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ORIGINAL PAPER

Genipin Inhibits LPS-Induced Inflammatory Response in BV2 Microglial Cells

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Abstract Genipin, an aglycon of geniposide, has been reported to have anti-inflammatory effect. However, the anti-inflammatory activity of genipin on LPS-stimulated BV2 microglial cells has not been reported. In this study, we investigated the molecular mechanisms responsible for the anti-inflammatory activity of genipin both in vivo and in vitro. The levels of TNF-α, IL-1β, NO and PGE_2 were detected by ELISA. The expression of Nrf2, HO-1, and NF-κB were detected by western blot analysis. In vivo, genipin significantly attenuated LPS-induced memory deficit in the Morris water maze and passive avoidance tasks. Genipin also inhibited LPS-induced TNF-α and IL-1β expression in brain tissues. In vitro, our results showed that genipin inhibited LPS-induced TNF-α, IL-1β, NO and PGE_2 production in a concentration-dependent manner. Genipin also suppressed LPS-induced NF-κB activation. In addition, the expression of Nrf2 and HO-1 were up-regulated by treatment of genipin. Furthermore, the inhibition of genipin on inflammatory mediator production was attenuated by transfection with Nrf2 siRNA. In conclusion, genipin inhibited LPS-induced inflammatory response by activating Nrf2 signaling pathway in BV2 microglia.

Keywords Genipin · LPS · NF-κB · Nrf2 · Microglia

Introduction

Microglia, a type of resident macrophage in the brain, has been reported to play critical roles in defensing against pathogens in the central nervous system [1]. Once stimulated by injury or infection, microglia secreted inflammatory mediators and inflammatory cytokines such as NO and PGE_2, TNF-α, and IL-1β [2]. However, persistent activation of microglia may lead to various neuronal disorders, such as Alzheimer's disease and Parkinson's disease [3, 4]. Overproduction of these inflammatory mediators may lead to neuronal damage and death [5]. Previous studies showed that regulation of inflammatory mediators production could attenuate the severity of neurodegenerative diseases [6]. NF-κB, a critical signaling molecule in inflammation, has been reported to play critical roles in the regulation of inflammatory mediator production [7, 8]. A large body of evidences demonstrated that many natural products had the ability to inhibit NF-κB activation in LPS-stimulated BV2 microglia cells [9, 10].

Genipin, an aglycon derived from geniposide, has been reported to have antioxidant and anti-inflammatory effects [11]. Studies showed that genipin could attenuate LPS-induced persistent changes of emotional behaviors and neural activation [12]. Genipin has been reported to inhibit LPS-induced apoptotic liver damage and sepsis in mice [13, 14]. Furthermore, genipin has been reported to inhibit inflammatory mediator production in rat brain microglial cells [15]. However, the anti-inflammatory mechanism of genipin on LPS-stimulated BV2 microglial cells has not been reported. The aim of this study was to investigate the anti-inflammatory effect and mechanism of genipin on LPS-stimulated BV2 microglial cells.

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Materials and Methods

Materials

Genipin and LPS (Escherichia coli O55:B5) were purchased from Sigma (St. Louis, MO, USA). Griess reagent was obtained from Beyotime Institute of Biotechnology (Shanghai, China). Primary antibodies specific for Nrf2, HO-1, p65, p-p65, IκBα and p-IκBα were purchased from Cell Signaling Technology (San Francisco, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits of TNF-α, IL-1β, and PGE2 were purchased from R&D Systems (Minneapolis, MN, USA). All other reagents were of analytical grade.

Cell Culture

BV2 microglia cells were purchased from the Institute of Basic Medical Sciences of the China Science Academy. Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator under 5% CO_2.

Cell Viability Assay

BV2 microglia cells in log phase were seeded in 96 well plates. 12 h later, the cells were treated with different concentrations of genipin. 24 h later, 20 μL MTT (5 μg/mL) was added to each well and incubated for 4 h. Then the supernatants were removed and 200 μL DMSO was added to dissolve formazan crystals. Absorbance was determined at 570 nm.

Nitrite Measurement

BV2 cells were pretreated with genipin for 1 h and stimulated by LPS for 24 h. Then the culture supernatants were collected for measuring nitrite production by Griess reagent assay. The culture supernatants were mixed with an equal volume of Griess reagent and incubated at room temperature for 15 min. Absorbance at 540 nm of the reaction was monitored with a microplate reader.

ELISA Assays

BV2 cells were seeded in 24-well plate at a density of 1×10^5 cells/well and pretreated with different concentrations of genipin for 1 h. After stimulating by LPS for 24 h, the culture supernatants were collected. The levels of TNF-α, IL-1β, and PGE_2 in the culture medium were detected by ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

Western Blot Analysis

Total cellular proteins of BV2 cells were extracted using a protein extraction kit according to the manufacturer's instruction. Protein concentrations were determined by BCA protein assay kit. Proteins were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Bedford, MA). The membranes were blocked in 5% skim milk for 2 h and incubated with the specific primary antibodies (1:1000) for 2 h at room temperature. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. The proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions.

Transient Transfection with siRNA

BV2 microglia cells were transfected with Nrf2 siRNA (100 nM) or control siRNA (100 nM) using the Lipofectamine 2000 transfection reagent (Thermo, USA). The siRNA sequence targeting *NRF2* 5′-CAU UGA UGU UUC UGA UCU ATT-3′ was designed and supplied by Qiagen. 36 h later, the cells were treated with genipin and LPS. 24 h later, the production of TNF-α, IL-1β, NO and PGE2 were detected. The inhibition of siRNA on Nrf2 expression was detected by western blot analysis.

In Vivo Experiments

Male ICR mice (2 months old) were provided by the Center of Experimental Animals of Harbin Medical University (Harbin, China). The mice were fed a standard diet. All animal procedures were performed in accordance with NIH guidelines for the care and use of laboratory animals. The animal study proposal was approved by the Institutional Animal Care and Use Committee (IACUC) of the Harbin Medical University with the permit number: SYXK(Hei)2011-022. Sixty mice were randomly divided into five groups: control group, LPS group, LPS + genipin (1, 2.5, 5 mg/kg). LPS was dissolved in sterile saline and injected intraperitoneally (0.25 mg/kg). Genipin was orally administered for 3 weeks prior to LPS injection. The mice were examined 3 day after LPS treatment. The profiles for spatial learning were recorded. The Morris water maze and passive avoidance tasks were based on previous study (Choi et al. 2012). The brain tissues were collected and the expression of TNF-α and IL-1β were detected by Western blot analysis.

Statistical Analysis

Data were presented as the mean ± SD of three independent experiments. Differences between mean values of the data were analyzed using one-way ANOVA and Tukey's multiple comparison tests. Differences are considered to be significant when $p < 0.05$ or $p < 0.01$.

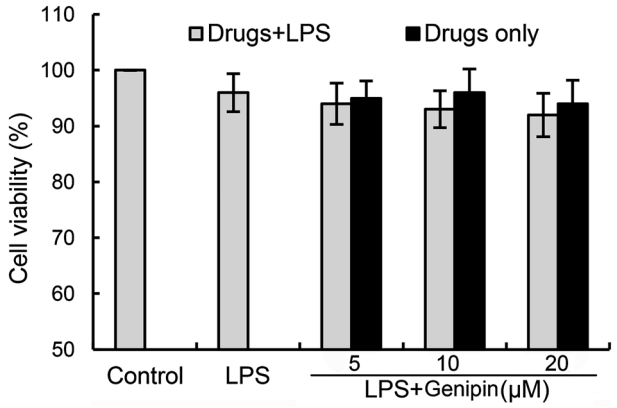

Fig. 1 Effects of genipin on the cell viability of BV2 microglial cells. Cells were cultured with different concentrations of genipin (5, 10, 20 μM) in the absence or presence of 0.5 μg/mL LPS for 24 h. The cell viability was determined by MTT assay. The values presented are the means ± SD of three independent experiments

Results

Genipin has No Cytotoxicity on BV2 Cells at Optimal Concentrations

An MTT assay was used to analyze the effect of genipin on BV2 cells viability. As shown in Fig. 1, the results showed that genipin had no cytotoxicity in the concentration range from 0 to 20 μM. Thus, genipin at the concentrations of 5, 10, 20 μM were used in the subsequent studies.

Genipin Attenuates LPS-Induced NO and PGE_2 Production

The effects of genipin on LPS-induced inflammatory mediators NO and PGE_2 production were detected in this study. Treatment of BV2 cells with LPS alone resulted in significant increases in NO and PGE2 production as compared to the control group. However, genipin concentration-dependently inhibited LPS-induced NO and PGE_2 production (Fig. 2a).

Genipin Suppresses LPS-Induced TNF-α and IL-1ß Production

The effects of genipin on LPS-induced inflammatory cytokines TNF-α and IL-1ß production were detected in this study. Treatment of BV2 cells with LPS alone resulted in significant increases in TNF-α and IL-1ß production as compared to the control group. However, genipin concentration-dependently inhibited LPS-induced TNF-α and IL-1ß production (Fig. 2b).

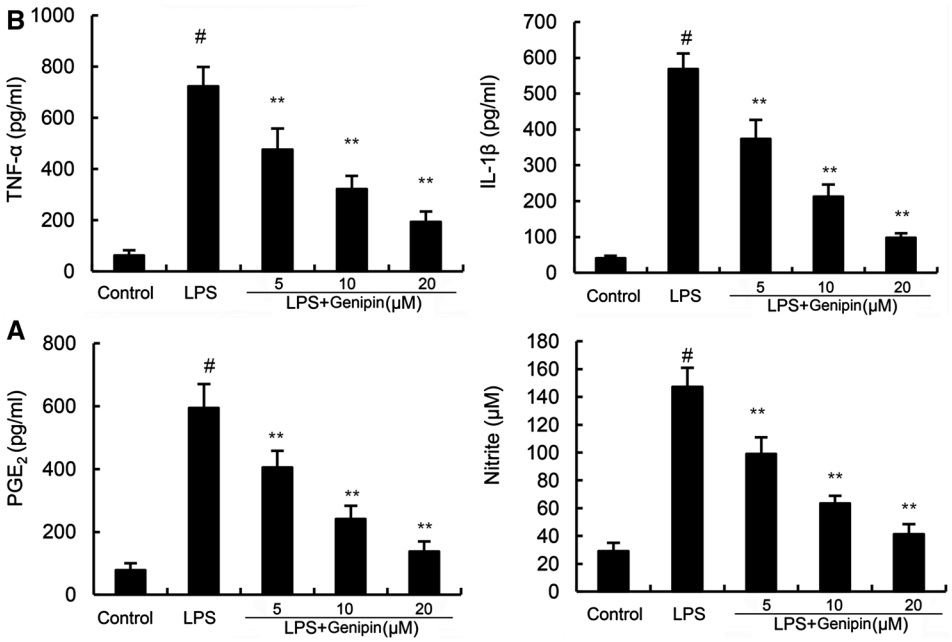

Fig. 2 **a** Effects of genipin on LPS-induced NO and PGE_2 production. **b** Effects of genipin on LPS-induced TNF-α and IL-1ß production. The data presented are the means ± SD of three independent experiments and differences between mean values were assessed by one-way ANOVA and Tukey's multiple comparison tests. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group

Effects of Genipin on LPS-Induced NF-κB Activation

NF-κB has been reported to play vital roles in LPS-induced inflammatory mediator production. In this study, we investigated the effects of genipin on NF-κB activation in BV2 cells. As shown in Fig. 3, LPS stimulation significantly increased the phosphorylation of IκB-α and NF-κB p65. Pretreatment with genipin inhibited LPS-induced NF-κB activation in a concentration-dependent manner.

Genipin Up-Regulates the Expression of Nrf2 and HO-1

Studies showed that Nrf2/HO-1 signaling was involved in NF-κB activation and inflammatory mediator production. Thus, the effects of genipin on Nrf2 and HO-1 expression were detected in this study. The results showed that treatment of genipin augmented the expressions of Nrf2 and HO-1 induced by LPS (Fig. 4).

Genipin Exerts Anti-inflammatory Activity Through Activating Nrf2

To further investigate the anti-inflammatory mechanism of genipin, Nrf2 was knockdown by siRNA. As shown in Fig. 5a, Nrf2 was significantly inhibited by siRNA. Furthermore, the inhibition of genipin on TNF-α, IL-1ß, IL-6 and PGE2 production was reversed by Nrf2 knockdown (Fig. 5b).

Genipin Attenuates LPS-Induced Memory Impairments

Compared with the control group, the escape distance and latency of LPS-treated group increased significantly. However, the increases were inhibited by treatment of genipin. Furthermore, the passive avoidance test were used to evaluate contextual memory of mice. The results showed LPS significantly decreased the step-through latency. However, treatment of genipin efficiently blocked the memory impairments (Fig. 6).

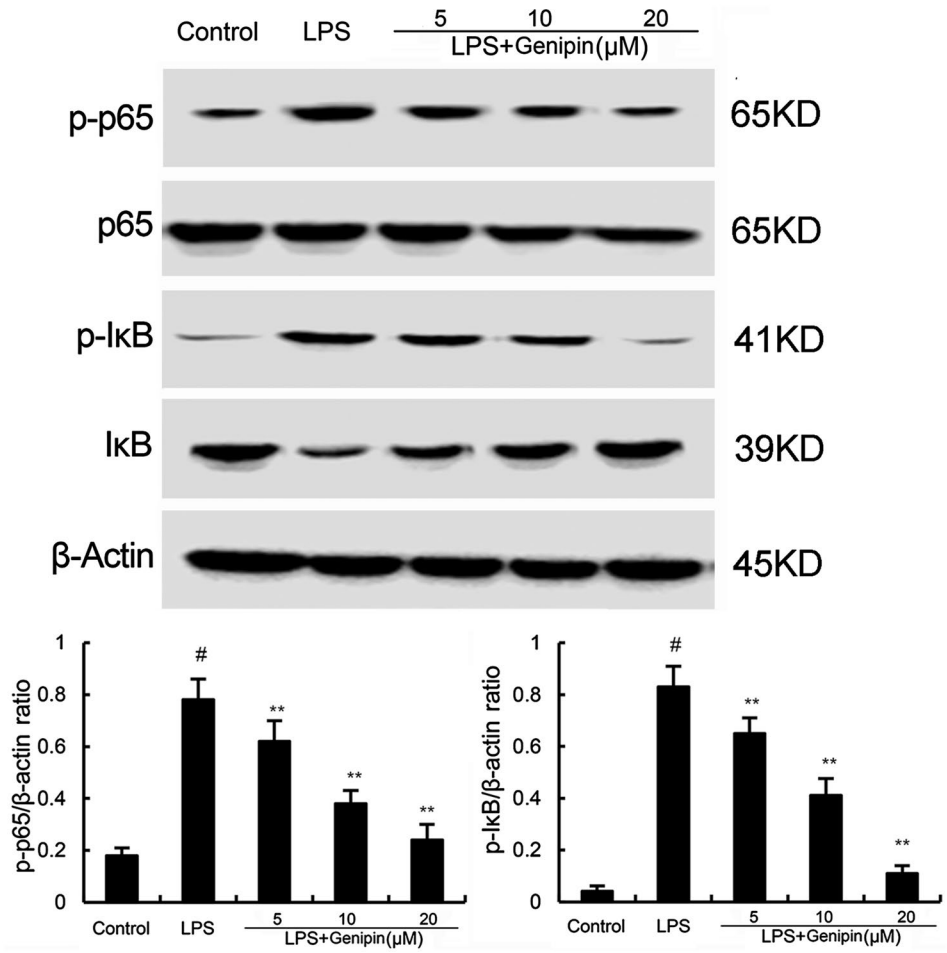

Fig. 3 Genipin inhibits LPS-induced NF-κB activation. The values presented are the means ± SD of three independent experiments and differences between mean values were assessed by one-way ANOVA and Tukey's multiple comparison tests. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group

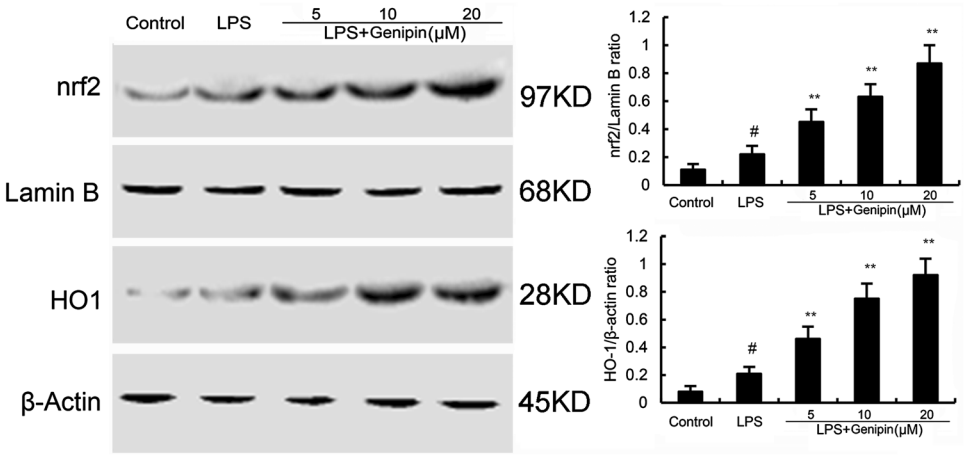

Fig. 4 Effects of genipin on the expression of Nrf2 and HO-1. The values presented are the means ± SD of three independent experiments and differences between mean values were assessed by one-way ANOVA and Tukey's multiple comparison tests. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group

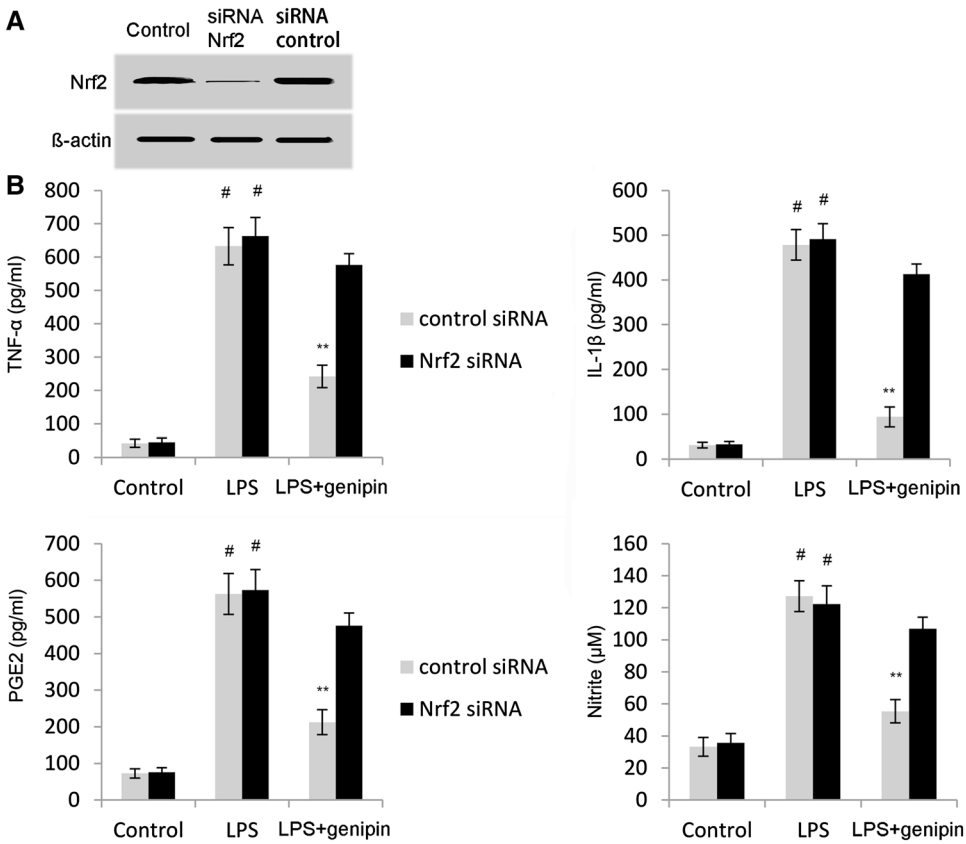

Fig. 5 **a** The inhibition of siRNA on Nrf2 expression. **b** Nrf2 knockdown reversed the anti-inflammatory effects of genipin. The values presented are the means ± SD of three independent experiments and differences between mean values were assessed by one-way ANOVA and Tukey's multiple comparison tests. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group

Genipin Inhibits LPS-Induced TNF-α and IL-1ß Expression in Brain Tissues

The expression of TNF-α and IL-1ß in brain tissues were detected in this study. As shown in Fig. 7, compared to the control group, the expression of TNF-α and IL-1ß increased significantly in LPS-treated group. However, the increases were dose-dependently inhibited by treatment of genipin.

Discussion

Previous studies indicated that natural compounds had the ability to treat neurological disorders by inhibition of microglial activation [16, 17]. Genipin has been reported to have anti-inflammatory effect. In the present study, we detected the anti-inflammatory effects of genipin on LPS-induced memory impairments and LPS-activated microglial cells. The results of this study showed that genipin

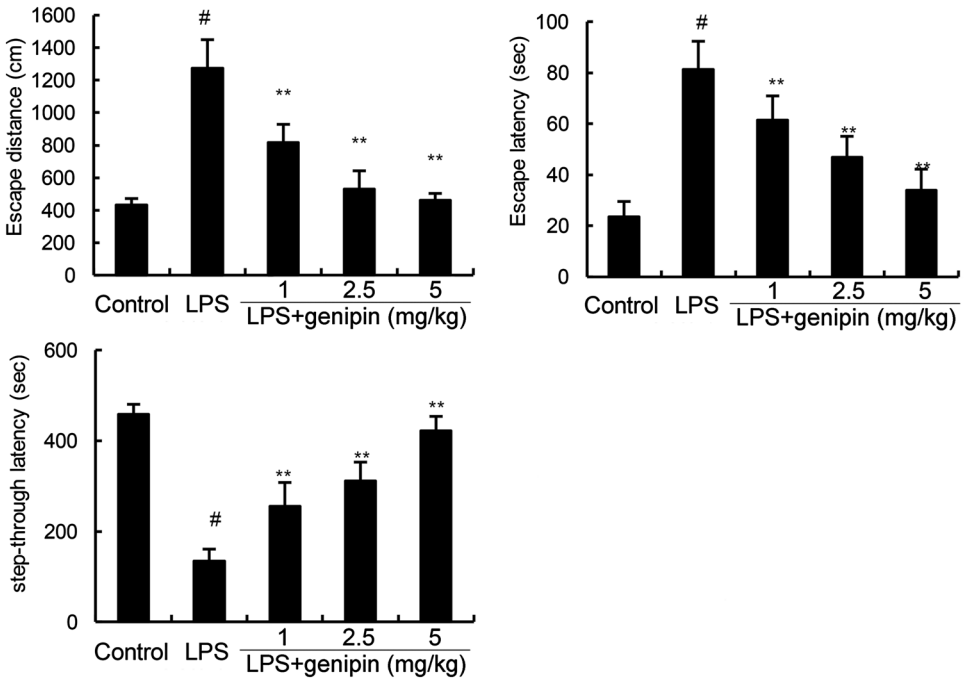

Fig. 6 Effects of genipin on LPS-induced memory impairments. The values presented are the means ± SEM of three independent experiments. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group

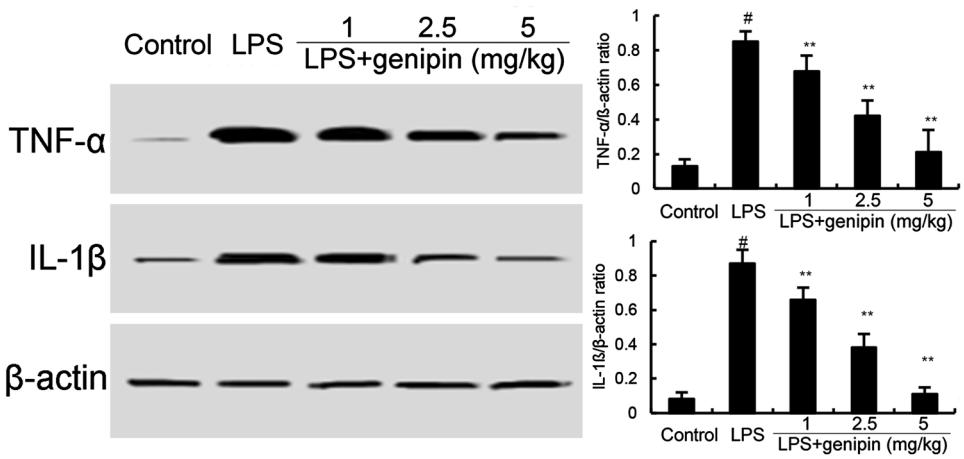

Fig. 7 Effects of genipin on LPS-induced TNF-α and IL-1ß expression in brain tissues. The data presented are the means ± SEM of three independent experiments. $^{\#}p < 0.05$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group

significantly attenuated LPS-induced memory impairments in vivo. In vitro, genipin inhibited LPS-induced inflammatory mediator production by activating Nrf2/HO-1 signaling pathway. Genipin may be a therapeutic agent for neurological disorders.

Neuroinflammation has been reported to be caused by immune response to pathogens or damaged cells within the brain [18]. Microglia are the resident macrophage-like cells in the brain [19]. Stimulation of microglia by LPS, the main virulence factor of Gram-negative bacteria, could induce the production of inflammatory mediators [20]. Overproduction of these inflammatory mediators lead to common neuronal diseases [21]. Previous studies showed that NO, PGE_2, TNF-α, and IL-1β levels increased in the cerebrospinal fluid of patients suffering from neurological diseases [22]. Controlling of these inflammatory mediators had the ability to attenuate the severity of neurodegenerative diseases [23]. In this study, we found that genipin remarkably suppressed LPS-induced inflammatory mediator production in BV2 microglial cells.

NF-κB, a critical signaling molecule, has been reported to play critical roles in the regulation of inflammatory mediators [24, 25]. Recent studies showed that stimulating of microglia cells with LPS could induce NF-κB activation, which subsequently induced the production of inflammatory mediators NO, PGE_2, TNF-α, and IL-1β [26]. Because NF-κB activation is closely related to the production of inflammatory mediators, we investigated whether VA inhibited inflammatory mediators by modulating NF-κB activation. Our results showed that VA suppressed LPS-induced

NF-κB activation in a concentration dependent manner. The transcription factor Nrf2 regulates the basal and inducible expression of numerous detoxifying and antioxidant genes, such as HO-1. Activating of Nrf2 could induce the expression of HO-1. Previous studies showed that Nrf2-null mice exhibited exacerbated brain inflammation in response to LPS. Activating Nrf2 could attenuate LPS-induced brain inflammation. These reports suggested that Nrf2 was a therapeutic target against brain inflammation. A large body of data suggests that activating of Nrf2 signaling pathway could inhibit LPS-induced inflammatory response in BV2 microglial cells. In the present study, we found that genipin concentration-dependently up-regulated the expression of Nrf2 and HO-1. Furthermore, the anti-inflammatory effects of genipin can be reversed by Nrf2 knockdown. Therefore, genipin may act through activating the Keap-Nrf2-ARE pathway, which subsequently up-regulated the expression of HO-1.

In summary, our results revealed that genipin activates Nrf2 signaling pathway, which lead to the inhibition of NF-κB activation and inflammatory mediators production in BV2 microglia cells. Genipin may be a therapeutic agent for the treatment of neurological diseases that associated with the overactivation of microglia.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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