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The effects of berberine on a murine model of multiple sclerosis and the SPHK1/S1P signaling pathway

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ABSTRACT

Berberine (BBR) has shown neuroprotective properties. The present study aims to investigate the effects of BBR on experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS), and SphK1/S1P signaling, which plays a key role in MS. EAE was induced in mice, followed by treatment with BBR at 50, 100, or 300 mg/kg/d. Neurophysiological function was evaluated daily; inflammation, cell infiltration, and the severity of demyelination were also examined. The SphK1, SphK2, and S1P levels in the animals and primary astrocyte culture were measured. We found that treatment with BBR reduced the loss of neurophysiological function and the degree of demyelination. Moreover, BBR was associated with a decrease in SphK1 and S1P levels both in the animals and in culture. These results indicated that BBR suppresses demyelination and loss of neurophysiological function by inhibiting the SphK1/S1P signaling pathway. The use of BBR as a treatment of MS warrant further exploration.

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1. Introduction

Multiple sclerosis (MS) is a central nervous system (CNS) disease characterized by demyelination as a result of chronic inflammation [1]. Although the exact mechanism of MS is not elucidated, evidence supports that MS is an autoimmune disease. Because of limited treatment options, the search for an effective pharmacological therapy for MS is a field of intense study [2]. Experimental autoimmune encephalomyelitis (EAE) has been developed as a model of MS and is commonly used in the study of MS as well as evaluating treatment for MS [3].

Astrocytes play a critical role in MS as they are activated in response to many CNS pathologies [4,5]. The severity of MS and EAE positively correlates with the degree of reactive gliosis, and inhibition of gliosis ameliorates the symptoms of MS and EAE [6]. Sphingosine-1-phosphate (S1P), a lipid that modulates the vascular and immune system [7], is important in the development of MS and EAE [8–10]. Binding of S1P with S1P1 in astrocytes is a key step in the pathogenesis of EAE, probably by promoting the release of interleukins and other cytokines that mediate inflammatory responses [11]. SphK1 is a kinase that phosphorylates and activates S1P [12]. Fischer et al. have reported that SphK1/S1P/S1PR3 signaling is upregulated in MS patients [13]. Thus, upregulation of SphK1/S1P/S1PR signaling is possibly a key factor in chronic inflammatory responses mediated by astrocytes.

BBR is a benzyl isoquinoline alkaloid and the active ingredient of a traditional Chinese medicine [14]. Recent studies have shown that BBR suppresses inflammation through various signaling pathways [15–17]. As BBR can cross the blood-brain barrier (BBB), administration of BBR could be beneficial to neurodegenerative diseases [18,19]. In fact, the anti-inflammatory effect of BBR has been shown to be neural protective in a murine model of traumatic brain injury [20].

Previous studies have reported that the possible mechanisms of BBR includes inhibiting the degradation of matrix metalloproteinase-9, maintaining the BBB, and modulating differentiation of Th1 and Th7 cells [21–23]. In addition, it has been reported that BBR inhibits the SphK1/S1P signaling pathway in the kidney cells [24]. The present study also aims to investigate the effects of BBR on EAE and SphK1/S1P signaling pathway in astrocytes.

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2. Methods

2.1. Experimental animals and EAE model

Female C57BL/6 mice (18–20 g) were obtained from the Center for Animal Experiments of Chongqing Medical University. The use of the animals was approved by the IACUC of Chongqing Medical University.

EAE was induced by using the methods described by Kataoka et al. [25]. Briefly, MOG35-55 (amino acid sequence: MEVGWYRSPFSRVVHLYRNGK, Sigma-Aldrich) was dissolved in 0.01 M phosphate-buffered saline (PBS) and mixed with equal volume of complete Freund's adjuvant (CFA, Sigma-Aldrich) to a final concentration of MOG35-55 at 300 μg/mL. The mixture was emulsified, and 0.2 mL of the emulsion was injected subcutaneously into the foot pads of each animal. Pertussis toxin (500 ng) was injected intraperitoneally at 0 and 48 h after the mice were immunized with the emulsion. The neurophysiological performance of the mice was scored daily.

2.2. Treatment with BBR

The mice were randomly divided into five groups: the EAE group, three BBR-treatment groups, and the CFA group. The EAE group of mice received intragastric administration of saline, and the BBR-treatment groups received daily intragastric administration of BBR at 50 mg/kg, 100 mg/kg, or 300 mg/kg. Intragastric administration of saline or BBR started 10 days after immunization with the MOG35-55 emulsion. The CFA group was immunized with an equal volume of CFA only.

2.3. Neurophysiological function evaluation

The neurophysiological functions were rated with the clinical score on a 0–5 scale, where 0 = unaffected, 1 = tail limpness, 2 = failure to right on attempt to roll over, 3 = partial paralysis, 4 = complete paralysis, and 5 = moribund. Two experimenters rated the mice daily, and the daily scores were the average of the two.

2.4. Histology

On day 35 post-immunization, mice were euthanized with intraperitoneal injection of chloral hydrate and perfused with 30 mL of saline followed by 100 mL of 4% paraformaldehyde (PFA). The spinal cord was dissected and fixed in 4% PFA for 24 h, and embedded in paraffin and sectioned. One 5 μm slice was taken every 25 μm. The slices were stained with either hematoxylin and eosin (H&E) for evaluation of inflammatory cell infiltration or Luxols fast blue (LFB) for evaluation of demyelination.

Infiltration of inflammation was rated on a 0–4 scale as described by Okuda et al. [26]. Briefly, inflammatory cells were counted under a microscope (400 × magnification; NikonTS100; Nikon, Japan), and the inflammatory cell infiltration was rated as follows: 0 = no infiltration, 1 = inflammatory cells were observed only in peri-vascular areas, 2 = 1–10 inflammatory cells per visual field, 3 = 11–100 inflammatory cells per visual field, and 4 = more than 100 inflammatory cells per visual field. The final scores for each animal were the average of the scores of five visual fields.

Demyelination was rated on a 1–4 scale as described by Zappia et al. [27]. Briefly, 1 = traces of subpial demyelination, 2 = pronounced subpial and perivascular demyelinating, 3 = confluent perivascular or subpial demyelination, and 4 = massive perivascular and subpial demyelination.

2.5. Primary astrocyte culture

The cortex was dissected from C57BL/6 mice (postnatal day 3 or younger) and triturated in serum-free Dulbecco's modified Eagle medium (DMEM)/F12 (Thermo-Fisher) with a glass pipette. The mixture was incubated with trypsin (0.25%, in PBS) at 37 °C for 10 min, and then diluted with 6 mL DMEM and filtered with 400-Mesh Scribble (pore size 37 μm). The resultant solution was then centrifuged at 1500 rpm, and the pellet was resuspended in 2 mL DMEM. The cell density was adjusted to 5–10 × 10^5 cells/mL and incubated in culture flasks that were pretreated with polylysine at 37 °C. To purify astrocytes, when astrocytes could be observed visually under a microscope on culture day 7–9, the culture flasks were gently shaken on a shaker (250 rpm) at 37 °C twice for 2 h (separated by a 1-h incubation period) and incubated at 37 °C overnight. The cultured cells were washed with PBS three times and incubated with 0.25% trypsin (in PBS) at 37 °C for 3 min. The cells were then washed, resuspended, and cultured in a polylysine-treated culture flask at 37 °C. The procedure was repeated once to obtain astrocyte culture (95% purity). Further experiments were performed on culture day 20–22.

For lipopolysaccharide (LPS) treatment, the cultured cells were incubated with LPS (100 ng/mL) at 37 °C for 24 h. For BBR treatment, the cells were incubated with LPS (100 ng/mL) and BBR at 1 μM, 3 μM, or 10 μM at 37 °C for 24 h.

2.6. Immunohistology

Frozen sections were fixed by using the acetone fixation method, and then incubated with a mixture of primary antibodies (SphK1, SphK2, and GFAP [all 1:1000 dilution, Abcam]) at 4 °C overnight, followed by incubation with the appropriate mixture of secondary antibodies in the dark at room temperature for 50 min. After washing, the sections were treated with DAPI for 10 min in the dark at room temperature. The sections were then sealed with an anti-fade mounting medium (Jinhgkehuaxue, Co.). Images were acquired with a Nikon inverted fluorescence microscope (model TS100). Integrated optical density (IOD) was measured with Image-Pro Plus 6.0 (Media Cybernetics, USA).

Measurement of S1P with enzyme-linked immunosorbent assay (ELISA.

S1P in astrocytes was measured with ELISA. Astrocytes after LPS and BBR treatment were centrifuged at 1500 rpm, and 37 °C for 5 min. The pellet was then incubated with radioimmunoprecipitation assay (RIPA) lysis buffer at room temperature for 30 min, and then centrifuged at 1300 rpm at room temperature for 10 min. The supernatant was collected and transferred to a microwell plate. ELISA was performed with an ELISA kit (ELISA Kits, S1P [MICE], Marburg, Germany), according to the manufacturer's instructions.

2.7. Western blot

Cultured astrocytes were centrifuged at 1500 rpm at 4 °C, and resuspended in RIPA buffer (250 μL/10^6 cells) containing 500 μM protease inhibitor (Jiangsu KeyGEN BioTECH Corp, Ltd). The mixture was incubated on ice for 30 min, and centrifuged at 12 000 g for 5 min. The supernatant was collected, and the protein concentration was measured with the bicinchoninic acid method. The sample was denatured by boiling with an appropriate volume of 2 × sample buffer at 100 °C. The protein samples were subjected to electrophoresis, and then transferred to nitrocellulose membrane. The membrane was blocked with 5% fat-free milk dissolved in tris-buffered saline containing Tween 20 for 1 h at 37 °C. The membrane were then incubated with the appropriate primary

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