < 0.05$ PD + puerarin group versus PD group.

Puerarin downregulates the MPTP-induced expression of apoptosis-associated proteins in the substantia nigra

Further western blot analyses revealed that the expression levels of cleaved Caspase-3 (Figure 3C) and Bax (Figure 3D) in the substantia nigra of the midbrains of the PD mice were >3.5 and >6.5 times higher, respectively, than those in the corresponding tissues of the control group animals ($P < 0.05$). The expression levels of cleaved Caspase-3 and Bax in PD mice were shown to be reduced by >30% following puerarin treatment ($P < 0.01$). These findings demonstrate that puerarin protects dopaminergic neurons in the substantia nigra of the C57/BL6 mice from MPTP-induced apoptosis by downregulating cleaved Caspase-3 and Bax (Figure 3E and 3F).

DISCUSSION

The damage to dopaminergic neurons in the substantia nigra of C57/BL6 mice following MPTP administration is unlike the progressive damage to the dopaminergic neurons observed in the brains of PD patients (Korecka et al., 2013) in that the PD-like damage to dopaminergic neurons induced by MPTP can be self-repaired. It is this damage to dopaminergic neurons in the substantia nigra that results in the repeated motor functions associated with PD patients (Hami et al., 2015). The TH enzyme is a rate-limiting enzyme in the dopamine synthesis process and reduced TH expression is considered a direct indicator for PD (Tan et al., 2014).

In this study, it was shown that the travel distance in 10 min and the motion duration of the mice assessed were shown to be significantly higher and the rest period was shown to be significantly lower in PD mice treated with puerarin compared with untreated PD mice. Puerarin may correct disorders of the neurotransmitters in the brain by raising the dopamine content in the substantia nigra corpus striatum system. The spontaneous activities of the animals may therefore be increased by treatment with puerarin. The results of the immunohistochemical and western blot analyses in this study indicate that puerarin effectively inhibits the MPTP-induced damage to the substantia nigra corpus striatum system. The mechanism by which MPTP selectively induces dopaminergic neuron loss in the substantia nigra is unclear; however, MPTP is known to interfere with the normal operation of the mitochondrial respiratory chain by inhibiting the activity of NADH dehydrogenase. Decreased generation of 6U and ATP enhances the permeability of the mitochondrial membrane, thereby allowing the released effector to activate the apoptosis signaling pathway, finally leading to cell death. Bax, a member of the apoptosis-promoting Bcl-2 family, can form heterodimers with other Bcl-2 family proteins and enter into the mitochondrial outer membrane to form a special channel for cytochrome C transport, allowing cytochrome C to be released into the cytoplasm from the mitochondria. Cytochrome C subsequently associates with Apafl, which mediates the breakdown of Caspase-9 to produce activated Caspase-9. The activated Caspase-9 allows for lysis of the Caspase-3 precursor, yielding activated Caspase-3. The initiation of this apoptosis cascade reaction leads to cell apoptosis. Terminal-deoxynucleotidyl transferase mediated dUTP nick end labeling has been used to demonstrate that apoptosis occurs in the dopaminergic neurons in the substantia nigra of MPTP-induced subacute C57/BL6 PD model mice at 3 days after MPTP injection and peaks after 5 days (Tatton and Kish, 1997). The findings reported here show that the expression levels of Bax and activated Caspase-3 in the substantia nigra of C57/BL6 mice injected with MPTP were significantly higher than those in the normal saline group, indicating that the MPTP-induced loss of dopaminergic neurons in the substantia nigra of the PD model animals in this study were closely associated with apoptosis.

Puerarin can inhibit the apoptosis of dopaminergic neurons in the substantia nigra by inhibiting Bax and activated Caspase-3. The number of inflammatory cytokines in the cerebrospinal fluid and substantia nigra corpus striatum systems of PD patients exceeds that in control subjects. A large number of activated glial cells is generated soon after MPTP administration in animal PD models. The expression of iNOS is subsequently upregulated, after which the toxicity mechanism induced by the inflammatory response largely leads to the activation of iNOS. Only low levels of iNOS are expressed in normal brain tissue; however, iNOS expression is upregulated in response to pathological stimuli and inflammatory factors. Activated iNOS produces a large amount of NO, which reacts with superoxides to produce peroxynitrite (ONOO-), which in turn can cause direct DNA damage because of its strong

oxidation capacity. The ONOO- can lead to peroxidation damage or mediate other inflammatory factors as a signal medium to produce neurotoxicity. Research has shown that the expression of iNOS mRNA increases and peaks at 48 h after MPTP administration and that iNOS gene knockout mice exhibit partial resistance to MPTP neurotoxicity (Tatton and Kish, 1997).

It has been shown that the protein expression levels of iNOS are high in PD mice and that puerarin inhibits this iNOS protein expression in the substantia nigra of the PD mice and furthermore inhibits the intracerebral inflammation in the brains of PD animals (Liberatore et al., 1999). Early-stage microglial cells and late-stage astrocytes are also present in PD animals, indicating that astrocytes also participate in the cerebral injury process, during which the activated astrocyte-specific protein GFAP is upregulated. We have found that MPTP upregulates the protein expression of GFAP in the substantia nigra of mice, indicating that glial cells are activated in the brains of the mice after MPTP-induced damage. Puerarin was shown here to inhibit apoptosis by inhibiting the expression of the apoptosis-related proteins, Bax and cleaved-Caspase-3. Puerarin inhibits inflammatory responses by downregulating the expression of iNOS. These findings may be the mechanisms by which puerarin exerts its protective role on dopaminergic neurons in MPTP-induced murine PD.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the following project funds: Functional Magnetic Resonance Imaging (MRI) Combined PET/CT for Applied Research in Early Diagnosis of Parkinson's Disease (#2014MS0874); and the Cognitive Dysfunction of Parkinson's Disease and Applications of Functional Magnetic Resonance Imaging (#YKD2013).

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