The inflammatory injury of microglia involved in Tourette syndrome development

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Abstract

Tourette Syndrome (TS), a chronic neuroinflammatory disorder, is characterized by motor and vocal tics. Microglial inflammatory activity in TS is increased, while microglial-mediated clearance mechanisms are compromised. Our study aimed to investigate the effect of puerarin on microglial inflammation. Our study confirmed that 20 mg/mL of puerarin was the optimal intervention concentration by CCK8 analysis. Furthermore, we found that puerarin could inhibit proinflammatory factors and up-regulate anti-inflammatory factors at the protein and mRNA levels, thus playing a role in regulating microglial inflammation. This study provides a new strategy for regulating neuroinflammation in TS.

Keywords: Tourette Syndrome; Puerarin; Microglia; neuroinflammation

Introduction

Tourette Syndrome (TS) is characterized by motor and vocal tics, which can cause significant distress or severe impairment in social or other important areas of functioning. [1] Common motor tics include blinking, head, shoulder, trunk jerks, and facial expressions; vocal tics include grunts, throat clearing, nose sniffing, screaming, and, less commonly, calling out a word or phrase. [2]

The global prevalence of TS ranges from 0.3% to 1%. In addition, 1% to 3% of children and adolescents may have mild and unexplained symptoms of TS. Epidemiological studies suggest that 20% of children in the United States have tics during childhood, and the best estimate of the prevalence of TS is likely to be 4 to 8 cases per 1,000 children. [3] The average age at which the first episode of motor tics occurs is 4–6 years. TS is more common in boys than in girls, with a prevalence of 3–4:1. The maximum severity of TS manifestations occurs at 10–12 years old. Most patients will have complete or nearly complete remission of the disorder after 21 years of age. Several hypotheses about the cause of TS include genetic factors, neurotransmitter abnormalities, and neuroinflammation. In neuroinflammation, microglia activation and cytokine expression play an important role. [4][5][6]

Microglia are the central nervous system’s primary immune cells, which fulfill various tasks mainly related to immune response and maintaining homeostasis. Microglia can polarize to either an M1 proinflammatory phenotype or an M2 anti-inflammatory phenotype in response to different microenvironmental disturbances. M1 microglia release inflammatory cytokines and chemokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-1β, IL-12, and CC chemokine ligand (CCL) 2, leading to inflammation and neuronal death. M2 microglia produce anti-inflammatory cytokines, promote phagocytosis of cell debris and misfolded proteins, promote extracellular matrix reconstruction and tissue repair, and support neuron survival by neurotrophic factors.

Microglia play an important role in neurodegenerative diseases, such as Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS). Also, balancing microglia M1/M2 polarization had a promising therapeutic prospect in neurodegenerative diseases (Fig.1). [7][8][9]

Figure 1 Neurological disorders involving microglia polarization

Puerarin is the main bioactive ingredient isolated from the roots of Pueraria Mirifica (Willd.) and is available in common foods and alternative medicine. It has been widely used in the treatment of Parkinson’s disease and Alzheimer’s disease. The beneficial effects of Puerarin include pharmacological properties such as
neuroprotective, antioxidant, and anti-inflammatory properties. [10][11] However, the effect of Puerarin on improving TS is still unclear. Therefore, our goal is to investigate both the ameliorative effects and the molecular mechanisms of Puerarin on TS.

**Materials and methods**

**Determination of the cytotoxicity of puerarin by CCK-8 assay**

A CCK-8 kit (Vazyme Biotech, China) was utilized to examine the cytotoxicity of puerarin against BV2 cells. BV2 cells were divided into six groups, including the control group and puerarin (5, 10, 20, 40, and 80 mg/mL), and incubated for 24 h. Following 1 d of incubation, 5 μg/μL CCK-8 (10 μL) was added, and the incubation proceeded for an additional four h. The 450-nm absorbance (A450) was determined with a microplate reader. The assays were repeated three times to obtain an average value.

**Cell culture and treatment**

Cultivation of BV2 cells was accomplished using 10% FBS-containing DMEM supplemented with 1% glutamine, 20 mM HEPES, and antibiotics (Gibco). BV2 cells were cultured with/without LPS (100 ng/ml) puerarin (5, 10, and 20 mg/mL) for 12 h to stimulate inflammation.

**Western blot analysis**

For the lysis of protein samples from cells, RIPA buffer (Beyotime Biotechnology, China) supplemented with one mM Pierce™ phosphatase inhibitor and 0.1% Halt™ protease inhibitor cocktail (Thermo-Fisher-Scientific, China) was utilized. A BCA Assay Kit (Beyotime Biotechnology) was used for protein level quantification. After 10% SDS–PAGE separation, the protein samples were transferred onto PVDF membranes (Millipore, Germany) and subjected to 90 min of blockade at room temperature (RT) using 5% BCA. This was followed by overnight incubation using primary antibodies against IL-4, IL-10, NLRP3, phosphor-NF-κB p65, and phospho-IκB at four °C. After three rinses in TBST, the membranes were exposed to a secondary antibody (Biogot Technology, China) at RT for 90 min. All the bands were visualized using a western chemiluminescent HRP substrate (Immobilon, Beyotime Biotechnology). GAPDH was used for the normalization of protein data.

**RNA extraction and qRT–PCR assays**

A Qiagen RNeasy plus mini kit extracted total RNA from cells. RNA was reverse-transcribed using reverse transcriptase (Superscript II, Invitrogen). At the same time, Green PCR Master Mix (Power SYBR, Applied Biosystems, Thermo-Fisher-Scientific) was utilized to perform qRT–PCR assessment on duplicate samples. Data were analyzed with the aid of QuantStudio 5 (Thermo-Fisher-Scientific). The internal control adopted was GAPDH. E =2ΔΔCt was employed to assay the relative mRNA levels, while the CT values were determined for all reactions.

**Statistical analysis**

The results are expressed as the mean± standard deviation (SD). All statistical analyses were performed with GraphPad (Prism 9.00). Comparisons between two groups for statistical significance were assessed using a two-tailed Student’s t-test. P < 0.05 was considered statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001.). All data presented are from at least three independent experiments.

**Results**

Puerarin exerted good biocompatibility at a concentration of 20 mg/mL. When evaluating whether intracellular administration of puerarin is necessary for exerting its effects, biological compatibility must be considered a crucial index. Therefore, the in vitro cytotoxicity of puerarin was assessed by monitoring the survival rate of the cells incubated with them. As shown in Fig. 2, the cell viability of nontreated cells (without puerarin) was assigned as 100%. Puerarin showed increased biocompatibility at concentrations lower than 20 mg/mL (Fig. 2). Herein, we selected a concentration of 20 mg/mL for subsequent research in LPS-induced BV2 cells treated with puerarin.

**Figure 2 Puerarin exerted good biocompatibility at a 20 mg/mL concentration.**

Puerarin suppressed inflammation in the LPS-induced BV2 cells.

To further verify the associations of puerarin with inflammation regulation, an in vitro culture system, which
comprised LPS-treated BV2 cells, was created. Western blot analysis showed drastic decreases in IL-4 and IL-10 levels and elevations in NLRP3, p-IκB, and p-p65 levels in LPS-treated BV2 cells compared with the control group (Fig. 3). IL-4 acts as a cytokine response to inflammations at the beginning stage. Interleukin 10 (IL-10) is also used to mediate the immune response to inflammation. NOD-like receptor thermal protein domain-associated protein 3 (NLPR3) detects changing homeostasis and signals leading to pyroptosis when damage occurs. P65 is a monomer of the family of NF-κB, which is activated when exposed to proinflammatory signals. IκB can remove the NF-κB from the nucleus and inactivate NF-κB to transport the NF-κB complex back to the cytoplasm after IκB is phosphorylated as an inhibitor of NF-κB. Next, we detected the levels of inflammation-related indicators induced by different concentrations of puerarin in LPS-induced BV2 cells (Fig. 3). According to the results, puerarin dose-dependently suppressed inflammation-related indicator levels.

Figure 3 Puerarin suppressed inflammation in the LPS-induced BV2 cells.

Puerarin regulated the transcript expression levels of inflammatory cytokines in the LPS-induced BV2 cells. Consistent with protein expression, LPS treatment significantly upregulated proinflammatory cytokines, including NLRP3, p-IκB, and p-p65 at the mRNA level (Fig. 4). Treatment with puerarin almost completely suppressed the upregulation. In addition, IL-4 and IL-10 at the mRNA level were significantly lower in the LPS-induced BV2 cells than in normal controls. Puerarin could upregulate these anti-inflammatory indicators. TNF-α is related to mediating apoptosis and inflammatory responses; it can also activate the phosphorylation of IκB. Interleukin 6 (IL-6) acts as a cytokine to activate B and T cells; it is released when reacting to inflammations.

Figure 4 Puerarin regulated the transcript expression levels of inflammatory cytokines in the LPS-induced BV2 cells.

Discussion
This study clearly shows how the puerarin level will affect the inflammatory response of microglia in isolated conditions. As the inflammatory response is maintained at a desirable level, the TS can be prevented and mitigated exacerbation. However, this study does not show the real situation in the brain. Understanding the effect of puerarin remains at the primary level. Moreover, the possible interaction between puerarin, microglia, and other neural
cells is not fully investigated. Puerarin could cause other problems when it enters the brain as they are not properly degraded and may accumulate in the brain. Meanwhile, another concern is transporting the puerarin into the brain to the target, microglia. The permeability of puerarin through the blood-brain barrier has not yet been tested. So far, few effective methods have been used to transport puerarin into the brain while doing intravenous injection, and the removal of this chemical from the brain is not been completely studied. Furthermore, the extraction and purification of puerarin are not currently mature, so the price of puerarin is high enough.

Our understanding of TS remains limited. The complete mechanisms underlying the onset of TS remain unknown, so we do not have much target to eliminate TS other than reducing the possible features of TS while TS partially prevails. This points out the final targets of our study. We should finally bring about the possible models of TS to find and test its mechanisms.

Reference


