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Myricetin prevents dopaminergic neurons from undergoing neuroinflammation-mediated degeneration in a lipopolysaccharide-induced Parkinson's disease model

Bingxu Huang, Juxiong Liu, Dongxu Ma, Guangxin Chen, Wei Wang, Shoupeng Fu*

College of Veterinary Medicine, Jilin University, Changchun 130062, China

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ABSTRACT

Myricetin, one of the lipophenolic compounds present in red wine, has been reported to pass through blood–brain barriers and reduce dopaminergic neuron degeneration, but the mechanism remains unclear. We thoroughly investigated the underlying mechanisms in Parkinson's disease models both *in vivo* and *in vitro*. The neuroprotective effects of myricetin were assessed by performing behavioural test, immunohistochemistry and western blotting *in vivo*. The expression of pro-inflammatory mediators in the rat' substantia nigra was examined using qPCR and ELISA. The toxicity of conditioned medium from BV-2 cells towards SH-SY5Y dopaminergic neuronal cells was measured by MTT assay *in vitro*. The myricetin treatment suppressed the activation of microglia, the expression of pro-inflammatory mediators and the reduction in the number of dopaminergic neurons and ameliorated the rats' motor dysfunction. Mechanistically, myricetin inhibited the production of pro-inflammatory mediators and the activation of the MAPK and NF-κB pathways in activated microglia. Based on these results, myricetin prevents dopaminergic neuron degeneration by inhibiting microglial neuroinflammation.

1. Introduction

Parkinson's disease (PD), the second most common neurodegenerative disease, affects 1% of the population over 55 years of age and increases in prevalence to approximately 5% by the age of 85. PD is clinically characterized by rigidity, bradykinesia, resting tremor and postural instability (Blesa, Phani, Jackson-Lewis, & Przedborski, 2012). The loss of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNpc) accompanied with dopamine depletion in the striatum is the biochemical signature of PD (Tieu et al., 2003). Microglial cells are the resident macrophages of the brain, playing a crucial role in the innate immune response and serving as the first line of defence against microbial invasion and central nervous system (CNS) injury (Graeber & Streit, 1990). Based on accumulating evidence, uncontrolled, over-activated microglia plays an important role in neuroinflammation and dopaminergic neurodegeneration (McGeer & McGeer, 2004; Rojo et al., 2014). The inhibition of neuroinflammation by activated microglia has been reported to ameliorate the degeneration of dopaminergic neurons in lipopolysaccharide (LPS)-induced animal models of PD (Fu et al., 2015; Tai et al., 2013; Wang, Wang, et al., 2014).

LPS is an endotoxin found in the outer membrane of gram-negative bacteria and a potent stimulator of both peripheral immune cells (macrophages and monocytes) and brain glia (microglia and astrocytes). It promotes the release of various cytotoxic factors and pro-inflammatory cytokines; thus, LPS is often used to elicit neuroinflammation and generate cellular/animal phenotypes of PD (Iczkiewicz et al., 2010; Machado et al., 2011; Mastroeni et al., 2009). During the pathogenesis of LPS-induced PD, microglial activation is an essential event in the induction of dopaminergic neuron damage. In addition, microglia can become over-activated in response to neuronal damage, which is then toxic to neighbouring neurons, resulting in a perpetual cycle of neuron death (Block, Zecca, & Hong, 2007). Therefore, inhibition of the activation of microglia-mediated neuroinflammatory responses represents a potential strategy for the prevention and treatment of PD.

According to epidemiological studies, the incidence of idiopathic PD is relatively low in chronic users of anti-inflammatory drugs (Chen et al., 2003; Esposito et al., 2007). Given the potential role of neuroinflammation in the pathogenesis of PD, drug candidates with anti-inflammatory properties are urgently needed (Moore et al., 2010). Myricetin is a widespread naturally occurring flavonoid compound

* Corresponding author.
E-mail address: shoupengfu@163.com (S. Fu).

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found in plants such as vegetables, fruits, berries, tea and medicinal herbs. It is also one of the lipophenolic compounds present in red wine (Maggiolini et al., 2005). Myricetin has been reported to pass through blood-brain barriers (G. Wang, Wang, Tang, Du, & Li, 2016), possess antioxidative (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Ozgova, Hermanek, & Gut, 2003), anti-inflammatory (Ko, 2012) and anti-proliferative properties (Jose et al., 2016; Zang et al., 2014). Myricetin exerts neuroprotective effects on 6-hydroxydopamine (6-OHDA)-induced dopamine neuron degeneration in rats (Ma, Wang, Jiang, Liu, & Xie, 2007). As shown in the study by Francisca Molina-Jimenez et al., myricetin significantly diminishes the cytotoxicity of rotenone and the release of lactate dehydrogenase into the medium of SH-SY5Y cells (Molina-Jimenez, Sanchez-Reus, & Benedi, 2003). Myricetin may also prevent and treat Alzheimer's disease by suppressing the formation and accumulation of amyloid-β protein (Ono et al., 2003). However, researchers have not clearly determined whether myricetin-mediated inhibition of activated microglia relieves or slows the pathogenic process of PD. Here, we report the preventive effects of myricetin on LPS-induced PD models both *in vivo* and *in vitro* and elucidate the potential anti-inflammatory mechanism.

2. Materials and methods

2.1. Reagents

Dimethylsulfoxide (DMSO), LPS (*E. coli*: serotype O55:B5), TRIzol reagent, Poly-L-lysine (PLL), apomorphine, and 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Myricetin (> 98% purity; Pufei De Biotech, Chengdu, China) was dissolved in DMSO as a stock solution at 0.5 g/ml, stored at −20 °C and freshly diluted to the final concentration (0.1% DMSO) for the *in vitro* cell study. The 0.25% trypsin and penicillin-streptomycin (PS) solutions were purchased from Invitrogen (Carlsbad, CA, USA). Phosphate buffer saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and foetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). The PrimeScript® 1st Strand cDNA Synthesis Kit was obtained from TaKaRa Biotechnology (Dalian, China). The SYBR Green QuantiTect RT-PCR Kit was purchased from Roche (South San Francisco, CA, USA). Rat and mouse tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) ELISA kits were purchased from Biolegend (San Diego, CA, USA).

2.2. Animals and treatment

All experiments were performed in accordance with approved animal protocols and guidelines established by the Institutional Animal Care and Use Committee of Jilin University (approved on 27 February 2015, Protocol No. 2015047). Nine- to eleven-week-old female Wistar rats (290–320 g) were purchased from the Center of Experimental Animals of the Baiqiuen Medical College of Jilin University. The rats were housed in microisolator cages on a 12 h light/dark cycle and received food and water *ad libitum*. The temperature of the laboratory was maintained at 24 ± 1 °C, and the relative humidity was 40–80%. All rats were randomly divided into the following five groups (n = 18 in each group): control group, LPS group, and myricetin (2.5, 5 or 10 mg/kg) + LPS groups. Rats were anaesthetized with sodium pentobarbital (45 mg/kg, i.p.), secured in a stereotaxic apparatus (David Kopf Instruments, USA) and given injections of LPS (10 μg dissolved in PBS with a total injection volume of 2 μl) or PBS into the right SNpc (anteroposterior (AP) −5.2 mm, lateral (LAT) 2.2 mm and dorsoventral (DV) 7.8 mm) at a rate of 0.2 μl/min (Tai et al., 2013). The injection needle was lowered through a drill hole 5.5 mm posterior, 1.5 mm lateral, and 8.3 mm ventral to the bregma for the SN. After the injection was completed, the needle was left in place for 10 min to avoid reflux along the injection track. The intraperitoneal injections of myricetin (2.5, 5 or 10 mg/kg, dissolved in 5 μl of DMSO and then diluted with 1 mL of PBS, once daily) were performed on the 3 days prior to surgery. Rats in the control group received an equal volume of the vehicle solution. After surgery, the animals were administered myricetin for 25 days. After the last behavioural test, the rats (n = 6 in each group) were transcardially perfused with paraformaldehyde for the immunohistochemical analysis of tyrosine hydroxylase (TH) (1:1000; Abcam, Cambridge, CA, USA) and ionized calcium binding adaptor molecule-1 (Iba-1) (1:200, Proteintech, Chicago, IL, USA) expression. The brain were rapidly removed from the remaining rats (n = 12). Then, fresh SNpc tissues were isolated to measure the levels of the inflammatory mediators, TH and Iba-1.

2.3. Cell culture and treatment

The BV-2 murine microglia cell line and SH-SY5Y neuroblastoma cell line were purchased from the Cell Culture Center at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Peking, China). These two cell lines were cultured in DMEM supplemented with 10% FBS and 50 U/mL penicillin-50 μg/mL streptomycin at 37 °C in a humidified cell incubator with a 95%/5% (v/v) air and CO_2 atmosphere. The culture medium was changed once every two days, and these two cell lines were trypsinized (0.05%, w/v) when they reached approximately 80% confluence. SH-SY5Y cells were plated onto 96-well plates 24 h before the conditioned medium (CM) test. The CM from LPS-stimulated BV-2 cells was collected and transferred to SH-SY5Y cultures to induce inflammation-induced cell death. After a 24 h treatment, the viability of SH-SY5Y cells was determined using the MTT assay.

2.4. Motor function test with the rotarod

An accelerating rotation test is widely used to assess the motor balance and coordination of the PD rats (Monville, Torres, & Dunnett, 2006). LPS-induced PD model rats were subjected to a rotational test at two and four weeks after LPS injection to measure the effect of the myricetin treatment on motor dysfunction. The apomorphine-induced rotational test is a classical method used to evaluate the behavioural dysfunction of PD model rats and detect damage to the dopaminergic system (Iancu, Mohapel, Brundin, & Paul, 2005). To adapt the test, rats were placed onto cylinders for a training session (10 rpm for 10 min) before treatment. After an intraperitoneal injection of 0.5 mg/kg apomorphine, rats were placed onto the cylinders for 30 min to measure their functional motor activity with minimal external stimuli. The number of turns was counted throughout the test.

2.5. Immunohistochemical staining

Dopaminergic neurons were labelled with a rabbit anti-TH polyclonal antibody (1:1000; Abcam, Cambridge, CA, USA) and microglia were labelled with the rabbit anti-Iba-1 polyclonal antibody (1:200, Proteintech, Chicago, IL, USA) using previously described (Haji Ghasem Kashani, Ghorbanian, & Hosseinpour, 2013). The number of Iba-1 and TH-positive cells, were counted by three researchers who were blinded to the experimental treatments. The ratios of these scores were determined.

2.6. Western blot analysis

After the last behavioural test, the SNpc was rapidly dissected, frozen, and stored in a deep freezer at −80 °C until further assay. The western blot assay was performed using standard protocols. The SNpc and BV-2 cells were lysed in RIPA lysis buffer (Beyotime Inst. Biotech, Beijing, China) containing phenyl-methylsulfonyl fluoride (PMSF). After 30 min, clarified lysates were obtained by centrifugation at 12,000 rpm for 10 min at 4 °C. The protein concentrations of the samples were then determined using a bicinchoninic acid protein assay kit (Beyotime Inst. Biotech, Beijing, China) and bovine serum albumin as