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Preventive and therapeutic effects of the selective Rho-kinase inhibitor fasudil on experimental autoimmune neuritis

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ABSTRACT

We studied the effects of fasudil, a selective Rho-kinase inhibitor, on experimental autoimmune neuritis (EAN). Continuous parenteral administration of fasudil prevented the development of EAN induced by P0 peptide 180–199 in Lewis rats while it also reduced EAN severity when administered after disease onset. Immunohistochemical examination disclosed a marked decrease in the amount of inflammatory cell infiltration and attenuation of demyelination and axonal degeneration. Specific proliferation of lymphocytes from fasudil-treated rats in response to P0 peptide was significantly reduced as compared with those from phosphate-buffered saline (PBS)-treated rats. Fasudil treatment was associated with a significant reduction in secretion of IFN-γ; by contrast, secretion of IL-4 was almost the same in the fasudil- and PBS-treated groups. As a result, the IFN-γ/IL-4 ratio in the supernatant was significantly deceased in fasudil-treated rats compared with PBS-treated ones. Therefore, our results indicate a beneficial effect of selective blockade of Rho-kinase in animals with autoimmune inflammation of the peripheral nerves, and may provide a rationale for the selective blockade of Rho-kinase as a new therapy for Guillain-Barré syndrome.

1. Introduction

Guillain-Barré syndrome (GBS) and its animal model experimental autoimmune neuritis (EAN) are representative of the autoimmune diseases that affect the peripheral nervous system (PNS). EAN can be induced in susceptible animals by active immunization with PNS myelin or proteins such as P2 and P0, combined with Freund's complete adjuvant (FCA) [1–3]. Blood–nerve barrier (BNB) breakdown, immunoglobulin leakage, infiltration with activated T cells and macrophages, and predominantly perivenular demyelination of nerve roots are observed in EAN [4]. The immunopathogenesis of EAN involves the integrated attack of T-cells, B-cells and macrophages [5,6]. Inflammatory cell infiltrates in the PNS of GBS patients are also composed of lymphocytes and macrophages, which exert their effects through proinflammatory cytokines, such as TNF-α [7,8] while abnormal cellular responses to P2 and P0 proteins have been reported in some patients with GBS [9]. Thus, cellular immunity may also play a pivotal role in GBS pathogenesis.

Statins, which downregulate cholesterol synthesis through inhibition of 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, have anti-inflammatory effects and are protective in animal models of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE) [10–14]. Although the exact mechanism underlying this protection is still unclear, it is partly attributable to the prevention of isoprenylation of Rho GTPase, which occurs downstream of the mevalonate pathway and is required for the membrane translocation and activation of GTPase proteins [15]. The Rho family GTPases (Rho, Rac and Cdc42) act as key regulators of the actin cytoskeleton. Rho-kinase is the major effector molecule for a variety of functions of Rho GTPase [16]. Activation of Rho-kinase by GTP-bound Rho (the activated form) leads to phosphorylation of ERM, myosin light chain, collapsin response mediator protein-2 (CRMP-2), LIM kinases 1 and 2, adducin and intermediate filament [17,18]. Inhibition of Rho-kinase activity induces suppression of cell proliferation and motility. Thus, statins may inhibit the cellular function of various cell types, including immunocytes, by inducing accumulation of the inactive form of Rho in the cytosol and thereby inhibiting downstream Rho-kinase signaling. Protein prenyltransferase inhibitors and flavonoids, which down-regulate Rho GTPase, have also been shown to be protective in EAE [19,20]. Thus, blockade of the rho/rho kinase system is considered to be beneficial for CNS inflammatory demyelination.

We previously reported that fasudil, a selective Rho-kinase inhibitor, has both protective and therapeutic effects in EAE animals [21]. In EAN animals, infiltration of Rho-positive macrophages and T cells into the spinal roots has been shown, and was correlated with the clinical severity of EAN [22]. Thus, the rho/rho kinase system could also be an important therapeutic target for peripheral nerve inflammatory demyelination. We therefore aimed to extend our study to EAN to

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explore if the drug is also useful for treating peripheral nerve inflammatory demyelinating diseases, such as GBS. In this paper, we demonstrate that fasudil acts in both a preventive and therapeutic fashion in EAN, in part through inhibition of P0-specific T-cell proliferation with a marked reduction in secretion of IFN-γ and suppression of the IFN-γ/IL-4 ratio.

2. Materials and Methods

2.1. Animals

Male Lewis rats, aged 7–8 weeks, with body weights of 250–300 grams, were purchased from Charles River Japan Inc. All animal protocols were approved by the Committee on Ethics in Animal Experiments of Kyushu University and were performed according to the Guidelines for Animal Experiments of Kyushu University and the Japanese Government.

2.2. Antigen and Antibodies

The P0 peptide 180–199 (SSHRGRQTPVLYAMLDHSRS) was synthesized using a peptide synthesis system (Applied Biosystems, MA, USA), based on the 9-flourenylmethyloxycarboneyl (Fmoc) strategy, and purified by C18 reverse-phase high performance liquid chromatography (HPLC). The purity of the peptide was 95% as determined by HPLC analysis [23]. The following primary antibodies were used for immunohistochemistry and western blot analysis: anti- ezrin/radixin/moesin (ERM) antibody, anti-phospho-ERM antibody (Cell Signaling Technology, MA, USA), anti-myelin basic protein (MBP) antibody (Acris Antibodies, Herford, Germany), and anti-neurofilament heavy chain (NF-H) 200 kD antibody (Chemicon, MA, USA).

2.3. Induction and clinical evaluation of experimental autoimmune neuritis in Lewis rats

Experimental autoimmune neuritis (EAN) was induced in Lewis rats by immunization with 200 μg of P0 peptide 180–199 emulsified in an equal volume of complete Freund's adjuvant containing 4 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco, KS, USA). The P0 emulsion (0.1 ml) was injected subcutaneously in both sides of a tail base. Every day, the rats were weighed and examined for clinical signs of EAN and scored as follows: 0, normal; 1, limp tails; 2, impaired righting reflex; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, moribund or dead.

2.4. Fasudil treatment using ALZET Mini Osmotic Pump

Fasudil (Asahi Chemical Industries, Tokyo, Japan) was administered continuously via a subcutaneously implanted ALZET mini-osmotic pump (DURECT Corporation, CA) with a dose of 100 mg/kg/day according to our study on experimental autoimmune encephalomyelitis (EAE) [21]. Briefly, in the preventive study, rats immunized with 200 μg of P0 peptide 180–199 were continuously administered fasudil from day −2, while in the therapeutic study, fasudil was started at the onset of neurological illness. Control animals were given phosphate-buffered solution (PBS) using the same osmotic pumps.

2.5. Antigen specific T-cell proliferation assays

Splenocytes were harvested and processed into single cell suspensions. Cells (2×10^5 cells/well) were distributed into 96-well round bottom plates (Falcon, Becton Dickinson, NJ, USA) and cultured with P0 peptide 180–199 (0.1, 0.5, 1, 5, 10 μM), phytohemagglutinin (PHA; 10 μg/ml), or medium alone. After 48 h of culture, 1 μCi of [^{3}H] thymidine was added to each well and cultures were harvested 18 h later and assessed for incorporation of [^{3}H] thymidine. All assays were performed in triplicate.

2.6. Cytokine analysis

The supernatants from cultures of splenocytes were harvested at 72 h and 120 h: 72 h for IFN-γ assays and 120 h for IL-4 assays. Both cytokines were analyzed using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions, as described previously [24]. All assays were performed in triplicate.

2.7. Western blot analysis for ERM phosphorylation

To quantify Rho-kinase activity in the liver (day 10) and lymph nodes (LNs) (day 10), western blot analysis of phosphorylated ERM (ezrin T567, radixin T564 and moesin T558) and total ERM was performed as described previously [25]. ERM is phosphorylated by Rho-kinase at T567 (ezrin), T564 (radixin) and T558 (moesin). Equal amounts of extracted proteins were separated by SDS-PAGE and subjected to immunoblot analysis. The regions containing ERM family proteins were visualized by electrochemiluminescence. Band intensities from western blots were quantified densitometrically by ImageJ 1.34 s downloaded from http://rsb.info.nih.gov/ij. The extent of ERM phosphorylation was normalized to the levels of total ERM.

2.8. Histopathology and immunohistochemistry

Rats were anesthetized and perfused with PBS and 4% buffered paraformaldehyde. Sciatic nerves were collected on day 18 after antigen immunization in the preventive study, and on day 35 in the therapeutic study. The tissues were dissected and post-fixed in 4% buffered paraformaldehyde solution and embedded in paraffin. After embedding, 6-μm-thick sections were prepared. For routine neuropathological evaluation, sections were stained with hematoxylin-eosin (H-E) stain. Because the function of NF-H is to maintain axonal structural integrity and disruption of axonal membrane integrity results in neurofilament proteins being released into the extracellular space [26], immunohistochemistry for NF-H was used for evaluation of axonal damage. MBP immunohistochemistry was used for evaluation of demyelination because it is a highly abundant protein in the PNS myelin. For immunohistochemical analysis, sections were deparaffinized in xylene, hydrated in ethanol, and incubated in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to inhibit endogenous peroxidase. After rinsing in tap water, the sections were completely immersed in distilled water and autoclaved for 15 min to enhance the immunoreactivity of MBP and NF-H. Subsequently, sections were incubated with primary antibody diluted in 5% non-fat milk in 25 mM Tris-HCl pH 7.6 containing 0.5 M NaCl, 0.05% NaN_3 and 0.05% Tween 20 (TBST) for 1 h at room temperature. As a secondary antibody, peroxidase-labeled anti-rabbit IgG (Vector Laboratories, CA, USA) was used. The colored reaction product was developed using Simple Stain DAB solution (Nichirei, Tokyo, Japan). The sections were counterstained lightly with hematoxylin. Inflammatory cell infiltrates were graded by hematoxylin and eosin (H-E) stain as: 0, no abnormality; 1, cellular infiltration adjacent to a vessel; 2, cellular infiltration in immediate proximity to a vessel; 3, cellular infiltration around a vessel and in more distant sites [27]. The severity of demyelination was graded by MBP immunostaining as: 0, none; 1, isolated demyelinated axons perivascular or scattered; 2, many foci of perivascular demyelination; 3, extensive demyelination, perivascular and confluent [28].

2.9. Statistical analysis

Disease frequency was compared using Fisher's exact probability test. Ratios of phosphorylated ERM against total ERM, proliferation of T cells and cytokine production were compared using the Student's *t* test. All other statistics were analyzed using the Mann-Whitney *U* test. A value of $p<0.05$ was considered significant.

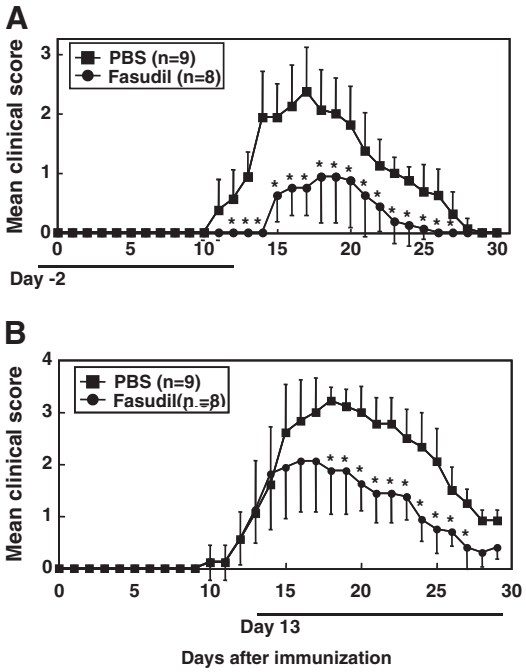

Fig. 1. Fasudil treatment suppresses P0-induced EAN. (A) Rats immunized with 200 μg of P0 peptide 180–199 were subcutaneously administered fasudil (100 mg/kg/day), continuously from day −2. Fasudil-treated rats have significantly fewer clinical symptoms than PBS-treated control rats (*, P<0.05). (B) In fasudil-treated rats, clinical symptoms are significantly reduced on days 12–27 (*, P<0.05) when fasudil treatment began on day 13. Fig. 1A and B show the sum of two independent experiments with essentially the same results.

3. Results

3.1. Preventive and therapeutic treatment with fasudil suppresses P0-induced EAN

In the preventive study, ALZET osmotic mini-pump delivery of fasudil prior to immunization significantly reduced the incidence of EAN in Lewis rats immunized with P0 180–189 ($p = 0.007$). All PBS-treated rats developed neurological symptoms with an average onset at day 12.1 and a peak at day 17, while in fasudil-treated rats, only 75% of the rats developed neurological symptoms with the average onset at day 15 and the peak at day 18. The severity of the disease in fasudil-treated rats was significantly reduced on days 12–27 compared with that in PBS-treated rats ($p<0.05$) (Fig. 1A). For the treatment group, in which the ALZET osmotic mini-pump was started at the onset of neurological illness, PBS-treated rats had higher clinical scores than fasudil-treated ones, and the severity of disease was significantly reduced on days 18–27 ($p<0.05$) (Fig. 1B).

3.2. Rho-kinase activity in rats with EAN with or without fasudil treatment

ERM is one of the major substrates of Rho-kinase. To confirm that fasudil inhibited the Rho-kinase pathway *in vivo* we measured the extent of ERM phosphorylation by western blot analysis in the liver and LNs. In the liver and LNs, 10 days after antigen immunization, the ratio of phosphorylated to total ERM in fasudil-treated animals also decreased significantly compared with that in PBS-treated animals and normal animals ($p<0.05$, and $p<0.01$ respectively) (Fig. 2). These data indicate that fasudil suppresses Rho-kinase activity *in vivo*.

3.3. Fasudil decreases infiltration of inflammatory cells into the peripheral nerves

Histopathological examination of the sciatic nerves of the animals at day 18 in the preventive study (administration before immunization) revealed that inflammatory infiltrates and demyelination severity were significantly reduced in fasudil-treated rats compared with PBS-treated ones (inflammatory index: 1.33 ± 1.03 vs. 2.67 ± 0.82, $p<0.05$, demyelination index: 0.5 ± 0.84 vs. 1.75 ± 1.04, $p<0.05$) (Fig. 3A and B). In the therapeutic study, we performed a histopathological study at day 35. In the chronic phase, demyelination severity was significantly decreased in fasudil-treated rats compared with that in PBS-treated ones (demyelination index: 1.5 ± 0.55 vs. 2.67 ± 0.52, $p<0.01$) (Fig. 3C). Axonal degeneration was more severe in PBS-treated rats than in fasudil-treated ones.

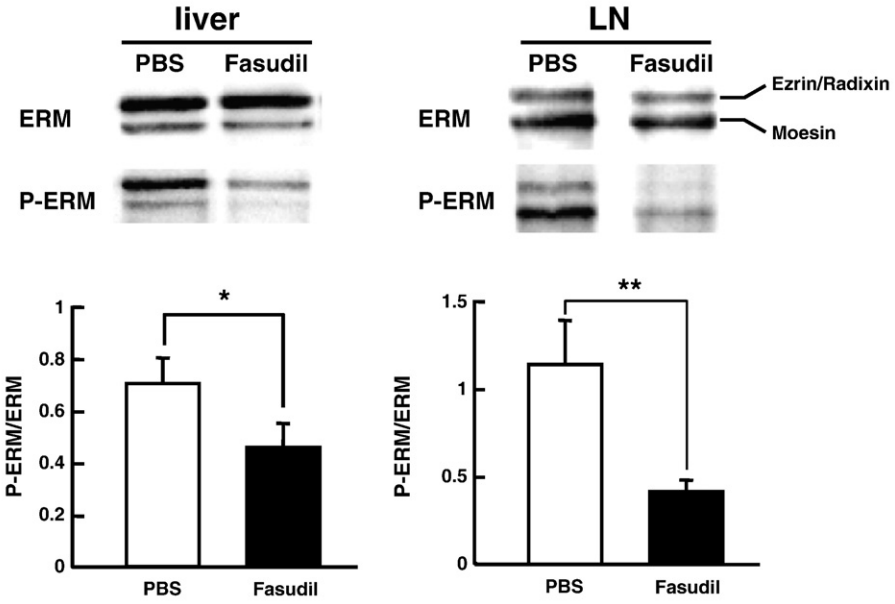

Fig. 2. Rho-kinase activity in EAN. Western blot analysis for ERM phosphorylation in the lymph node and liver of animals treated with or without fasudil. In lymph node and liver, ERM phosphorylation is significantly decreased in fasudil-treated EAN animals (n = 3) compared with control animals (n = 3) (*, $p<0.05$; **, $p<0.01$). Results are expressed as means ± SEM.

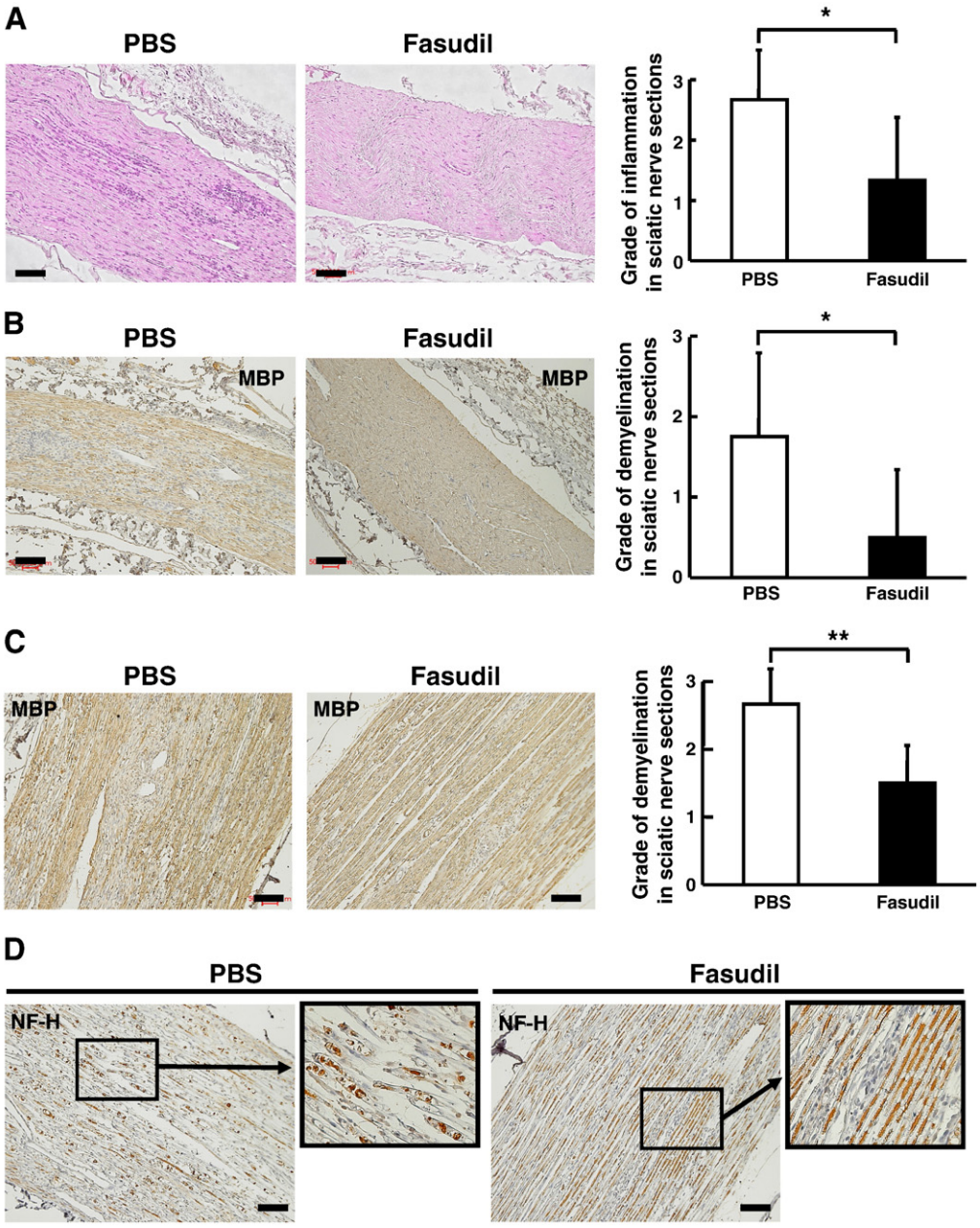

Fig. 3. Decreased inflammation in the sciatic nerves of fasudil-treated rats with EAN. Inflammatory cell infiltration (A) and demyelination (B) in sciatic nerve sections in the preventive study (day 18). The inflammatory infiltrates and demyelination severity are significantly reduced in fasudil-treated rats (n = 6) compared with PBS-treated rats (n = 6) (*, $p<0.05$). Demyelination (C) and axonal degeneration (D) in the therapeutic study (day 35). When fasudil treatment began at day 13 after sensitization, demyelination severity is significantly reduced in fasudil-treated rats (n = 6) compared with PBS-treated rats (n = 6) (**, $p<0.01$). Axonal degeneration is more severe in PBS-treated rats than in fasudil-treated rats. Scale bars, 100 μm.

3.4. Fasudil treatment induces a reduction of P0-specific T cell proliferation

To gain insights into the mechanisms underlying the inhibitory effects of fasudil, splenocytes were removed from fasudil-treated and PBS-treated rats on day 10, and the cells were re-stimulated with P0 peptide 180–199 at different concentrations; their cytokine production and antigen-specific proliferation were measured. Fig. 4A shows the results of the proliferation of splenocytes. These results indicate that treatment of rats with fasudil suppressed the proliferative response to the antigen, although the proliferative response in PBS-treated rats was not marked (a two-fold increase in PBS-treated rats vs. practically no increase in fasudil-treated rats). Culture supernatants were then examined for cytokines. Fasudil treatment was associated with a marked reduction in secretion of IFN-γ ($p<0.05$), while in contrast, secretion of IL-4 was almost the same in the two groups (Fig. 4B). As a result, the IFN-γ/IL-4 ratio in the supernatant was decreased in fasudil-treated rats compared with PBS-treated ones ($p<0.05$) (Fig. 4C).

4. Discussion

The present study is the first to demonstrate that the specific Rho-kinase inhibitor fasudil is preventive of EAN in model animals when administered before immunization and therapeutic when administered after the onset of disease. These results are consistent with the results of our previous study on EAE, which also revealed both preventive and therapeutic effects of the drug [21].

The known beneficial effects of statins on EAE and MS can partly be explained by inhibition of the isoprenylation of Rho GTPase [11,15], which results in a Th2 shift acting on both T cells and antigen-presenting

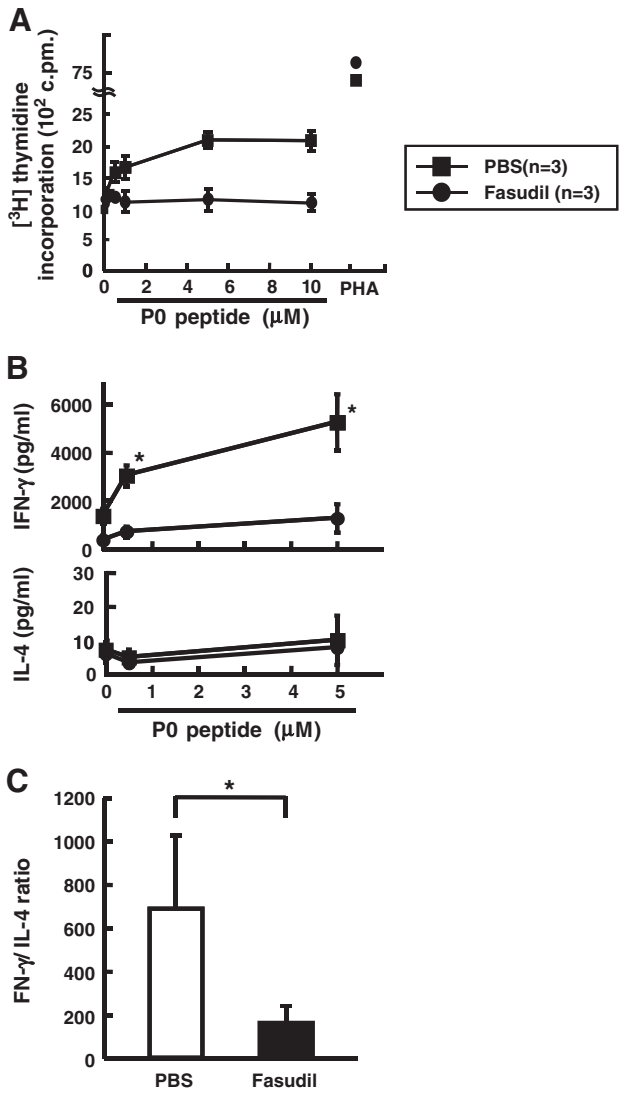

Fig. 4. Fasudil suppresses antigen-dependent proliferation of and cytokine production by splenocytes from sensitized rats. (A) P0 peptide 180-199-specific proliferation of splenocytes from fasudil-treated rats (closed circle, n = 3) is lower than that of splenocytes from PBS-treated rats (closed square, n = 3). (B) Cytokine assay of supernatants from cultures of splenocytes from rats treated with P0 180–199. Fasudil treatment markedly reduces secretions of IFN-γ (*, $p<0.05$), while in contrast, secretion of IL-4 is almost the same in the two groups. (C) The IFN-γ/IL-4 ratio in the culture supernatants of splenocytes from P0-sensitized rats stimulated with P0 peptide 180–199 is greatly decreased in fasudil-treated rats compared with that in PBS-treated rats (*, $p<0.05$).

cells (APCs) [10,12,14], and inhibition of T cell migration to the CNS acting on both T cells [29] and brain endothelial cells [11]. In EAE, flavonoids and 17β-estradiol have also been shown to be protective through down-modulating the activity of Rho GTPase [20]. Protein prenyltransferase inhibitors partially suppressed EAE when administered before the onset of disease, whereas no therapeutic effect was found when administration was started after disease onset [19]. Based on our previous [21] and present studies showing that fasudil has preventive and therapeutic effects on EAE and EAN, the direct inhibition of Rho-kinase itself appears to be more beneficial in treating inflammatory demyelination of the CNS and PNS than prenyltransferase inhibitors.

In the present study, although LN cell proliferation following relevant antigenic stimulation was mild in PBS-treated rats, IFN-γ production in culture was evident. By contrast, in fasudil-administered animals, the production of IFN-γ was significantly reduced while there was practically no proliferation of LN cells in response to P0 peptide 180–199. These findings are consistent with a report of another Rho-kinase inhibitor, Y-27632, which strongly suppressed the production of IFN-γ but only weakly suppressed that of IL-4 and IL-5 in human peripheral blood T cells [30]. In our study, production of IL-4 was not evident, even in PBS-treated rats. This might be a result of insufficient incubation time to observe a response, or a poor Th2 response in P0-sensitized animals. Nonetheless, because there was a marked reduction in IFNγ secretion in fasudil-treated rats, we assume that down-regulation of IFNγ is at least in part responsible for fasudil's beneficial effects on EAN. The possibility that regulatory T cells may be modulated by fasudil should be examined in future studies.

The temporal and spatial alteration of ERM activity, regulated by its dephosphorylation and rephosphorylation, is suggested to be critical for immunological synapse formation and T cell activation through its binding to many transmembrane proteins critical for lymphocyte trafficking to the nervous tissues, such as CD43 [31]. Inhibition of ERM function is known to decrease production of IFN-γ and IL-2 [32]. IFN-γ promotes T cell homing to the PNS, enhances vascular permeability, and induces MHC antigens, especially MHC class II expression on macrophages and cultured Schwann cells, and adhesion molecules on endothelial cells, macrophages, T cells and Schwann cells, thereby playing a crucial role in the inflammation accompanying both EAN and GBS [33]. Thus, suppression of ERM by fasudil *in vivo*, as shown in the present study, may contribute to the amelioration of EAN partly through a down-regulation of Th1 cytokines and inhibition of lymphocyte trafficking. Rho-kinase inhibitor has also been shown to influence on macrophage activation and migration [34,35], and such effects of the drug in EAN requires future studies.

Increasing evidence suggests that Rho-kinase induces retraction of axons while fasudil facilitates axonal growth through inhibition of the kinase [36,37]. Fasudil may thus offer the possibility of functional recovery from EAN through facilitation of axonal growth, in addition to suppression of inflammatory cell infiltration into the PNS.

Fasudil has been used with minimal side effects in more than 30,000 patients with subarachnoid hemorrhage [36] and has been safely used to treat vasospasm following subarachnoid hemorrhage since 1995 in Japan [38]. Thus, our results indicate a beneficial effect of selective blockade of Rho-kinase in animals with autoimmune inflammation of the PNS, and may provide a rationale for oral use of fasudil in the treatment of GBS.

Acknowledgments

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