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The effects of fasudil on the permeability of the rat blood–brain barrier and blood–spinal cord barrier following experimental autoimmune encephalomyelitis

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ABSTRACT

Dysfunction of the blood–brain barrier (BBB) and blood–spinal cord barrier (BSCB) is a primary characteristic of multiple sclerosis (MS). We evaluated the protective effects of fasudil, a selective ROCK inhibitor, in a model of experimental autoimmune encephalomyelitis (EAE) that was induced by guinea-pig spinal cord. In addition, we studied the effects of fasudil on BBB and BSCB permeability. We found that fasudil partly alleviated EAE-dependent damage by decreasing BBB and BSCB permeability. These results provide rationale for the development of selective inhibitors of Rho kinase as a novel therapy for MS.

1. Introduction

The blood–brain barrier (BBB) and blood–spinal cord barrier (BSCB) maintain the internal environment and stability of the central nervous system (CNS). The pathophysiological changes in the CNS during multiple sclerosis (MS) and other autoimmune diseases are often characterized by structural and functional changes to these barriers.

Under normal physiological conditions, lymphocyte movement into the CNS is minimal. In an individual with MS, as well as in the animal model of experimental autoimmune encephalomyelitis (EAE), numerous circulating immunocompetent cells readily gain access to the CNS. Although peripheral blood leukocyte infiltration plays an essential role in MS lesion development (Lees et al., 2006; Song et al., 2008; Ahn et al., 2009; Brini et al., 2009; Peruga et al., 2011), there is also evidence suggesting that BBB dysfunction precedes immune cell infiltration (Bennett et al., 2010).

The intercellular space within tight and impermeable biological barriers is sealed by large molecular complexes called tight junctions (TJs). In the BBB and BSCB, TJs are composed of large multi-protein complexes that mediate tight intercellular contacts between adjacent cells and serve as "molecular fences" (Paris et al., 2008; Zlokovic, 2008). TJs consist of three principal types of trans-membrane proteins: occludin, claudins and junctional adhesion molecules (Vorbrodt and Dobrogowska, 2003; Ballabh et al., 2004). Analysis of CNS vessels in MS tissue found alterations in the expression of TJs and associated proteins within lesions (Plumb et al., 2002; Kirk et al., 2003; Padden et al., 2007; Alvarez et al., 2010). Several lines of evidence have revealed that alterations in BBB permeability are a primary initiating factor in MS and EAE (Kirk et al., 2003; Hawkins and Davis, 2005; Persidsky et al., 2006; Leech et al., 2007; Wosik et al., 2007; Reijerkerk et al., 2008). A recent study demonstrated that disease severity during the acute phase of EAE directly correlated with the extent of BBB permeability (Bennett et al., 2010). Therefore, a potential therapeutic strategy to treat MS and EAE is to prevent the infiltration of inflammatory cells into the CNS by enhancing BBB and BSCB permeability (Luccarini et al., 2008).

Although MS pathology is not fully understood, some therapeutics may slow down disease progression and help control symptoms. The Rho/ROCK signaling pathway is important for the regulation of endothelial permeability. The Rho family, including RhoA, Rac and Cdc42, disrupts the barrier function of TJs (Jou et al., 1998; Bruewer et al., 2004), Fasudil is a specific inhibitor of ROCK that has been clinically used in Japan since 1995 for the treatment of vasospasm following subarachnoid hemorrhage (Tachibana et al., 1999). Previous studies have shown that fasudil treatment (parenteral and oral administration) prevents the development of EAE induced by proteolipid protein p139-151 in SJL/J mice (Sun et al., 2006) and decreases the development of EAE in 57BL/6 mice induced by the MOG_{35-55} peptide (Yu et al., 2010). Matrix metalloproteinases (MMPs) and other enzymes promote the degradation and remodeling of occludin and other junctional complex associated molecules (Agrawal and Yong, 2007; Yang et al., 2007; Bauer et al., 2009; Sozen et al., 2009). Interestingly, a recent study found that fasudil inhibits MMP-2 mRNA and protein expression in a dose-dependent manner (Deng et al., 2010).

The effects of fasudil on EAE and MS led us to further investigate the mechanism by which fasudil regulates these barriers. We hypothesize

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that the effects of fasudil are attributed to the prevention of BBB and BSCB disruption. In this study, we confirm fasudil-dependent therapeutic effects in a Lewis rat model of EAE induced by guinea pig spinal cord. In addition, we extended these findings by using a systematic approach to assess BBB and BSCB permeability and the expression of occludin. Furthermore, we examined the function of TJs in BBB permeability and measured the ROCK expression. More importantly, we demonstrated that fasudil promotes its preventive and therapeutic effects on EAE by affecting BBB and BSCB integrity. These results provide a rationale for the use of selective inhibitors of Rho kinase as a novel therapy for MS.

2. Methods

2.1. Animals

Fifty-four pathogen-free female Lewis rats (6–8 weeks old; Laboratory Animal Research Institute of Chinese Medical University) were acclimated to the experimental apparatus for at least 1 week before immunization. The animal protocols were approved by the Committee on Ethics in Animal Experiments of the Experimental Animal Research Institute of the Second Affiliated Hospital at Harbin Medical University. Food and tap water were provided ad libitum, and the animals were housed in a climate-controlled environment.

2.2. Whole spinal cord (WSC) induction of EAE

EAE was induced as described previously (Pozza et al., 2000) with slight modification. The inoculum consisted of a homogenous mixture of 1-g guinea pig spinal cord, 1-ml 0.9% saline, 1-ml incomplete Freund's adjuvant (Sigma-Aldrich Co.), and 10-mg Bacillus Calmette-Guerin BCG (Beijing Institute of Biological Products, China). The solution was emulsified and frozen at −20 °C until use. On day 0, the rats were anesthetized with 3.5% chloral hydrate and subcutaneously injected with the inoculum (400 μl) bilaterally in proximity to the axillary and inguinal lymph nodes.

2.3. Animal grouping and management

Fifty-four animals were randomly divided into three groups: a) the control group(n = 18), which was immunized with saline emulsified with an equal volume of IFA and 5 mg/ml of BCG without WSC; b) the vehicle-treated group (n = 18), which was immunized with a mixture containing WSC and treated daily with saline from day 0 post immunization (0DPI) until sacrifice; and c) the fasudil-treated group (n = 18), which was immunized with a mixture containing WSC and treated daily with fasudil from 0DPI until animal sacrifice. Fasudil (40 mg/kg; Tianjin Chase Sun Pharmaceutical Co., Tianjin, China) was administered (i.p.) once a day. Group B rats received saline (i.p.). Each experiment was repeated three times, and each experimental group had eighteen samples per replicate.

2.4. Neurological evaluation

Two independent investigators performed neurological evaluations of twice daily during the EAE course in a single-blind fashion. EAE symptoms were scored on a seven-point scale as follows (Scott et al., 2001): 0 = normal, 1 = piloerection and tail weakness, 2 = tail paralysis, 3 = tail paralysis plus hind limb weakness, 4 = tail paralysis plus partial hind limb paralysis, 5 = complete hind limb paralysis, 6 = hind and forelimb paralysis and 7 = moribund or dead.

2.5. Neuropathological evaluation (hematoxylin–eosin and myelin stains)

Six rats from each group were randomly selected on the third day for evaluation by two independent investigators in a single-blind manner. Rats were anesthetized and transcardially perfused with physiological saline and 4% buffered paraformaldehyde. The cerebellum and lumbar enlargement were collected. Tissue was dissected and post-fixed in a 4% buffered paraformaldehyde solution and embedded in paraffin. After embedding, 2–3-μm and 5-μm thick sections were prepared; the 2–3-μm sections were stained with hematoxylin–eosin (H–E) stain, whereas the 5-μm sections were stained with Chromotrope 2R and Brilliant Green glacial acetic acid. The number of immune cells in each section was counted using Image Plo Plus 5.1 software (Media Cybernetics, Silver Spring, MD) by double-blinded reader (Yu et al., 2010).

2.6. Assessment of BBB and BSCB permeability

BBB and BSCB permeability was determined by counting the number of cells stained with Evans Blue (EB) dye. Six rats from each group were randomly selected and anesthetized at the onset of neurological signs. According to our pilot studies, the onset of neurological signs occurs upon changes in the BBB and BSCB permeability. EB dye (2%; 4 ml/kg body weight; Sigma, St. Louis, MO, USA) was slowly administered through the tail vein and allowed to circulate for 1 h. The rats were then transcardially perfused with saline to flush excess dye in the blood vessels. The cerebellum and lumbar enlargement were dissected, and each sample was immediately weighed. The EB dye was extracted by first homogenizing the sample in 2.5 ml of 0.1 M phosphate buffered saline at pH 7.4. To precipitate protein, 2.5 ml of 60% trichloroacetic acid was added. The mixture was then vortexed for 2 min and cooled for 30 min. The sample was subsequently centrifuged for 40 min at 4000 rpm to pellet the tissue. The absorbance of the supernatant was then measured at 610 nm using a spectrophotometer (Beijing Purkinje General Instrument Co.; Beijing, China). The EB dye content was calculated as μg/g of tissue using a standard curve.

2.7. Immunohistochemistry

Paraffin embedded tissue from each group was cut into 3-μm thick sections and repaired with high voltage. The sections were warmed to room temperature and rinsed twice with deionized water and then three times with PBS (5 min per wash). The sections were incubated with the AQP4 (H-80) primary antibody (1:400 dilution; sc-20812; Santa Cruz., CA, USA) diluted in 2% BSA at 4 °C overnight. `Sections were then rinsed twice in phosphate buffered saline (PBS) (2 min per wash), incubated in non-biotinylated goat anti-rabbit IgG secondary antibody (ZSGB-BIO; Beijing, China) for 20 min at room temperature, and then rinsed three times with PBS (5 min per wash). The chromophore product was developed using a Simple Stain DAB solution (ZSGB-BIO).

2.8. Western blot analysis of ROCK2, P-MLC and occludin expression

Six rats from each group were randomly selected on the third day of onset. Under deep anesthesia, tissue samples were taken from the cerebellum and lumbar enlargement and immediately frozen in liquid nitrogen. Samples were stored at −80 °C until further use. Upon experimentation, samples were homogenized in liquid nitrogen and diluted in RIPA Lysis Buffer (Beyotime Institute of Biotechnology; Jinan, Shandong, China), which contained 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40 (Fisher), 0.5% sodium deoxycholate, 0.1% SDS, phosphatase inhibitor cocktail (Roche), and 5% complete protease inhibitor cocktail (Roche). Tissue homogenates were centrifuged at 12,000 rpm for 10 min at 5 °C and the supernatant containing the protein lysate was collected. Protein concentration was subsequently determined by a using a spectrophotometer (Bio-RAD, America). Equivalent protein samples were resolved on 8% (ROCK2, occludin) and 12% (P-MLC and occludin) SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked overnight at 4 °C in Tris-buffered