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2-BFI attenuates experimental autoimmune encephalomyelitis-induced spinal cord injury with enhanced B-CK, CaATPase, but reduced calpain activity

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ABSTRACT

The lack of disease-modifying pharmacological agents for effective treatment of multiple sclerosis (MS) still represents a large and urgent unmet medical need. Our previous studies showed that ligands to type 2 imidazoline receptors (I_2R) were effective in protecting spinal cord injury caused by experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. In this study, we further examined the protective property of a very selective ligand of I_2R, 2-(2-benzofuranyl) 2-imidazoline (2-BFI) against EAE. Importantly, a mechanism of 2-BFI-mediated protection was investigated which possibly involves an I_2R binding protein, brain-creatine kinase (B-CK), as well as CaATPase and calpain. The enzymatic activity of B-CK and CaATPase was significantly reduced in EAE injured spinal cord. Reduction of B-CK activity in EAE spinal cord may lead to energy reduction and dysfunction in cellular calcium homeostasis. Increased intracellular calcium evokes elevation of calpain activity occurring in EAE spinal cord which causes further tissue damage. Indeed, EAE injured spinal cord showed significant reduction in CaATPase and increase calpain activities. Remarkably, spinal cord tissue from mice treated daily with 2-BFI during the progression of EAE significantly restored B-CK and CaATPase enzymatic activities and showed no induction in calpain activity. Moreover, EAE spinal cord from 2-BFI treated mice also demonstrated better preservation of myelin; reduced axonal injury, as evidenced by the lower level of β-APP expression, and above all, highly improved neurobehavioral scores ($p < 0.01$; $n = 10$). These findings suggest that 2-BFI can be further developed as a therapeutic drug for MS treatment.

1. Introduction

The lack of disease-modifying pharmacological agents effective for multiple sclerosis (MS) treatment represents a large and urgent unmet medical need. As such, management of MS symptoms appears to be the only common clinical practice for MS treatment [25]. MS is a chronic inflammatory autoimmune disease of the central nervous system [12,25]. Mechanistically, the interplay between susceptibility genes and environmental factors contributes to the pathogenesis of MS [21]. Experimental autoimmune encephalomyelitis (EAE) in rat or mouse is widely used as an animal model for MS [4]. This animal model, induced by immunizing the rat or mouse with myelin oligodendrocyte glycoprotein (MOG_{33-55}), not only produces histopathological, structural and clinical features similar to those of the human disease, but more importantly, it exhibits a similar profile of disturbances for many biochemical processes, including changes in ion homeostasis, mitochondrial dysfunction, and perturbation of neurotransmitter-related enzymes [3,8]. Understanding the disturbances of these biochemical events may shed light on mechanisms for MS pathogenesis.

Brain-creatine kinase (B-CK) is a key enzyme in brain cellular energy production. Through converting phosphocreatine to creatine, B-CK mediates the process to generate ATP as a cellular energy source. Energy produced is used for the maintenance of cellular ion homeostasis, such as through CaATPase to maintain the balance of intracellular Ca^{2+}, which is critical for normal neuronal function [1]. Specifically, B-CK has been shown to be expressed at the same time as myelin basic protein during cerebellum development indicating its involvement in myelination [23]. CaATPase, such as the plasma membrane calcium ATPase isoform 2 (PMCA2), plays an important role in removing intracellular calcium and modulating vesicular exocytosis. These processes are critical in maintaining calcium homeostasis and normal neuronal function. Reduction in PMCA2 activity has been shown during the pathogenesis of EAE and MS, which may cause delays in calcium clearance resulting in neuronal damage [2]. In fact, PMCA2 is essential for the survival and proper function of spinal cord neurons, especially motor neurons [20]. Disturbance of calcium removal and increasing intracellular calcium evoke calcium-dependent proteases, such

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as calpain, to breakdown key intracellular structural proteins, thereby causing neuronal death.

Recent studies identified B-CK as the binding protein of type 2 imidazoline receptors (I_2R). Molecular modeling revealed the existence of an I_2 binding site within this enzyme [18]. Furthermore, 2-(2-benzofuranyl)2-imidazoline (2-BFI), a very selective ligand to the I_2 site and is abundantly expressed in the brain, binds to B-CK with high specificity [18]. These interesting studies led us to believe that our recent findings where Idazoxan, another ligand to I_2R, was effective in attenuating EAE-induced spinal cord injury [26], transient cerebral ischemia [6], and excitotoxicity [16], may indeed involve B-CK. To this end, we investigated B-CK, CaATPase and calpain in the pathogenesis of EAE. We show that 2-BFI treatment provided potent protection against EAE-induced neuronal injury, possibility through enhanced activities of B-CK and CaATPase, as well as reduced activation of calpain activity.

2. Materials and methods

2.1. Mouse model of EAE

All animal procedures were conducted following an institutionally approved protocol in accordance with guidelines set by the National Institutes of Health Guideline for the Care and Use of Laboratory Animals. EAE was induced by immunizing 6–8 week old female C57BL/6 mice with MOG_{35-55} emulsified in incomplete Freund's adjuvant (CFA) supplemented with 8 mg/ml mycobacterium tuberculosis H37Ra (MOG_{35-55}-CFA). Each mouse received a subcutaneous injection in the flanks with 200 μg MOG_{35-55}-CFA (Sigma–Aldrich). In addition, we administered 200 ng pertussis toxin (Sigma–Aldrich) intravenously on the initial day as well as the second day of immunization. Both body weight and clinical signs of disease were examined daily.

2.2. Drug treatment

A total of 25 mice were randomly assigned to three treatment groups as follows: (1) saline–saline group (control group, $n = 5$); saline was injected into the mice as a negative control; (2) EAE-saline group (control group, $n = 10$). Mice were treated with saline immediately after the MOG_{35-55}-CFA immunization; and (3) EAE-2BFI group (experimental group, $n = 10$). Mice were treated daily with 2-BFI (20 mg/kg body weight) via i.p. for 14 days twice daily immediately after the initial MOG_{35-55}-CFA immunization. The dose of 2-BFI was selected empirically based on previous studies showing protective effect from our own laboratory.

2.3. Behavioral evaluation for EAE

The day of MOG_{35-55}-CFA immunization was marked as day 0 dpi. We monitored the mice for neurological deficits and scored these deficits twice a day until the time the animal was killed. The following scoring system for neurological deficits was used: (1) tail signs: 0 = no signs; 1 = half paralyzed tail; 2 = complete limp tail; (2) signs from the hind- and forelimbs (each assessed separately): 0 = no signs; 1 = a weak or altered gait; 2 = paresis; 3 = a fully paralyzed limb. The sum of the state of the tail and all of the four limbs at three levels was from 0 to 15. Thus, a fully paralyzed quadriplegic mouse would attain a score of 14. Mortality equals a score of 15. Mice were killed after the appearance of a peak in neurological deficits for histological and biochemical analysis.

2.4. Spinal cord tissue fixation and sectioning

Mice were sacrificed and spinal cords were dissected out. Isolated cervical cords were immediately frozen in liquid nitrogen, and then stored at −80 °C for further analysis. The rest of the spinal cord was fixed in 4% paraformaldehyde and finally embedded in paraffin wax for standard tissue sectioning as previously described [6,26]. Ten micrometer thickness sections were cut and mounted on pre-leaned glass slides.

2.5. Histochemical and immunostaining of tissue sections and their analysis

2.5.1. H&E staining

The sections were stained with hematoxylin and eosin using a previously published protocol [6,26]. The level of infiltration of inflammatory cells were semi-quantified. Specifically, the severity of inflammatory cell infiltration was assessed as 0 = no inflammation; 1 = cellular infiltrates only around blood vessels; 2 = mild cellular infiltrates in parenchyma (1–10 cells/section); 3 = moderate cellular infiltrates in parenchyma (11–100 cells/section); and 4 = severe cellular infiltrates in parenchyma (>100 cells/section).

2.5.2. Luxol fast blue staining for myelin

Spinal cord sections were stained with Luxol fast blue to detect myelin damage using a previously published protocol [17]. Briefly, after a defatting step, sections were left in Luxol fast blue solution in a 56 °C oven overnight (14 h). After a rinse with 95% ethanol and distilled water to remove excess stain, the slides were differentiated in lithium carbonate solution for 30 s and then in 70% ethyl alcohol for 30 s. The slides were then rinsed in distilled water. Differentiation was verified under microscope to ensure that myelin was sharply stained. Sections were then mounted for examination under a light microscope.

2.5.3. Immunostaining of β-APP and calpain

The protocol for immunostaining was used exactly according previously published method [6,14]. Either rabbit anti-mouse β-APP antibody (Wuhan Boster Bio-engineering Co. LTD., PR China, 1:200 dilution), calpain I (Santa Cruz, USA), or calpain II (Abcam, USA) was used. Sections were reacted with ABC reagent and diaminobenzidine for visualization. As a negative control, sections were stained without the presence of primary or secondary antibodies. Sections were examined under a light microscope blinding the treatment of the sample to the observer.

2.6. Analysis of enzymatic activity of B-CK, CaATPase and calpain

Total protein from the cervical segment of the spinal cord was isolated to detect the enzymatic activities of B-CK, Ca^{2+}ATPase and calpain using commercial kits from Shanghai Jiemei GenMed Co. Ltd. (PR China) and Nanjing Jiancheng Bio-engineering Institute. To determine the B-CK enzyme activity, 100 mg of tissue was lysed and centrifuged exactly according to the manufacture's protocol. The intensity of B-CK activity was finally measured using a spectrophotometer. To measure the activities of CaATPase, tissue homogenates were centrifuged using a cold bench-top centrifuge. The supernatant went through a G250 column. The appropriate reagent supplied from the commercial kit was added and the final reaction was measured in a plate reader. Calpain activity was measured exactly as previously described [14,16].

2.7. Image analysis

To quantify the expression levels of myelin, images of Luxol fast blue staining were taken under identical fluorescent intensity settings. Areas of the spinal cord were selected on digitized grayscale images with resolutions of 1300 × 1030 pixels. Intensity was measured using NIH Image J software (http://rsb.info.nih.gov/ij/). At least four measurements were taken from non-overlapping areas.

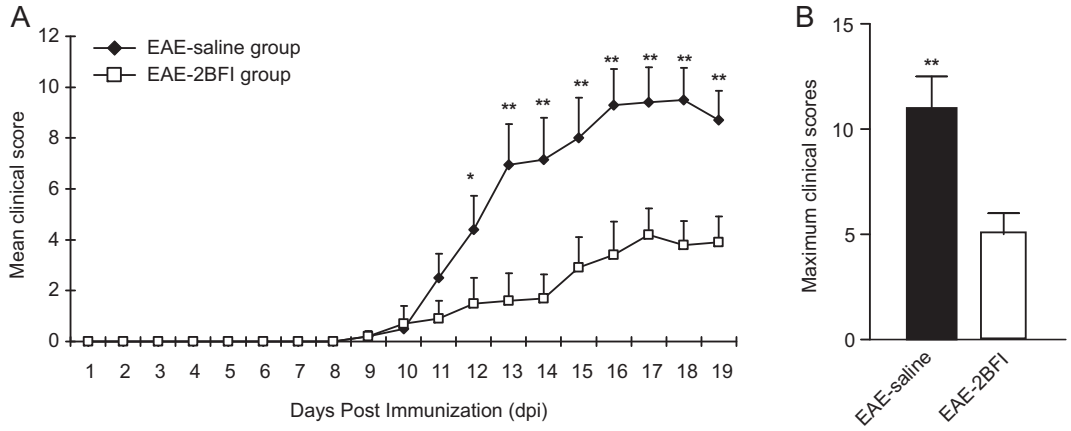

Fig. 1. Treatment with 2-BFI ameliorates neurological deficit in EAE mice. Mice (n = 10 per group) were treated daily with 2-BFI (20 mg/kg, i.p.) for 14 days twice daily. Neurological deficit (clinical scores) was measured twice daily. (A) Comparison of the mean clinical scores between mice in the EAE-saline (n = 10) and the EAE-2BFI (n = 10) groups after immunization with MOG_{33-35}. (B) A histogram comparing the maximum clinical scores between the EAE-saline and the EAE-2BFI groups. Data represents mean ± S.E.M with $*$ and $**$ indicating $p < 0.05$ and $p < 0.01$, respectively.

The average intensity was calculated using Prism 3.0 as previously described [10]. To quantify β-APP and calpain expression, the number of positive cells was counted from at least four areas on the spinal cord section under 20× magnifications and plotted using Prism 3.0.

2.8. Statistical analysis

Data was analyzed using the SPSS statistical program. All values were expressed as Means ± S.E.M. Statistical significance was determined using two-tailed Student's t-test for two groups, or

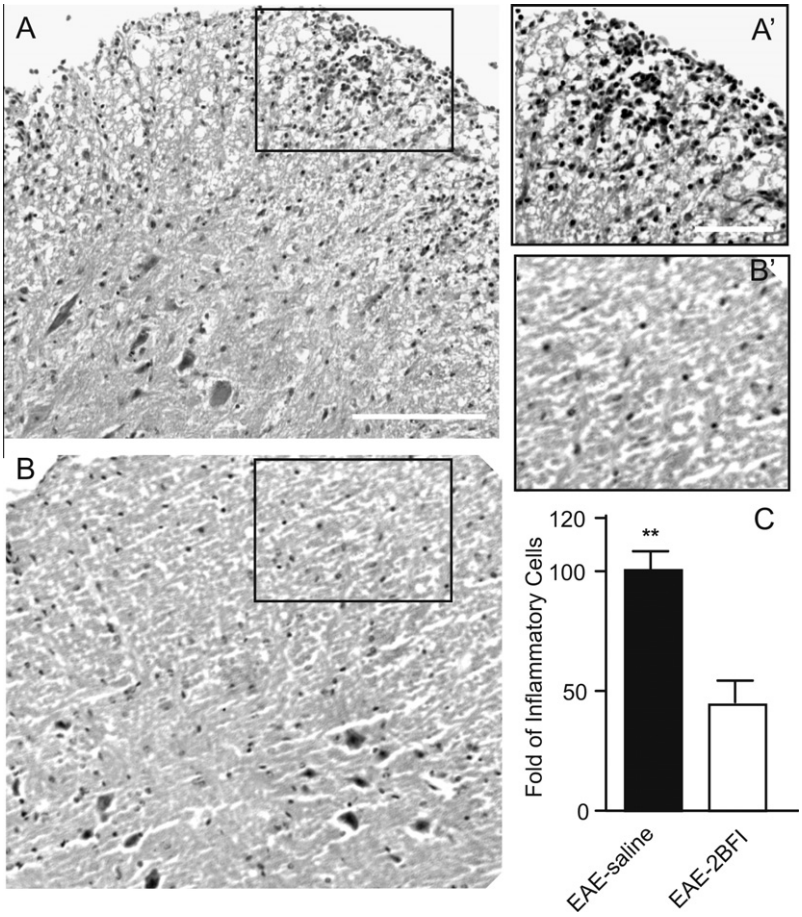

Fig. 2. Reduced inflammatory response in the spinal cord of 2-BFI treated EAE mice. Mice were treated twice daily with either 2-BFI (20 mg/kg body weight) or saline for 14 days, immediately following immunization with MOG_{33-35} as described in the methods section. Animals were sacrificed at 19 dpi. The cervical segment of the spinal cord was sectioned and stained with H&E to compare the severity of infiltration of inflammatory cells between the EAE-saline (panel A and the boxed area is highlighted in panel A′) and the EAE-2BFI (panel B and the boxed area is highlighted in panel B′) groups. The degree of inflammatory cell infiltration in the spinal cord was semi-quantified and the fold of change was presented in panel C. Scale bar = 50 μm.

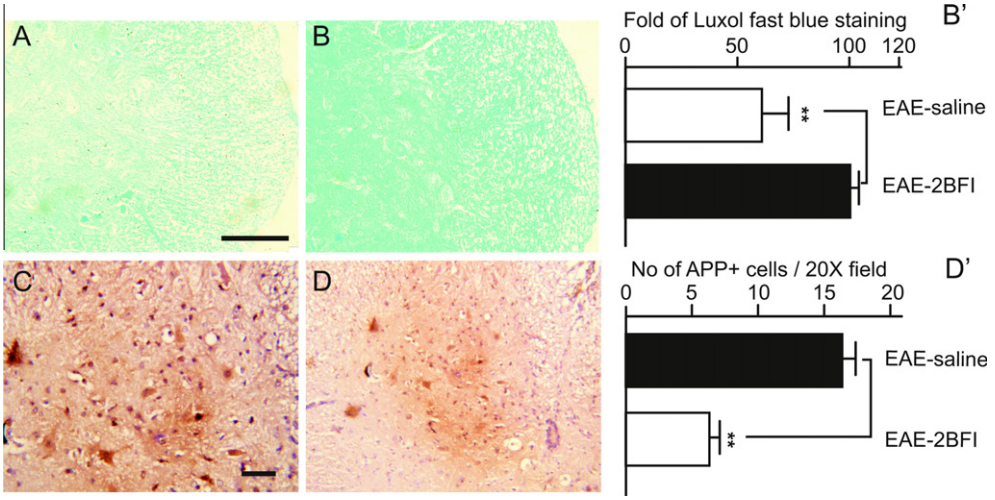

Fig. 3. Preservation of myelin and reduction in axonal injury by 2-BFI treatment. Luxol fast blue staining was performed on spinal cord sections to compare the dramatically reduced myelin staining in the EAE-saline group (A) with the much better preserved myelin in 2-BFI treated EAE-2-BFI group mice (B). The intensity of Luxol fast blue staining from digitized images was converted to grayscale and quantified using Image J (B′). Immunostaining was performed also on the adjacent sections to detect increased β-APP expression (C and D). The number of β-APP positive cells were counted from selected 20× microscopic field and plotted in panel D'. Data represents mean ± S.E.M with ** indicating $p < 0.01$. Scale bar = 50 μm.

by ANOVA with *post hoc* multiple comparison analysis for multiple groups. * and ** indicate statistical significant with $p < 0.05$ and $p < 0.01$, respectively.

3. Results

3.1. 2-BFI attenuates EAE-induced morbidity and neurobehavioral deficits in mice

Mice immunized with MOG_{33-35} began to display neurobehavioral deficit starting at 8 days post-immunization (dpi). The level of exploratory activities and feeding frequencies were reduced resulting in the loss of body weight. At 9 dpi, mice started to show signs of the onset of EAE characterized by the appearance of a flaccid tail. These symptoms became gradually worse after 10 dpi leading to the complete paralysis, tetraplegia, and even moribund (Fig. 1A). Clinical signs were evaluated and scored which showed a significant difference at 13 dpi compared with the 12 dpi mice (Fig. 1A). The escalation of clinical signs of EAE lasted for another 4–5 days before the clinical scores gradual declined at 19 dpi (Fig. 1A).

Amongst the 10 mice assigned to the EAE-saline group (control group, $n = 10$), nine of them displayed clinical signs of EAE as shown in Fig. 1A. On the other hand, none of the mice developed any clinical signs of EAE in the saline–saline group ($n = 5$), serving as a background control (not shown). Importantly, the EAE-2BFI group (experimental group; $n = 10$) showed a significant reduction in the intensity of clinical scores of EAE after 13 dpi compared with the EAE-saline group. Furthermore, the maximum clinical score in EAE-2BFI group was also significantly lower compared with that of the EAE-saline group (Fig. 1B), strongly suggesting that 2-BFI conferred protection to MOG_{33-35}-induced EAE.

3.2. 2-BFI reduces inflammatory cell infiltration, demyelination and axonal injury in the spinal cord of EAE mice

The effect of twice daily treatment with 2-BFI on reducing EAE-induced spinal cord injury was further evaluated in terms of infiltration of inflammatory cells, the degree of demyelination and axonal injury in 14 dpi mice. Infiltration of inflammatory cells in the spinal cord parenchyma and perivascular cuffing with mononuclear cells have previously been shown to be typical pathological signs of EAE. H&E staining showed that the saline–saline group showed no inflammatory cell infiltration (data not shown); while in contrast, EAE-saline group had almost 100% occurrence of infiltration of inflammatory cells in the injured spinal cord (Fig. 2A and A′), consistent with the MOG_{33-35}-induced pathology. Spinal cord sections from mice that were treated twice daily with 2-BFI showed significantly reduced numbers of infiltrating inflammatory cells when compared with the EAE-saline group (Fig. 2B, B' and C).

The severity of demyelination was evaluated using Luxol fast blue staining (Fig. 3A and B). In the EAE-saline group, demyelination was shown to be the most extensive around the site of inflammatory cell infiltration in the injured spinal cords. The intensity of Luxol fast blue staining was significantly reduced due to the loss of myelin content (Fig. 3A and B'). In contrast, spinal cord sections from mice treated twice daily for 14 days with 2-BFI after MOG_{33-35} immunization showed much improved preservation of Luxol staining indicating the reduction in demyelination (Fig. 3B and B').

Axonal injury was determined using immunostaining to detect increased expression of β-Amyloid Precursor Protein (β-APP). β-APP deposits increase in axons after focal blockage of axonal transport during injury, and elevation of β-APP has been widely used as a marker for axonal injury. As shown in Fig. 3C and D, β-APP staining was strong and clear in the spinal cord section from the EAE-saline group (Fig. 3C). Interestingly, the spinal cord section from mice treated with 2-BFI (EAE-2BFI group) showed reduced β-APP immunostaining and the number of β-APP positive cells (panel D'), strongly supporting the suggestion that 2-BFI treatment conferred neuroprotection to the spinal cord during EAE.

3.3. Treatment with 2-BFI restores B-CK and CaATPase activities in EAE mouse spinal cord

In order to better understand the molecular mechanism of 2-BFI-mediated protection to EAE-induced spinal cord injury, we examined the activities of B-CK and CaATPase in the spinal cord from mice treated twice daily with 2-BFI for 14 days during the course of development of EAE. As shown in Fig. 4A and B, the EAE-saline group demonstrated significantly reduced B-CK (Fig. 4A) and CaATPase (Fig. 4B) enzymatic activities; while in

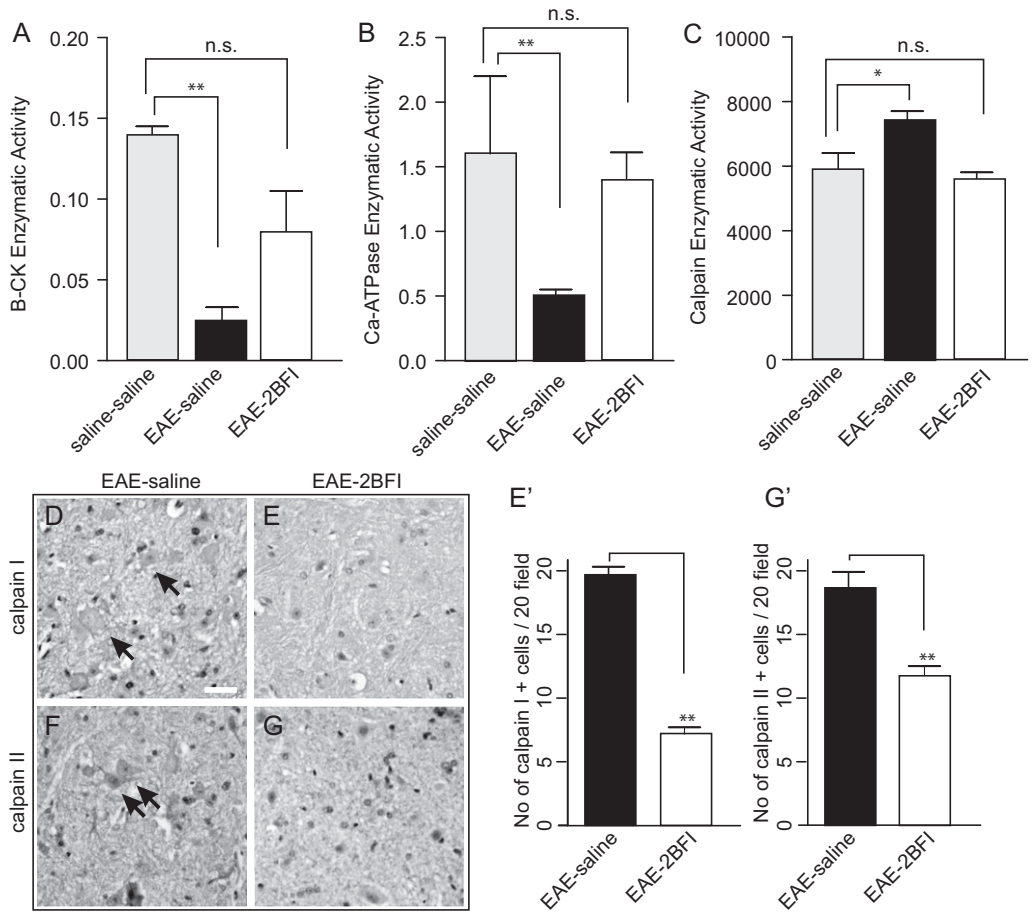

Fig. 4. Altered enzymatic activities of B-CK, CaATPase and calpain in response to 2-BFI treatment in EAE mice. Mice were treated twice daily with 2-BFI for 14 days immediately following immunization with MOG_{35-55} as described in the methods section and killed at 19 dpi. Spinal cords from the cervical segment were lysed and proteins were subjected to an enzymatic activity assay using commercial kits to determine B-CK (A), CaATPase (B) and calpain (C) activities. Spinal cord transverse sections were also immunostained with antibodies to calpain I (D and E) and calpain II (F and G) which detect both the latent and active calpains. Arrows indicate calpain positive cells. The numbers of calpain positive cells were also counted from selected 20× microscopic field and plotted in panels E′ and G′. Data represents mean ± S.E.M with ∗∗indicating $p < 0.01$. n.s. indicates not statistical significant. Scale bar = 20 μm.

comparison, B-CK and CaATPase activities from EAE-2BFI group were significantly restored to near the level of saline–saline control group (Fig. 4A and B). This data indicates that B-CK and CaATPase are important in EAE-induced spinal cord injury and that enhancing B-CK and CaATPase activities during 2-BFI treatment may be responsible for the protection of the spinal cord.

3.4. EAE spinal cord from mice treated with 2-BFI shows no calpain activity

Calpain is a calcium dependent protease which has been shown to be involved in neuronal damage in a variety of neurodegenerative conditions [9,14–16]. As shown in Fig. 4C, calpain activity increased significantly in the EAE-saline group compared with that of the saline-saline group, suggesting that increased intracellular calcium may be responsible for triggering the activation of calpain. Spinal cord from mice treated with 2-BFI showed no significant induction of calpain activity. In addition, spinal cord sections were also immunostained with antibodies to calpain I and II, which detect both the latent and active calpain I and II. As shown in Fig. 4D and F, the EAE-saline group exhibited strong calpain staining in spinal cord neurons (arrows), while the level of staining was low in the EAE-2BFI treated group (E and G). The number of neurons positive for calpain I or II immunostaining were counted and shown in panels E′ and G′, respectively. Indeed, the EAE-2BFI treated group demonstrated significantly reduced number of calpain positive cells. Taken together, these data strongly supports for a role of calpain in EAE-induced spinal cord pathogenesis and argues for the necessity for further studies which may establish calpain as a target for EAE therapeutics.

4. Discussion

Here we show, for the first time, that 2-BFI, a very specific and selective ligand to I_2R, attenuated spinal cord injury in a mouse MS model of EAE. Twice daily treatment of EAE mice with a 20 mg/kg body weight of 2-BFI showed no signs of toxicity; and remarkably, these mice exhibited significantly reduced neurological deficits and tissue damage as evidenced by the better preservation of myelin, less axonal damage and reduced infiltration of inflammatory cells. Mechanistically, 2-BFI treated mice were able to resort the enzymatic activities of B-CK and CaATPase which are important in maintaining cellular energy and intracellular calcium homeostasis. As such, calcium-dependent calpain activation was maintained at the basal level in the EAE-2BFI group, in contrast to the elevated calpain activity occurring in the EAE-saline group.

Our studies also confirmed several previously published works that ligands targeting I_2R is beneficial to EAE-induced spinal cord injury. For example, treatment with idazoxan in mice with a 1.5–4.5 mg/kg body weight significantly attenuated EAE-induce spinal

cord injury [26]. Amiloride, another I_2R ligand, was also found to be neuroprotective against EAE, possibly through blocking both the acid ion sensing channel and I_2R [5]. However, questions remain as to whether the protective action of these ligands was through the I_2R and that I_2R is a legitimate molecular target for EAE therapeutics.

Nevertheless, we explored the possibility that 2-BFI protection may involve its binding protein B-CK. The activity of this enzyme is a key in maintaining the balanced supply of energy, critical for many biochemical processes such as influencing membrane potentials and neurotransmitter release. Indeed, through a proteomic approach, B-CK changes have been detected in EAE brain and spinal cord [13]. We directly measured the level of enzymatic activities of B-CK from both the EAE-saline and EAE-2-BFI groups. The protection to the spinal cord by 2-BFI was associated with the restored B-CK activity, which lends further support to the argument that 2-BFI may directly interact with B-CK to modulate its stability.

Plasma membrane calcium pumps, such as the plasma membrane CaATPase (PMCA), require energy to maintain calcium homeostasis [19]. Imbalanced intracellular calcium is known to cause neuronal dysfunction and even death in a number of neurodegenerative diseases [11]. A decrease in PMCA2 expression causes neuronal death during EAE through the reduction in collapsin response mediator protein 1 levels [19]. Our results provided further evidence confirming the importance of CaATPase, in that CaATPase activity was significantly reduced in EAE spinal cord and treatment with 2-BFI restored B-CK-mediated energy level, thereby maintaining CaATPase activity.

The present study also confirmed the involvement of calpain in EAE-induced brain injury [22]. Through examining the white matter plaques from human patients with MS, it was found that calpain expression was up-regulated by 462.5% in MS plaques compared with the controls. This increase in calpain activity may cause demyelination in EAE and MS [7,24]. Together with our results, it is plausible to hypothesize that EAE perturbs intracellular calcium homeostasis, thereby triggering the activation of calpain damaging myelin and neuronal function.

Acknowledgments

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