Fasudil Ameliorates Disease Progression in Experimental Autoimmune Encephalomyelitis, Acting Possibly Through Antiinflammatory Effect

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SUMMARY

Aim: The purpose of this investigation was to further explore the mechanism(s) underlying the amelioration in EAE caused by Fasudil, particularly focusing on anti-inflammatory effect. **Methods:** We induced a chronic-progressive experimental autoimmune encephalomyelitis (EAE) in B6 mice immunized with myelin oligodendrocyte glycoprotein_{35–55} and performed Fasudil intervention in early and late stages of the disease. **Results:** The administration of Fasudil (40 mg/kg, i.p) had a therapeutic effect in delaying the onset and ameliorating the severity of EAE, accompanied by the improvement in myelination and the decrease in inflammatory cells in spinal cords. Fasudil inhibited TLR-4, p-NF-κB/p65, and inflammatory cytokines (IL-1β, IL-6, and TNF-α) and enhanced IL-10 production in spinal cords. The ratio of arginase/iNOS was enhanced mainly in the spinal cords of EAE mice treated with Fasudil, reflecting a shift toward the M2 (antiinflammation) macrophage/microglia phenotype. The administration of Fasudil also induced the upregulation of CB2 receptor in spinal cords, but did not significantly trigger CB1 receptor. Levels of neurotrophic factors NGF, BDNF, and GDNF in the CNS were not altered by Fasudil. **Conclusion:** Fasudil ameliorates disease progression in EAE, acting possibly through antiinflammatory pathway.

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Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease in the central nervous system (CNS). The etiology of MS is still not known, a combination of several factors may be involved, including genetics and environment [1]. During development of MS, the involvement of CNS inflammation is associated with demyelination and axonal loss that leads to neurodegeneration [2]. In MS and experimental autoimmune encephalomyelitis (EAE), autoreactive T cells are activated in the periphery and migrated through the blood–brain barrier. Upon entering the CNS, the T cells are reactivated by antigen-presenting cells (APCs) such as infiltrating macrophages and resident microglia, in which chronic inflammation is propagated by continuous

generation of APCs and diversification of the myelin-specific T-cell response [3,4].

Rho-kinase (ROCK), a serine/threonine kinase of molecular mass 160 kDa, is activated by binding to the active GTP-bound form of the small GTPase Rho and is expressed both centrally and peripherally where it is implicated in fundamental cellular processes including migration, proliferation, and survival [5]. A series of studies have demonstrated that ROCK inhibitor Y-27632 reduced leukocyte infiltration in several models of inflammation such as ischemic heart injury [6], endotoxic liver [7], and lung injury [8]. Fasudil, a selective ROCK inhibitor, has been used to treat cerebral vasospasm [9], stroke [10], and traumatic spinal cord injury [11]. Recent studies have highlighted the potential of Fasudil in the treatment of EAE or MS.

In addition, the inhibition of ROCK enhanced oligodendrocyte precursor maturation into the oligodendrocyte lineage [12,13]. The blockade of Rho/ROCK is considered to be beneficial for CNS inflammatory demyelination [14]. Thus, targeting ROCK inhibition is a promising strategy to achieve neuroprotection, axonal regeneration, oligodendrocyte generation, and suppression of inflammation [14].

The goal of this study was to explore novel potential mechanism(s) of Fasudil in suppressing the development of EAE in mice. Here, Fasudil delayed EAE onset and ameliorated its clinical severity, accompanied by the improvement in myelination and the decrease in inflammatory foci and cells that infiltrated into the CNS during disease. Most importantly, Fasudil inhibited expression of TLR-4 and p-NF- κ B/p65 and production of inflammatory cytokines in the CNS, which may be related to the shift of macrophage/microglia phenotype from M1 to M2 and the upregulation of antiinflammatory CB2 receptor (CB₂R) in the CNS, but still remains to be determined.

Materials and Methods

Animals

Female C57BL/6 mice, 8–10 weeks old and 20–22 g, were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All experiments were conducted in accordance with the guidelines of the International Council for Laboratory Animal Science. The study was approved by the Ethics Committee of Shanxi Datong University, Datong, China. All mice were housed under pathogen-free conditions, received food and water *ad libitum*, and maintained in a reversed 12:12-h (h) light/dark cycle in a temperature-controlled room ($25 \pm 2^\circ\text{C}$) for 1 week prior to experimental manipulation.

Induction and Clinical Evaluation of EAE

Mouse myelin oligodendrocyte glycoprotein peptide_{35–55} (MOG_{35–55}, MEVGWYRSPFSRVVHLYRNGK) was produced in an automatic synthesizer (CL Bio-Scientific Company, Xian, China). The purity of the peptide was >95% as determined by HPLC.

Chronic EAE was induced by subcutaneous immunization on the upper dorsal flanks with 300 μg of MOG_{35–55} in Freund's complete adjuvant (Sigma, St. Louis, MO, USA) supplemented with 3 mg/mL of *M. Tuberculosis* H37Ra (BD Difco, Detroit, MI, USA) (400 μg /mice). Mice were injected intraperitoneally with 400 ng of pertussis toxin (Enzo Life Sciences, Ann Arbor, MI, USA) at the same time of immunization and again 48 h later. Animals were weighed and evaluated for clinical score every other day in a blinded fashion by at least two investigators. Once clinical score of EAE was 3, we gave special care, which is necessary to soften the food with water in dish, add nutrients such as egg, and put dish in the bottom of the cage, making it easy to obtain food, water and nutrition.

Clinical scores of EAE were graded according to the following criteria: 0. healthy; 1. limp tail; 2. ataxia and/or paresis of hindlimbs; 3. paralysis of hindlimbs and/or paresis of forelimbs; 4. tetraparalysis; and 5. moribund or death.

Administration of Fasudil

Fasudil (Tianjin Chase Sun Pharmaceutical Co., Ltd., Tianjin, China) dissolved in normal saline (NS) was injected intraperitoneally at 40 mg/kg/day every other day on day 3 postimmunization (p.i.) (Fasudil early treatment, $n = 23$) or at onset of clinical symptoms of EAE (Fasudil late treatment, $n = 23$) till day 27 p.i. The injection of NS was set up as control (EAE control, $n = 26$) in a similar manner.

Histology and Immunohistochemistry

On day 21 p.i., mice were anesthetized and perfused with PBS and 4% buffered paraformaldehyde. The spinal cords of EAE mice in different groups were sliced (10 μm), and the pathological changes were detected by luxol fast blue and hematoxylin/eosin (H&E) staining. For immunohistochemistry, nonspecific binding was blocked with 3% bovine serum (Serotec, Bicester, UK) and permeabilized with 0.3% Triton X-100 in 1% BSA-PBS for 30 min. The sections were incubated at 4°C overnight with anti-CD4 (1:1000; clone A60, Sigma), anti-CD68 (1:1000, Serotec), anti-CD11b (1:1000, eBioscience, San Diego, CA, USA), anti-GFAP (1:1000, Millipore, Bedford, MA, USA), and anti-p-NF- κ B/p65 (1:1,000, Cell Signaling, Boston, MA, USA), then incubated with corresponding secondary antibodies at room temperature (RT) for 2 h. As a negative control, additional sections were treated similarly, but the primary antibodies were omitted. Total white matter in luxol fast blue was manually outlined, and color photograph in H&E stain was automatically converted to black and white. Pixel area (%) of demyelination in total white matter and number of inflammatory foci (>20 mononuclear cells/focus) in whole spinal cord were calculated by Image-Pro Plus software. Six mice $\times$ 3 slides (6×3) in spinal cords were examined in a blinded fashion.

Cytokine ELISA

Mice were anesthetized and perfused with PBS only, and spinal cords were homogenized on ice with a extraction kit supplemented with protease inhibitors (Millipore) in a microcontent motor-operated tissue homogenizer (Kimble Kontes Glass Co., Vineland, NJ, USA). Lysates were centrifuged at $10,000 \times g$ for 20 min at 4°C , and protein concentration was determined by a Bradford protein assay. The levels of IL-1 β (Invitrogen Inc., Carlsbad, CA, USA), IL-6, TNF- α , and IL-10 (Pepro tech Inc., Hawthorne, NJ, USA) were measured by a sandwich ELISA kits following the manufacturer's instructions. Determinations were performed in duplicate in three independent experiments. The results were expressed as pg/10 mg protein.

Western Blot Analysis

Mice were anesthetized and perfused with PBS only, proteins from brains and spinal cords were extracted using the above-mentioned protocol. Extract of proteins (30 μg) was separated by SDS-PAGE and electroblotted onto nitrocellulose membrane (Immobilon-P, Millipore). After nonspecific antibody binding was blocked with 5% nonfat dry milk, membranes were incubated at 4°C overnight with anti-iNOS (1:200, Cayman Chemicals Com-

pany, Ann Arbor, MI, USA), anti-Arginase-1 (Arg-1) (1:300, Cayman Chemicals Company), anti-CB₁R (1:500, Cayman Chemicals Company), anti-CB₂R (1:500, Cayman Chemicals Company), anti-TLR-4 (1:2,000, Eptomics Inc., Burlingame, CA, USA), anti-p-NF- κ B/p65, and anti-GAPDH (1:1,000, Eptomics). After washing in TBST, the immunoblots were incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) at RT for 1 h. The immunoblots were developed with an enhanced chemiluminescence (ECL) system (GE Healthcare Life Sciences, Niskayuna, NY, USA) and measured with Quantity Software (Bio-rad, Hercules, CA, USA). To compare protein loading, antibody directed against GAPDH was used.

Statistical Analysis

All the experiments were repeated two or three times, and GraphPad Prism software was used for statistical analysis. One-way ANOVA, where applicable, was performed to determine whether an overall statistically significant change existed before Student's *t*-test to analyze the difference between any two groups. Correlation was analyzed with Spearman rank correlation analysis. A statistically significant difference was assumed at $P < 0.05$.

Results

Fasudil Delays Onset and Ameliorates Severity of EAE

To observe the therapeutic effect of Fasudil in induction and onset phases of EAE, Fasudil was injected intraperitoneally on day 3 p.i. or at onset of clinical symptoms of EAE. As shown in Supplement 1 and Figure 1(A), the incidence of EAE (8/23, 34.7%) in Fasudil early-treated mice was decreased as compared with EAE control (25/26, 96.1%) and Fasudil late-treated mice (18/23, 78.3%). In EAE group, mean onset date was 12.76 ± 1.86 , mean maximum score was 2.41 ± 1.20 . The treatment of Fasudil at onset phase of

EAE slightly delayed onset (14.00 ± 2.09 , $P < 0.05$) and declined maximum clinical score (1.35 ± 0.98 , $P < 0.01$). Obviously, the administration of Fasudil in induction phase of EAE is able to delay its onset (16.50 ± 3.16 , $P < 0.01$) and declined maximum clinical score (0.45 ± 0.78 , $P < 0.001$). There is a relationship between loss of body weight and severity of clinical score during the development of EAE. The treatment of Fasudil at induction or onset phases of EAE had less body weight loss as compared with that of EAE mice (Figure 1B). In our experiments, no mortality was observed most likely due to our special care in mice with clinical score 3.

Fasudil Inhibits Inflammation and Improves Myelination

We evaluated the pathology of CNS inflammation and demyelination. As shown in Figure 2(A) and (B), mice with EAE showed extensive myelin loss (demyelination) associated with infiltration of immune cells (inflammation) in spinal cord. When compared with EAE control, Fasudil-treated mice had a significant improvement in the extent of demyelination (Figure 2A, $P < 0.01$ as compared with late and early treatment, respectively) and inflammation (Figure 2B, $P < 0.05$ and 0.01 as compared with late and early treatment, respectively) in spinal cords.

As shown in Figure 2(C), mice with EAE showed extensive infiltration of inflammatory cells (CD4+ T cells and CD68+ macrophages) and relative activation of microglia and astrocytes (CD68+ microglia and GFAP+ astrocytes) in spinal cords. The ability of Fasudil to reduce the formation of inflammatory foci in spinal cords (Figure 2B) was paralleled by a similar reduction in the infiltration of CD4 T cells and macrophage as well as the activation of microglia and astrocytes (Figure 2C).

Fasudil Inhibits the Inflammatory Molecules

The therapeutic effect of Fasudil in EAE prompted us to investigate its possible mechanisms and to identify the target molecules

Figure 1 Fasudil delays onset and ameliorates severity of EAE. Fasudil was injected (intraperitoneally) at 40 mg/kg/day every other day on day 3 p.i. (Fasudil early treatment, $n = 23$) or at onset of clinical symptoms of EAE (Fasudil late treatment, $n = 23$). The injection of normal saline was set up as control (EAE control, $n = 26$) in a similar manner. (A) clinical score of EAE mice treated with Fasudil early or late treatment. (B) body weight of EAE mice treated with Fasudil early and late treatment. * $P < 0.05$; ** $P < 0.01$.

Figure 2 Fasudil inhibits inflammation and improves myelination in spinal cord. On day 21 p.i., spinal cords were used for luxol fast blue, H&E and immunohistochemistry staining. **(A)** Demyelination stained with luxol fast blue. Total white matter in luxol fast blue was manually outlined, and pixel area (%) of demyelination in total white matter was calculated by Image-Pro Plus software. **(B)** Inflammation stained with H&E stain. Color photograph in H&E stain was automatically converted to black and white, and a number of inflammatory foci (>20 mononuclear cells/focus) in whole spinal cord were calculated by Image-Pro Plus software. **(C)** CD4 T cells, CD68 macrophage/microglia and GFAP astrocytes. Quantitative results are analyzed for 6 mice in each group and are representative of three experiments with similar results. * $P < 0.05$, ** $P < 0.01$.

through which Fasudil ameliorates the severity of EAE. It is postulated that inflammation may be primed via TLR-4 that stimulates NF- κ B activation, which may contribute to inflammatory micro-environment [15]. We first measured the expression of TLR-4 in brains and spinal cords by Western blot. The expression of TLR-4 in spinal cords was obviously higher than that of brains (Figure 3A). Fasudil treatment inhibited the expression of TLR-4 in brains ($P < 0.05$, vs. Fasudil early treatment) and spinal cords ($P < 0.05$, EAE vs. Fasudil early and late treatment), especially in spinal cords (Figure 3A). These data provide strong evidence that Fasudil directly inhibits the expression of TLR-4 as compared with EAE control mice.

Accumulating evidence demonstrates that TLR-4–NF- κ B signaling contributes to inflammatory responses [16,17]. We next examined the expression of p-NF- κ B/p65 in brain and spinal cord of EAE, demonstrating that expression of NF- κ B/p65 in spinal cords was obviously higher than that of brains (Figure 3A). Fasudil

treatment significantly inhibited the expression of p-NF- κ B/p65 in brains and spinal cords of mice treated with Fasudil in both late- and early-treated groups (Figure 3A, $P < 0.05$, respectively).

Besides NF- κ B, other transcription factor AP-1 is known to affect inflammatory cascades, manifested by the ample production of inflammatory mediators. Figure 3(A) shows that the expression of AP-1 in spinal cords was inhibited in mice treated with Fasudil in both late- or early-treated groups as compared with EAE control ($P < 0.05$, respectively).

Because Fasudil has been shown to suppress p-NF- κ B/p65 in the CNS (mainly in spinal cord), we next measured the levels of proinflammatory molecules such as IL-1 β , IL-6, and TNF- α among three groups. As expected, the results showed that IL-1 β , IL-6, and TNF- α were suppressed in spinal cords from mice treated with Fasudil in late- or early-treated groups (Figure 3(B), $P < 0.05$ and 0.001, respectively). In parallel, the levels of IL-10 were enhanced

Figure 3 Fasudil inhibits inflammatory molecules. On day 28 p.i., brains and spinal cords were collected, and proteins were extracted by protein extraction kit. **(A)** Representative bands of Western blot for TLR-4, p-NF-kB/p65, and AP-1 in brains and spinal cords by Western blot. Quantitative analysis of TLR-4, p-NF-kB/p65, and AP-1 was performed from 5 to 6 mice in each group. **(B)** The levels of IL-1 β , IL-6, TNF- α , and IL-10 in spinal cords by ELISA. Quantitative analysis was performed from 5 to 6 mice in each group. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

in spinal cord of EAE treated with Fasudil early treatment (Figure 3B, $P < 0.05$).

We further examined the cellular localization of NF expression in spinal cord, finding that p-NF-kB/p65 was observed in both white matter and gray matter (Figure 4). However, p-NF-kB/p65 was expressed mainly in white matter after Fasudil treatment (Figure 4). Double immunohistochemistry showed that astrocytes, but not microglia, overlap with p-NF-kB/p65 (Figure 4), suggesting that astrocytes are main cell source of p-NF-kB/p65 in EAE. Fasudil treatment (late and early) inhibited p-NF-kB/p65 in astrocytes as compared with EAE control (Figure 4).

Fasudil shifts M1 to M2 phenotype

The classical M1 polarization of macrophage/microglia has been linked with promoting inflammation, whereas M2 phenotype is antiinflammatory and promotes tissue repair [18]. Arg-1 is

responsible for metabolism of arginine to ornithine and polyamines in M2 macrophages, thereby diminishing expression of iNOS by M1 cells [19]. Fasudil inhibited the expression of iNOS in spinal cords (Figure 5A and B) and elevated the expression of Arg-1 in mice treated with Fasudil in both late- or early-treated groups (Figure 5A and C). The ratio of Arg-1/iNOS was significantly enhanced as compared with EAE control (Figure 5D), indicating an obvious shift toward an M2 macrophage/macrophage phenotype, which is consistent with the decrease in inflammatory cytokines as described earlier.

Fasudil Induces the Expression of CB2 Receptor, not CB1 Receptor

Cannabinoids are involved in neuroprotection via NF-kB inhibition [20]. Thus, we detected the changes of CB₁R and CB₂R among three groups. After Fasudil early treatment, the expression

Figure 4 Expression and location of p-NF-kB/p65 in spinal cord. On day 28 p.i., slices from spinal cords were stained with anti-p-NF-kB/p65, anti-GFAP, and anti-CD11b. In EAE mice, p-NF-kB/p65 was observed in both white matter and gray matter. Double immunohistochemistry showed that astrocytes, but not microglia, overlap with p-NF-kB/p65.

of CB₂R was upregulated in spinal cords (Figure 6A and C), but the expression of CB₂R did not exhibit significant difference in Fasudil late treatment compared with EAE control. In brains, we did not find significant difference on CB₂R among three groups (Figure 6A and C). As for CB₁R, we did not find any significant differences in brains and spinal cords among three groups (Figure 6A and B).

Fasudil Did Not Trigger The Production of Neurotrophic Factors

It has been demonstrated that NF- κ B is associated with the pathophysiology of ischemic/traumatic injuries and neurodegenerative diseases, where it modulates the expression of target genes involved in cell growth, survival, adhesion, and death [21]. We finally observed the changes in neurotrophic factors in mice treated with Fasudil, in which NF- κ B was inhibited. The results indicate that the levels of NGF, BDNF, and GDNF from brains and spinal cords of mice treated with Fasudil in both late- or early-treated groups were not altered by Fasudil (Supplement 2). It is possible that the regulation of these neurotrophic factors is mediated possibly via indirect mechanisms.

Discussion

In the present study, Fasudil delayed the onset of EAE and ameliorated the severity of symptoms when administered in induction or onset phases of EAE, but better therapeutic effect could be observed at the initial phase of EAE (on day 3 p.i.), which represents the immunological incipience of the disease. Consistent with previous studies, Fasudil inhibited the migration of inflammatory cells into the CNS. It has been reported that ROCK is required for efficient T-cell polarization and migration on endothelial cells and transendothelial migration by affecting both actomyosin contractility and microtubules stability [22,23].

Figure 5 Fasudil shifts M1 to M2 phenotype. On day 28 p.i., brains and spinal cords were collected, and proteins were extracted for Western blot. (A) Representative bands of Western blot for iNOS and arginase in brains and spinal cords, (B) quantitative analysis of iNOS, (C) quantitative analysis of arginase, and (D) quantitative analysis of arginase/iNOS ratio. * $P < 0.05$.

Figure 6 Fasudil enhances the expression of CB₂R in brains and spinal cords. On day 28 p.i., brains and spinal cords were collected, and proteins were extracted for Western blot. (A) Representative bands of Western blot for CB₁R and CB₂R in brains and spinal cords, (B) quantitative analysis of CB₁R, and (C) quantitative analysis of CB₂R. Quantitative analysis was performed from 5 to 6 mice in each group. **P* < 0.05.

Toll like receptors (TLRs) are transmembrane proteins that play an important role in mediating inflammatory response. In this sense, TLR-4 is inducible and associated with downstream NF- κ B activation and proinflammatory cytokine release, which may contribute to neuronal death [13,24]. Constitutive activity of NF- κ B in myeloid cells drives the pathogenicity of monocytes and macrophages during EAE [25]. Our results show the inhibition and the relationship of TLR-4 and p-NF- κ B/p65 in EAE treated with Fasudil. NF- κ B is an essential element of the TLR-mediated response in cells of immune system. In macrophages, one of the physiological consequences of TLR stimulation is the release of inflammatory cytokines such as TNF, IL-1 β , and IL-6 [26]. In spinal cords, Fasudil treatment inhibited the production of these inflammatory cytokines possibly through TLR-4 and/or NF- κ B suppression. Although the relationship of TLR-4 and NF- κ B in other groups has been confirmed, the TLR-4 and NF- κ B pathway in this study is still need to be defined.

Encephalomyelitis is initiated through the peripheral activation and proliferation of myelin-specific CD4 T cells, which then migrate into the CNS. Once inside, microglia are activated, and a large number of macrophages are recruited from the circulating blood, resulting in CNS inflammation [27,28]. In recent years, a population of myeloid cells suppressing immune responses has been described [29–31]. These cells mainly exert their functions through iNOS and/or arginase activity [32,33]. In TLR-4 deficient mice, arginase activity in response to *L. major* is enhanced, implicating that TLR-4 negatively controls arginase induction [34]. Arginase⁺-cells were detected exclusively when the switch from proinflammatory to antiinflammatory conditions occurs, limiting the immune response [33,35]. Arginase⁺-myeloid-derived cells from the spinal cords of EAE limit the inflammation by promoting T-cell apoptosis [36]. Our data show that ratio of arginase/iNOS is enhanced in mice treated with Fasudil, which may be differentially regulated by TLR-4/NF- κ B pathway. In turn, the upregulation of arginase significantly decreased macrophage infiltration and inflammation, whereas downregulation of arginase aggravated these adverse effects [37]. In addition, cytokines can also influence the balance between arginase and iNOS in

inflammation, whereas Th 1 cytokine, IFN- γ , induces iNOS, the Th2 cytokines IL-4 and IL-10 induce arginase [38]. Based on previous studies and our results, we postulate that the functional ramification of arginase and NOS induction and their reciprocal regulation likely depend on the disease process, TLR4 and/or NF- κ B signaling, and cytokine milieu.

After many years of continuous research, it is becoming more evident that cannabinoid compounds and the endocannabinoid signaling system offer a promising novel form of therapy for MS. This appears reasonable that, in addition to the potential of CB₂R in antiinflammatory effect, CB₁R may also represent an important protective target able to improve neuronal damage in the progression of MS [20,39]. CB₂R knockout mice exacerbated EAE symptoms, which was associated with CD4⁺ T-cell infiltration and myeloid cell recruitment into the inflamed CNS, whereas the opposite action was observed upon CB₂R-selective agonist administration [39]. A selective pharmacological CB₂R agonist HU-308 improved EAE symptoms and reduced spinal cord lesions and microglial activation [40]. CB₂R stimulation suppressed the proliferation and attenuated proinflammatory cytokine production [41–43], indicating that CB₂R reduces inflammation and could be beneficial for the treatment of MS [44]. Studies in CB₁R knockout mice showed more rapid development of neurodegeneration during EAE [39,45], raising the possibility that neurotrophic factors and CBR interact to regulate multiple functions in the brain. Neuroprotective effects driven by CB₁R may involve control of excitotoxic glutamate activity and toxic ion influxes, antioxidant properties and neurotrophic factors [39,46,47]. The level of BDNF was decreased in the brain of mice lacking CB₁R [48], whereas activation of these receptors increased BDNF production in rodents [49] and in humans [50]. Furthermore, BDNF release triggered by CB₁R stimulation mediated the neuroprotective effects [51]. It is by now established that MS is not simply an autoimmune disease, and that in addition to inflammation and demyelination, axonal injury and neuronal loss underlie the accumulation of disability and the disease progression. Thus, we try to explore whether Fasudil-mediated amelioration of clinical score in EAE is related with neurotrophic factors. In this study,

Fasudil early treatment upregulated the expression of CB₂R in spinal cords. However, the role of CB₂R in Fasudil-treated mice still needs further exploration.

In conclusion, Fasudil delays the onset and ameliorates the severity in different phases of EAE, accompanied by the improvement in myelination and the inhibition of inflammatory responses in the CNS. Fasudil suppresses CNS inflammatory responses possibly through inhibiting TLR-4 and/or NF- κ B activation, which may be related to the shift of macrophage/microglia phenotype from M1 to M2 and the upregulation of neuroprotective CB₂R in the CNS, but still remains to be determined.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The clinical evaluation of EAE mice.

Fig S1. Fasudil did not influences the levels of neurotrophic factors.

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