**Ramulus Cinnamomi** extract attenuates neuroinflammatory responses via downregulating TLR4/MyD88 signaling pathway in BV2 cells

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**Graphical Abstract**

**Abstract**

**Ramulus Cinnamomi** (RC), a traditional Chinese herb, has been used to attenuate inflammatory responses. The purpose of this study was to investigate the effect of RC extract on lipopolysaccharide (LPS)-induced neuroinflammation in BV2 microglial cells and the underlying mechanisms involved. BV2 cells were incubated with normal medium (control group), LPS, LPS plus 30 μg/mL RC extract, or LPS plus 100 μg/mL RC extract. The BV2 cell morphology was observed under an optical microscope and cell viability was detected by MTT assay. Nitric oxide level in BV2 cells was detected using Griess regents, and the levels of interleukin-6, interleukin-1β, and tumor necrosis factor α in BV2 cells were determined by ELISA. The expression levels of cyclooxygenase-2, Toll-like receptor 4 and myeloid differentiation factor 88 proteins were detected by western blot assay. Compared with the LPS group, both 30 and 100 μg/mL RC extract had no significant effect on the viability of BV2 cells. The levels of nitric oxide, interleukin-6, interleukin-1β, and tumor necrosis factor α in BV2 cells were significantly increased after LPS induction, and the levels were significantly reversed after treatment with 30 and 100 μg/mL RC extract. Furthermore, RC extract significantly inhibited the protein expression levels of cyclooxygenase-2, Toll-like receptor 4 and myeloid differentiation factor 88 in LPS-induced BV2 cells. Our findings suggest that RC extract alleviates neuroinflammation by downregulating the TLR4/MyD88 signaling pathway.

**Key Words:** nerve regeneration; Ramulus Cinnamomi; BV2 cells; lipopolysaccharide; neuroinflammation; pro-inflammatory factors; TLR4/MyD88 signaling pathway; nitric oxide; interleukin-6; interleukin-1β; tumor necrosis factor α; neuronal regeneration

**Introduction**

Microglia are the tissue macrophages of the brain and are crucial for maintaining tissue homeostasis and for the scavenging of pathogens, dying cells, and molecules through microbial-associated molecular pattern receptor dependent and independent mechanisms (Sid et al., 2014). Activated microglia induced by stimuli release various pro-inflammatory factors, such as interleukin-6 (IL-6), interleukin-1β (IL-1β),...
and tumor necrosis factor (TNF)-α (Innamorato et al., 2008; Lu et al., 2011; Lafrenaye, 2016). The excessive production of these inflammatory mediators can act as neurotoxins and damage the brain (Chao et al., 1992; McGuire et al., 2001). Therefore, for the treatment of many neuroinflammation-mediated diseases, such as stroke, Alzheimer’s disease, and Parkinson’s disease, controlling microglial activation and inhibiting the release of pro-inflammatory factors are of great significance.

Lipopolysaccharide (LPS) stimulates the release of pro-inflammatory cytokines and nitric oxide (NO) production in BV2 microglial cells (Kang et al., 2004; Piao et al., 2004; Lyu et al., 2006; Lu et al., 2007). Toll-like receptors (TLRs) play important roles in initiating immune responses; for example, TLR4 in the immune system binds to LPS to stimulate pro-inflammatory cytokine release (Jack et al., 2005; Takeuchi and Akira, 2010). Myeloid differentiation factor 88 (MyD88) plays a crucial role in signal transduction in the TLR4 signaling pathway (O’Neill and Bowie, 2007) and it was reported that the TLR4/MyD88 signaling pathway plays an important role in neuroinflammation (Qin et al., 2013).

Ramusus Cinnamoni (RC, GuiZhi in Chinese), a traditional Chinese herb is used to treat inflammation based on its purgative, antipyretic, anti-inflammatory, and antineoplastic activities (Zheng et al., 2015; Kwon et al., 2016). However, how RC extract affects neuroinflammation in microglial cells is poorly understood. Here, the effect and possible mechanisms of RC extract against LPS-induced inflammation in BV2 microglial cells were investigated.

Materials and Methods
Preparation of RC extract
Dried RC extract was extracted from GuiZhi and identified by Professor Hai-lin Qin (Department of Phytochemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College). In brief, dried RC (30 g) was soaked in 95% aqueous ethanol (500 mL) for 2 hours and then refluxed by water-bath heating for 1 hour. The extracts were filtered and concentrated by a rotator evaporator (Heidolph, Schwabac, Germany). Then, they were placed in a 500 mL of separatory funnel, and extracted with petroleum ether (Hennet al., 2009). In the current study, BV2 cells were purified from Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China) and maintained in Dulbecco’s modified Eagle’s medium (Gibco BRL, Gaithersburg USA) containing 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco BRL), and 10% fetal bovine serum (FBS; Gibco BRL) at 37°C in a 5% CO₂ humidified cell incubator.

RC extract treatment and cell viability study
BV2 cells were cultured in a 96-well plate (2 × 10⁵ cells/well). At 70–80% confluence, BV2 cells were pre-incubated with RC extract 30 or 100 μg/mL for 1 hour and then exposed to 200 ng/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for another 24 hours. After treatment, BV2 cell morphology was observed and photographed under an optical microscope (Olympus X71, Tokyo, Japan). Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide (MTT; Sigma, St. Louis, MO, USA) (Han et al., 2013). Briefly, 10 μL of the MTT solution was added to each well of the 96-well plate and incubated at 37°C for 4 hours. After removing the medium, the formazan product was dissolved in 200 μL dimethyl sulfoxide in each well. Absorbance values were measured at 560 nm with a microplate reader (Spectramax M5 microplate reader, Molecular Devices, Sunnyvale, CA, USA).

Cell supernatant collection
BV2 cells were added to a 6-well plate (4 × 10⁵ cells/well). At 70–80% confluence, the medium was replaced with DMEM free of FBS and cells were pre-incubated with different concentrations of RC extract for 1 hour and then exposed to LPS (200 ng/mL) for another 24 hours. Then, the BV2 cells were collected and centrifuged at 4,000 ×g for 5 minutes, and the supernatant was collected.

NO assay
Nitrite in the cell culture media was measured as an index of NO production using the Griess assay kit (Promega, Madison, WI, USA) (de Oliveira et al., 2014). Then, 50 μL of supernatant was mixed with the Griess reagents for 10 minutes at room temperature. Absorbance values at 540 nm were measured using a microplate reader (Spectramax M5 microplate reader, Molecular Devices) and nitrite concentrations were determined by comparison to the Nitrite Standard reference curve.

Cytokine quantification by ELISA
The levels of IL-6, IL-1β and TNF-α in the supernatant were determined using ELISA kits (Genetimes, ExCell Biology, Shanghai, China) in accordance with the manufacturer’s instructions. First, the ELISA plate was coated with anti-mouse antibody. After overnight incubation at 4°C, the plates were washed three times with sterile PBS, and the uncoated sites were blocked with 200 μL of PBS containing 10% FBS for 2 hours at 37°C. After subsequent washing, 100 μL of the supernatant obtained from the culture samples was added, and the plates were incubated for 2 hours at 37°C. The plates were subsequently washed and the biotinylated anti-mouse antibody solution was added to each well except for the blank control, and incubated for 1 hour at 37°C. After incubation and additional washing, the horseradish peroxidase (HRP)-streptavidin conjugated secondary antibody was added to each well and incubated for 30 minutes at 37°C. After washing, 3,3′,5,5′-tetramethylbenzidine (TMB) solution was added and incubated at room temperature, followed by the addition of stop solution to each well. The absorbance value at 450 nm was measured using a microplate reader. The cytokine concentration was determined using a standard curve.

Western blot assay
After treatment, the cells were lysed with Radio Immunoprecipitation Assay (RIPA) buffer in the presence of cocktail
Figure 1 Effect of RC extract on cell morphology and cell viability of BV2 cells. (A) Morphology of cells treated with normal medium (a), LPS (b), LPS + RC extract 30 μg/mL (c), LPS + RC extract 100 μg/mL (d) under an optical microscope (original magnification, 40×). (B) Viability of BV2 cells determined by MTT assay. Data are expressed as the mean ± SD. Experiments were performed in triplicate. RCE 30: RC extract at 30 μg/mL; RCE 100: RC extract at 100 μg/mL; LPS: lipopolysaccharide; RC: Ramulus Cinnamomi.

Figure 2 Effect of RC extract on NO production and pro-inflammatory factors in the supernatants of BV2 cells as detected by ELISA. (A) NO level; (B) interleukin-1β level; (C) interleukin-6 level; (D) tumor necrosis factor-α level. Data are expressed as the mean ± SD. Experiments were performed in triplicate. Intragroup comparisons were conducted using one-way analysis of variance and Student-Newman-Keuls test. *P < 0.01, vs. control; #P < 0.05, ##P < 0.01, vs. LPS group. RCE 30: RC extract at 30 μg/mL; RCE 100: RC extract at 100 μg/mL; LPS: lipopolysaccharide; RC: Ramulus Cinnamomi; NO: nitric oxide.

Figure 3 Effect of RC extract on the expression levels of COX2, TLR4, and MyD88 in LPS-treated BV2 cells detected by western blotting. (A–C) COX2, TLR4, and MyD88 protein expression levels. Data are expressed as the mean ± SD. Experiments were performed in triplicate. Intragroup comparisons were conducted using one-way analysis of variance and the Student-Newman-Keuls test. *P < 0.01 vs. control group; #P < 0.05, ##P < 0.01, vs. LPS group. RCE 30: RC extract 30 μg/mL; RCE 100: RC extract 100 μg/mL; LPS: lipopolysaccharide; RC: Ramulus Cinnamomi; COX2: cyclooxygenase-2; TLR4: Toll-like receptor 4; MyD88: myeloid differentiation factor 88; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
protease inhibitors (pepsatin, leupeptin, aprotinin) in an iced water bath for 30 minutes and then centrifuged at 4°C for 12,000 g for 15 minutes. The supernatant was collected and the protein concentration was determined using BCA assay. Then, loading buffer was added into samples, boiled for 5 minutes and used for the following assay.

Total protein from the supernatant was separated by 10% SDS-polyacrylamide gel electrophoresis, and the protein bands were transferred to nitrocellulose membranes. The membranes were blocked by incubation with 5% bovine serum albumin (BSA) in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween-20) for 1 hour at room temperature and then incubated with different primary antibodies: rabbit anti-COX-2 (Rt, 1:1,000, Abcam, Cambridge, UK), rabbit anti-TLR4 (Rt, 1:1,000, Abcam), rabbit anti-MyD88 (Rt, 1:1,000, Abcam), and anti-GAPDH (Rt, 1:1,000, Abcam) overnight at 4°C. After washing, the membranes were incubated with HRP-conjugated rabbit anti-secondary antibody (1:2,000, Abcam) for 2 hours at 37°C, followed by washing. The bands were revealed using the ECL system (Beijing ComWin Biotech Co., Ltd., Beijing, China). The signal densities of the bands were measured with Gel-pro software (Molecular Imager ChemiDoc XR+ System, Bio-Rad, CA, USA) and normalized using anti-GAPDH as an internal control (optical density detected protein/optical density internal control).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 6.02 (GraphPad Software Inc., CA, USA) Data are expressed as the mean ± SD. Measurement data between groups were compared using one-way analysis of variance and the Student-Newman-Keuls test. \( P < 0.05 \) was considered statistically significant.

**Results**

**Effect of RC extract on BV2 cell viability**

After treatment, BV2 cell morphology was observed under an optical microscope. As shown in Figure 1, RC extract at concentrations of 30 and 100 μg/mL had no significant effect on the viability of BV2 cells following LPS induction. Therefore, RC extract at concentrations of 30 and 100 μg/mL was used for all subsequent experiments.

**RC extract reduced NO, IL-6, IL-1β, and TNF-α production in the supernatants of LPS-induced BV2 cells**

As shown in Figure 2, 200 ng/mL LPS induction for 24 hours significantly increased the levels of NO, IL-1β, IL-6, and TNF-α in the supernatants of BV2 cells compared with the control group (all \( P < 0.01 \)). However, after treatment with RC extract for 24 hours, both 30 and 100 μg/mL RC extract significantly decreased the levels of NO (all \( P < 0.05 \)), as well as IL-6, IL-1β and TNF-α (all \( P < 0.05 \)) in the supernatants of LPS-induced BV2 cells.

**RC extract downregulated protein expression levels of COX2, TLR4, and MyD88 in LPS-induced BV2 cells**

As shown in Figure 3, western blot assay results showed that LPS stimulation significantly increased the protein expression levels of COX2, TLR4, and MyD88 in BV2 cells (all \( P < 0.01 \)). However, treatment with 30 and 100 μg/mL RC extract inhibited the protein expression of COX2, TLR4, and MyD88 in LPS-induced BV2 cells (all \( P < 0.01 \)).

**Discussion**

RC is commonly used for gastritis, dyspepsia, blood circulation disturbances and inflammation (Liao et al., 2012). RC extract was reported to relax vascular smooth muscle by inhibiting Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels and inositol triphosphate-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (Kang and Shin, 2012). Moreover, Jung et al. (2011) found that RC extract exhibited antioxidant activity *in vitro* and protected against gastric damage *in vivo* by the stimulation of mucus secretion. Hwang et al. (2009) reported that RC exhibited anti-inflammatory effects by downregulating the expression of various genes related to inflammatory responses in LPS-stimulated BV2 cells. In the current study, we report for the first time that RC extract inhibits NO, IL-6, IL-1β, and TNF-α production in BV2 microglial cells, possibly by inhibiting the TLR4/MyD88 signaling pathway.

During central nervous inflammation, levels of the inducible form of NOS are increased, and the persistent overproduction of NO is mediated by appropriate stimuli such as LPS or pro-inflammatory cytokines (MacMicking et al., 1997; Lyu et al., 2006). The overproduction of NO may induce nerve injury. Furthermore, pro-inflammatory factors (IL-1β, IL-6 and TNF-α) in the ischemic brain are upregulated from resident brain cells and infiltrating immune cells after ischemic insult (Waje-Andreassen et al., 2005; Zeng et al., 2012; Jin et al., 2013). In our study, we found that the levels of NO, IL-1β, IL-6, and TNF-α were increased after LPS induction, and that RC extract significantly reduced the LPS-induced NO, IL-6, IL-1β, and TNF-α production.

COX2, the key enzyme responsible for the synthesis of inflammation-related prostaglandin, is closely associated with chronic inflammation (Lee et al., 2002; Kim et al., 2004; Guo et al., 2006). Notably, RC extract was reported to suppress COX2 expression and decrease LPS-induced PGE2 production in RAW 264.7 macrophages (Park et al., 2005). Our results indicate that the COX2 protein expression level was also increased in BV2 microglial cells by the effect of LPS. However, RC extract treatment significantly decreased the protein expression level of COX2.

TLRs are widely expressed in a variety of immune cells and brain resident cells, such as microglia, cerebral endothelium, neurons, and astrocytes (Bisbisi et al., 2002; Jack et al., 2005; Marsh et al., 2009). Activation of TLR signaling pathways regulates cytokine and chemokine production. TLR4 is a specific pattern recognition receptor for LPS (Park et al., 2009; Takeuchi and Akira, 2010). TLR signaling involves MyD88-dependent and -independent pathways. It was reported that mice lacking either TLR2 or TLR4 were less susceptible to cerebral ischemic damage (Gao et al., 2007; Ziegler et al., 2007). Furthermore, TLR4-/- mice were protected from brain injury induced by global or permanent focal cerebral ischemia (Caso et al., 2007; Hua et al., 2007). In this study, our results indicated
that RC extract treatment significantly decreased the protein expression levels of TLR4 and MyD88, suggesting that RC extract alleviates inflammation in BV2 microglial cells by inhibiting the TLR4/MyD88 signaling pathway.

In summary, RC extract effectively inhibits neuroinflammation induced by LPS in BV2 microglial cells by downregulating the TLR4/MyD88 signaling pathway. This comprehensive understanding of RC extract in nervous system will provide novel insight into the development of therapeutic approaches against neuroinflammation-mediated diseases.

Author contributions: HY, XC, and YLY performed the research and analyzed the data. HY and YHW wrote the paper. YHW and GHD designed the research and revised the paper. All authors read and approved the final manuscript.

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References


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