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

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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 Spkh1, Sphkh2, 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 Spkh1 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]. Spkh1 is a kinase that phosphorylates and activates S1P [12]. Fischer et al. have reported that Spkh1/S1P/S1PR signaling is upregulated in MS patients [13]. Thus, upregulation of Spkh1/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 Spkh1/S1P signaling pathway in the kidney cells [24]. The present study also aims to investigate the effects of BBR on EAE and Spkh1/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: MEVG-WYRSPFSRVHLYRNGK, 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 Luxol 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 x 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 x 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 radio-immunoprecipitation 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 x 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
antibodies (SPHK1 and SPHK2 monoclonal antibodies [1:1000, Abcam, UK]) at 4 °C overnight. The membrane was washed, and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at 37 °C for 30 min (1:3000, Abcam, UK). The protein bands were visualized on a film, the images were scanned, and the optical density was measured with Alpha software (USA).

2.8. Statistical analysis

Statistical analyses were performed with SPSS 15.0 (IBM, USA). All data are presented as mean ± standard error unless otherwise noted. One-way analysis of variance (ANOVA) was used to test significance. A p < 0.01 was considered significant.

3. Results

3.1. The effects of BBR on the neurological outcomes in EAE model mice

Neurophysiological abnormalities of each mouse were rated daily with a clinical scoring system with 0 being affected and 5 moribund. All MOG peptide-injected mice developed neurophysiological abnormalities with an acute or subacute onset. Clinical symptoms were first observed between 8 and 12 days after injections, and progressed with time. Between day 12 and 14, the mice showed limpness and sagging in the tail as well as waddling gaits when walking on a flat surface, and from day 14 to day 20, all mice showed symptoms of hind limbs weakness. In untreated mice, three mice were partially paralyzed, two mice were completely paralyzed, and one was moribund at the end of the study period. Administration of BBR at high dosages showed protective effects: none of the mice treated with 100 mg/kg or 300 mg/kg BBR was completely paralyzed, and one mouse was partially paralyzed (100 mg/kg).

The mice that were treated with 100 mg/kg or 300 mg/kg BBR scored significantly lower than both the untreated group and the 50-mg/kg BBR group on the clinical score. In addition, the maximal score, the highest score observed in the study period, were 2.33 ± 0.154 in the 100-mg/kg group and 2 ± 0.164 in the 300-mg/kg group, significantly lower compared with the untreated group (3.33 ± 0.16), and the 50-mg/kg group, with a score of 3.17 ± 0.16 (p < 0.01). The cumulative scores, the sum of the clinical scores during the study period, were 36.99 ± 4.49 in the 100-mg/kg group and 33.41 ± 4.28 in the 300-mg/kg group, significantly lower compared with the untreated group (53.89 ± 4.76) and the 50-mg/kg group (51.24 ± 4.57, p < 0.01) (Fig. 1).

3.2. The effects of BBR on the histology of the spinal cord in EAE mice

The mice were sacrificed on day-35 post-immunization, and demyelination and infiltration of inflammatory cells in the spinal cord were evaluated. The nerve fibers appeared thin and sparse, with an uneven distribution and gaps between the fibers. In the most severe case, the nerve fibers were found to be damaged or

Fig. 1. BBR treatment at 100 or 300 mg/kg significantly improved the neurophysiological outcome in EAE mice. (A) The neurophysiological outcome was assessed daily for 35 days after EAE induction and expressed as the clinical score. Mice treated with 100 or 300 mg/kg BBR scored significantly lower than both untreated mice or mice treated with 50 mg/kg BBR, showing that 100 and 300 mg/kg BBR-treated mice had significantly better neurophysiological outcomes (n = 6 in each group, p < 0.01, one-way ANOVA). (B) At the end of the study period, the accumulative clinical scores of the 100- and 300-mg/kg BBR groups of mice were significantly lower than those of the untreated mice and 50-mg/kg BBR groups of mice (n = 6 in each group, p < 0.01, one-way ANOVA, post hoc Student–Newman–Keuls test). (C) On 17th day, the neurophysiological function as assessed by the maximum clinical score was significantly better in the 100- and 300-mg/kg BBR groups of mice compared with the untreated or 50-mg/kg BBR group of mice (n = 6 in each group, p < 0.01, one-way ANOVA, post hoc Student–Newman–Keuls test).

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ruptured. The severity of demyelination was rated on the demyelination scale, and the EAE group scored 2.78 ± 0.47 (n = 6), which was significantly higher than that of the CFA group (1.40 ± 0.17, n = 6, p < 0.01). EAE mice treated with 50 mg/kg BBR scored significantly less demyelination and less inflammatory cell infiltration.

**Fig. 2.** Mice treated with BBR at 100 or 300 mg/kg showed significantly less demyelination and less inflammatory cell infiltration. (A) Representative LFB staining of the spine in each group of mice. The bottom panel shows high magnification (400 × ) of the images shown in the top panel. (B) The degrees of demyelination in each experimental group were assessed and expressed as the pathology score. (C) Representative H&E staining of the spine in each group of mice. The bottom panel shows high magnification (400 × ) of the images shown in the top panel. (D) Inflammatory cell infiltration in each experimental group was assessed and expressed as the pathology score. (n = 6 for each group, p < 0.01 for 100 or 300 mg/kg group vs. untreated or 50 mg/kg group, one-way ANOVA, p < 0.01 for 100 or 300 mg/kg group vs. the CFA group, one-way ANOVA).

**Fig. 3.** Treatment with BBR suppressed SphK1 in spinal astrocytes. (A) Representative immunocytochemistry images of the spine. (B) SphK1 was labeled with fluorescent dye, and its expression was assessed and expressed as the IOD. Mice treated with 100 or 300 mg/kg BBR showed a lower expression of SphK1, compared with untreated mice or mice treated with 50 mg/kg BBR (n = 6 for each group, p < 0.01; 100- or 300-mg/kg BBR group vs. the untreated or 50-mg/kg BBR group, one-way ANOVA, followed by post hoc Student–Newman–Keuls test). SphK2 expression was not different among the experimental groups (p = 0.4863). (C) The S1P level in the 100- and 300-mg/kg BBR groups were significantly lower compared with the untreated or 50-mg/kg BBR group (n = 6 in each group, p < 0.01; 100- or 300-mg/kg BBR group vs. the untreated or 50-mg/kg BBR group).
2.58 ± 0.23 (n = 6), not significantly different from that of the untreated EAE mice (p > 0.01). In contrast, the demyelination scores were 1.50 ± 0.15 (n = 6) in mice treated with 100 mg/kg BBR and 1.47 ± 0.13 (n = 6, p < 0.01) in mice treated with 300 mg/kg BBR; both of the scores were significantly lower than the untreated EAE group (p < 0.01, 100 mg/kg vs. untreated; p < 0.01, 300 mg/kg vs untreated) (Fig. 2).

In the CFA group, infiltration of inflammatory cells was not detected and not evaluated. In contrast, the EAE groups showed significant infiltration of inflammatory cells. The degree of infiltration was rated with the Okuda method in each group. The EAE group (n = 6) scored 3.07 ± 0.25, the 50-mg/kg BBR group (n = 6) scored 3.00 ± 0.07, the 100-mg/kg BBR group (n = 6) scored 2.27 ± 0.09, and the 300-mg/kg BBR group (n = 6) scored 1.90 ± 0.09. The 100-mg/kg and 300-mg/kg groups scored significantly lower than the untreated EAE group (p < 0.01, 100 mg/kg vs. untreated; p < 0.01, 300 mg/kg vs untreated) (Fig. 2).

Thus, BBR at 100 mg/kg and 300 mg/kg, but not 50 mg/kg, exhibited protective effects in EAE mice.

3.3. The effects of BBR on the SPHK1/S1P signaling pathway

Next, we examined the effects of BBR on the SPHK1/S1P signaling pathway. The level of SPHK1 was significantly higher in EAE mice when compared with the CFA group; furthermore, the level of SPHK1 was significantly lower in mice treated with 100 mg/kg or 300 mg/kg BBR when compared with the EAE mice. In contrast, the levels of SPHK2 were not significantly different among groups. In addition, the level of S1P in the EAE mice was significantly higher in the spinal tissues when compared with the CFA group; daily treatment with 50, 100, or 300 mg/kg BBR antagonized this increase (Fig. 3).

Because the animals received direct gastric administration of BBR, it is possible that the effects we observed were mediated by metabolites. In order to test this possibility, we examined the effects of BBR in cultured astrocytes. The SPHK1/S1P signaling pathway was activated by incubation with LPS. The SPHK1 protein level in the LPS-treated astrocytes was significantly higher than in the untreated astrocytes, and LPS-treated astrocytes that were also treated with 1 (n = 6, p < 0.01), 3 (n = 6, p < 0.01), or 10 (n = 6, p < 0.01) μM BBR (Fig. 4). We then measured the S1P levels and found that the S1P level measured 28.87 ± 4.85 pmol/mg astrocyte in the untreated astrocytes (n = 6) and 79.26 ± 11.89 pmol/mg astrocyte in the LPS-treated astrocytes (n = 6, p < 0.01). Moreover, in LPS-treated astrocytes that were subsequently treated with 1 μM, 3 μM, or 10 μM BBR, the S1P level measured 62.25 ± 6.58 pmol/mg astrocyte (n = 6), 46.02 ± 3.80 pmol/mg astrocyte (n = 6), and 26.87 ± 2.24 pmol/mg astrocyte (n = 6), respectively, all significantly lower than that of the LPS-treated astrocytes (p < 0.01) (Fig. 4).

4. Discussion

MS is a chronic inflammatory disease that lacks an effective
treatment to date. In the present study, we reported that BBR exhibited neuroprotective effects in a murine model of MS as evidenced by improved neurophysiological function, and reduced severity of demyelination and infiltration of inflammatory cells in the spinal cord. Our finding is consistent with previous studies that demonstrated the protective effects of BBR and improved outcomes in BBR-treated EAE mice [21,23]. However, we found that this effect was unlikely to be dose-dependent. The neurophysiological abnormalities as assessed with the clinical scale decreased significantly in EAE mice treated with 100 or 300 mg/kg BBR, whereas 50 mg/kg BBR did not show an effect. Furthermore, the effects of BBR at 100 and 300 mg/kg did not differ. Similarly, BBR at 50 mg/kg did not affect demyelination, whereas 100 and 300 mg/kg BBR partially blocked demyelination to a similar degree. These results indicate that the effect of BBR is more likely to be dichotomic. However, suppression of inflammatory cell infiltration seemed to be dose-dependent. This difference in dose-dependence will be further explored as it might have implications in the pathogenesis of MS.

Activation of the SphK1/S1P signaling pathway is a biomarker of many autoimmune diseases [10,12,28], and upregulation of SphK1 has been implicated in the pathogenesis of MS [25]. We found that SphK1 and S1P were upregulated [11,25], and that BBR blocked the upregulated expression of SphK1 and partially blocked the increase in tissue S1P level. BBR has been reported to reduce protein synthesis [30]; however, this effect of BBR is not due to a general inhibition of protein synthesis, as BBR did not affect expression of FTY720 inhibits SphK1/S1P signaling by blocking S1P receptors, FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine-1-phosphate receptor 1 (S1P1) modulation, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 751–756.


P. Dinesh, M. Rasoul, Berberine, an isoquinoline alkaloid suppresses TNXIP mediated NLRP3 inflammasome activation in MSU crystal stimulated RAW 264.7 macrophages through the upregulation of NFκB transcription factor and alleviates MSU crystal induced inflammation in rats, Int. Immunopharmacol. 44 (2017) 26–37.


