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Journal of Neuroimmunology 180 (2006) 126 – 134

Journal of Neuroimmunology

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The selective Rho-kinase inhibitor Fasudil is protective and therapeutic in experimental autoimmune encephalomyelitis

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Received 20 April 2006; received in revised form 23 June 2006; accepted 29 June 2006

Abstract

We studied the role of fasudil, a selective Rho-kinase inhibitor, in experimental autoimmune encephalomyelitis (EAE). Both parenteral and oral administration of fasudil prevented the development of EAE induced by proteolipid protein (PLP) p139-151 in SJL/J mice. Specific proliferation of lymphocytes to PLP was significantly reduced, together with a downregulation of interleukin (IL)-17 and a marked decrease of the IFN-γ/IL-4 ratio. Immunohistochemical examination also disclosed a marked decrease of inflammatory cell infiltration, and attenuated demyelination and acute axonal transaction. These results may provide a rationale of selective blockade of Rho-kinase by oral use of fasudil as a new therapy for multiple sclerosis.

Keywords: Fasudil; Rho-kinase; Multiple sclerosis; Experimental autoimmune encephalomyelitis; IL-17

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), in which an autoimmune attack is supposed to be mediated by myelin antigen-specific Th1 cells. Increasing evidence suggests that statins, which downregulate cholesterol synthesis through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, have anti-inflammatory effects and are protective in animal models of MS, experimental autoimmune encephalomyelitis (EAE) (Aktas et al., 2003; Greenwood et al., 2003; Nath et al., 2004; Stanislaus et al., 1999; Youssef et al., 2002). Moreover, a recent open trial of simvastatin for MS revealed a significant reduction in the number of new brain lesions on magnetic resonance imaging (Vollmer et al., 2004). Nearly all of statins' pleiotropic effects on immune system are reversed by L-mevalonate, indicating that the inhibition of mevalonate pathway is crucial. Although the exact mechanism of the protective effects of statins in EAE or MS is still unclear, it is in part attributable to the prevention of isoprenylation of Rho GTPase, which occurs downstream of mevalonate pathway and is required for the membrane translocation and activation of Rho GTPase proteins (Neuhaus et al., 2004). Thus, statins may inhibit cellular functions of various cell types, including immunocytes, by inducing accumulation of the inactive form of Rho in the cytosol and thereby inhibiting downstream Rho-kinase/ROK/ROCK.

It has been reported that daily injections of protein prenyltransferase inhibitors, which prevent functional Rho GTPase, before disease onset attenuated the severity of EAE but failed to treat the disease when injections started after the onset of symptoms (Walters et al., 2002). Recently, flavonoids have also been shown to be protective in EAE through downregulating Rho GTPase activity (Hendriks et al., 2004). It is therefore

Abbreviations: APP, amyloid precursor protein; ERM, ezrin/radixin/moesin; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MBP, myelin basic protein; NF, neurofilament; PLP, proteolipid protein.

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doi:10.1016/j.jneuroim.2006.06.027

Table 1
Fasudil ameliorates the development of acute EAE

Treatment	n	EAE incidence (%)	Mean maximum clinical score	Mean day of onset
Intraperitoneal administration				
Vehicle	9	100.0	2.33	13.3
Fasudil	7	42.9 [a]	1.00	12.7
Oral administration				
Vehicle	8	100.0	2.88	12.9
Fasudil	11	54.5 [a]	1.64	12.7

[a] $p<0.05$, significantly different from vehicle-treated control group.

critical to know whether direct inhibition of Rho-kinase may have a protective effect on EAE.

On the other hand, since cholesterol is essential for myelination (Saher et al., 2005) and neurite growth and maintenance (Schulz et al., 2004), cholesterol-lowering effects of statins may be unfavorable for MS. We therefore studied the effects of the specific inhibitor of Rho-kinase fasudil, which has been safely used for vasospasm following subarachnoid hemorrhage since 1995 in Japan (Tachibana et al., 1999), in EAE. In this report, we demonstrate that fasudil acts both in a preventive and therapeutic fashion in EAE through a down-regulation of IL-17 producing T cells and Th1 cells.

2. Materials and methods

2.1. Animals

Female SJL/J mice, 6–7 weeks old, were purchased from Charles River Japan Inc. All animal protocols were approved by the Committee on Ethics in Animal Experiments of the Kyushu University and were performed according to the Guidelines for Animal Experiments of the Kyushu University and of the Japanese Government.

2.2. Antigen and antibodies

The PLP peptide p139-151 (HSLGKWLGHPDKF) was synthesized using a peptide synthesis system (Applied Biosystems), based on the 9-fluorenylmethyloxycarboneyl (Fmoc) strategy and purified by C18 reverse-phase HPLC. The purity of the peptide was >95% as determined by HPLC analysis (Minohara et al., 2001). The following primary antibodies were used for immunohistochemistry and Western blot analysis: anti-ERM antibody, anti-Phospho-ERM antibody (Cell Signaling Technology), anti-MBP antibody (Acris Antibodies, Germany), anti-mouse CD45 antibody (BD Biosciences), anti-NF 200 kD antibody and anti-APP antibody (Chemicon).

2.3. Induction and clinical evaluation of EAE in SJL/J mice

EAE was induced in SJL/J mice by immunization with 200 μg PLP p139-151 emulsified in an equal volume of complete Freund's adjuvant containing 4 mg/ml of heat-killed mycobacterium tuberculosis H37Ra (Difco). The PLP emulsion (0.1 ml) was injected subcutaneously in both sides of the rear flank. 200 ng of pertussis toxin (Sigma–Aldrich) was given intraperitoneally at the time of immunization and 48 h later. Every day, mice were weighed and examined for clinical signs of EAE and scored as follows: 0, normal; 1, limp tail; 2, impaired rightening reflex; 3, partial hind limb paresis; 4, total hind limb paralysis; 5, moribund or death.

2.4. Fasudil treatment

Fasudil (Asahi Chemical Industries, Tokyo, Japan) was administered orally or intraperitoneally. For oral treatment, fasudil was then dissolved in the drinking water to reach a

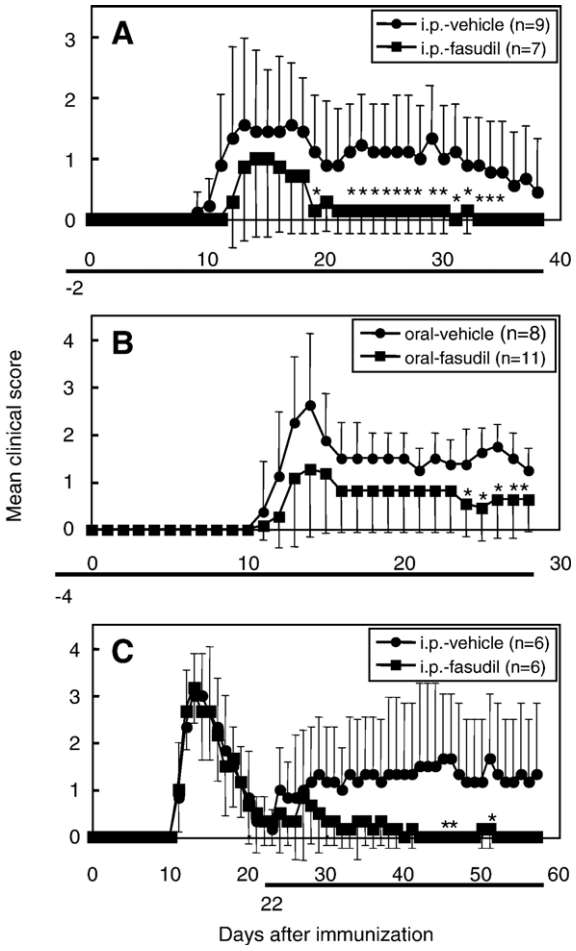

Fig. 1. Fasudil treatment inhibits acute and relapsing EAE. (A) Mice sensitized for EAE were injected intraperitoneally with fasudil (50 mg/kg/day) from day-2. EAE incidence and severity were significantly reduced in fasudil-treated mice ($n=7$) compared with vehicle (PBS)-treated controls ($n=9$) (*, $p<0.05$). (B) Oral fasudil administration ($n=11$) from day −4 also significantly prevented occurrence of EAE and decreased its severity compared with vehicle-treated mice ($n=8$) (*, $p<0.05$). (C) When fasudil treatment started at day 22, relapses were greatly inhibited and clinical symptoms showed significant alleviation after day 45 (*, $p<0.05$) compared with vehicle-treated mice ($n=6$ in each group). Data are presented as mean ± SD of clinical scores.

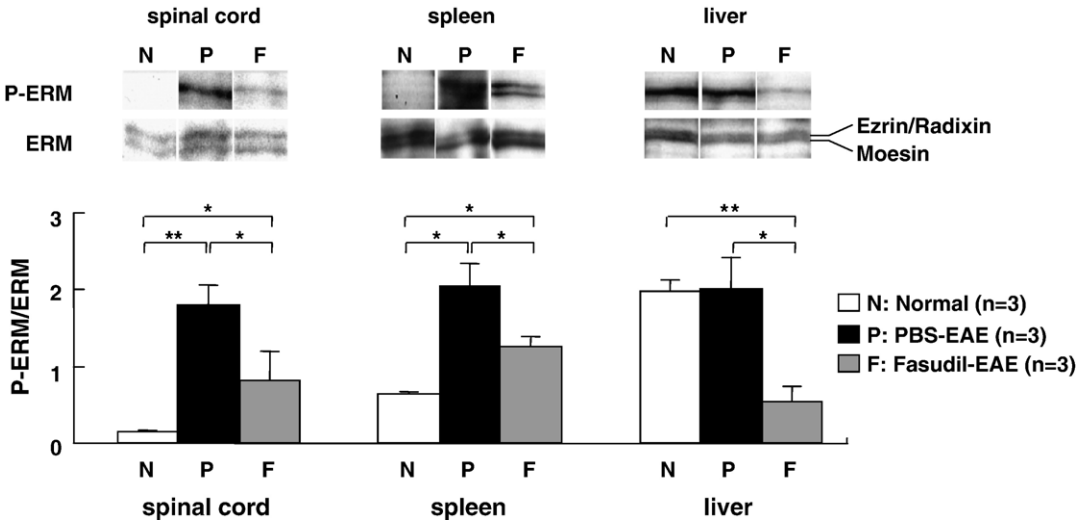

Fig. 2. Rho-kinase activity in EAE. Western blot analysis for phosphorylated ERM, a marker of Rho-kinase activity, normalized by total ERM, in spinal cord (day 14), spleen (day 10) and liver (day 10) of normal mice and of PLP-induced EAE mice. In spinal cord and spleen, ERM phosphorylation was significantly increased in PBS-treated EAE mice as compared with normal mice, which was suppressed by the fasudil treatment. (*, $p<0.05$; **, $p<0.01$). In liver, ERM phosphorylation was also suppressed by fasudil treatment as compared with PBS-treated EAE mice and normal mice. Results are expressed as mean±SEM.

final dosage of 100 mg/kg/day (Higashi et al., 2003) and the treatment was started 4 days before antigen immunization. To adjust the daily intake of fasudil, we measured the water intake and body weight on a daily basis. The fasudil-treated mice were able to freely access the water in which fasudil was dissolved. Mice with normal drinking water served as control. For intraperitoneal treatment, fasudil suspended in phosphate buffered saline (PBS) (50 mg/kg) was administered daily from 2 days before antigen immunization while PBS alone was administered as a control.

2.5. Antigen-specific T cell proliferation assays

Splenocytes or LN cells were harvested and processed into single cell suspensions. Cells (2×10^5 cells/well) were distributed into 96-well round-bottom plates (Falcon) and cultured with PLP p139-151 (0.1, 0.5, 1, 5, 10 μM), PHA (10 μg/ml), or medium alone. After 48 h of culture, 1 μCi of [^{3}H] thymidine was added per 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 supernatant from cultures of splenocytes and LN cells was harvested at 72 and 120 h: 72 h for IL-1β, IL-2, GM-CSF, IFN-γ, TNF-α and IL-17, and 120 h for IL-4, IL-5 and IL-10. Levels of IL-1β, IL-2, GM-CSF, IFN-γ, TNF-α, IL-4, IL-5 and IL-10, IL-17 were simultaneously analyzed using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories) according to the manufacturer's instructions as described previously (Ishizu et al., 2005). All assays were performed in triplicate.

2.7. Western blot analysis for ERM phosphorylation

To quantify Rho-kinase activity in the spinal cord (day 14 after immunization), liver (day 10) and spleen (day 10), Western blot analysis for phosphorylated ERM was performed by using antibodies that specifically recognize phosphorylated ERM (ezrin T567, radixin T564 and moesin T558) and total ERM as described previously (Higashi et al., 2003). ERM is phosphorylated by Rho-kinase at T567 (ezrin), T564 (radixin) and T558 (moesin). Equal amounts of extracted protein was loaded for SDS-PAGE/immunoblot analysis. The regions containing ERM family proteins were visualized by electrochemiluminescence. Band intensities from Western blots were quantified densitometrically by ImageJ 1.34s downloaded from http://rsb.info.nih.gov/ij. The extent of ERM phosphorylation was normalized by that of total ERM.

2.8. Histopathology and immunohistochemistry

Mice were anesthetized and perfused with PBS and 4% buffered paraformaldehyde. Spinal cords were collected on day 14 and 42 after antigen immunization in the preventive study, and on day 67 in the therapeutic study. The tissue was dissected and post-fixed in 4% buffered paraformaldehyde solution and embedded in paraffin. After embedding, 6-μm-thick section were prepared. For routine neuropathological evaluation, sections were stained with the hematoxylin-eosin (H–E) stain. For immunohistochemical analysis, sections were deparaffinized in xylene, hydrated in ethanol, and sections were 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

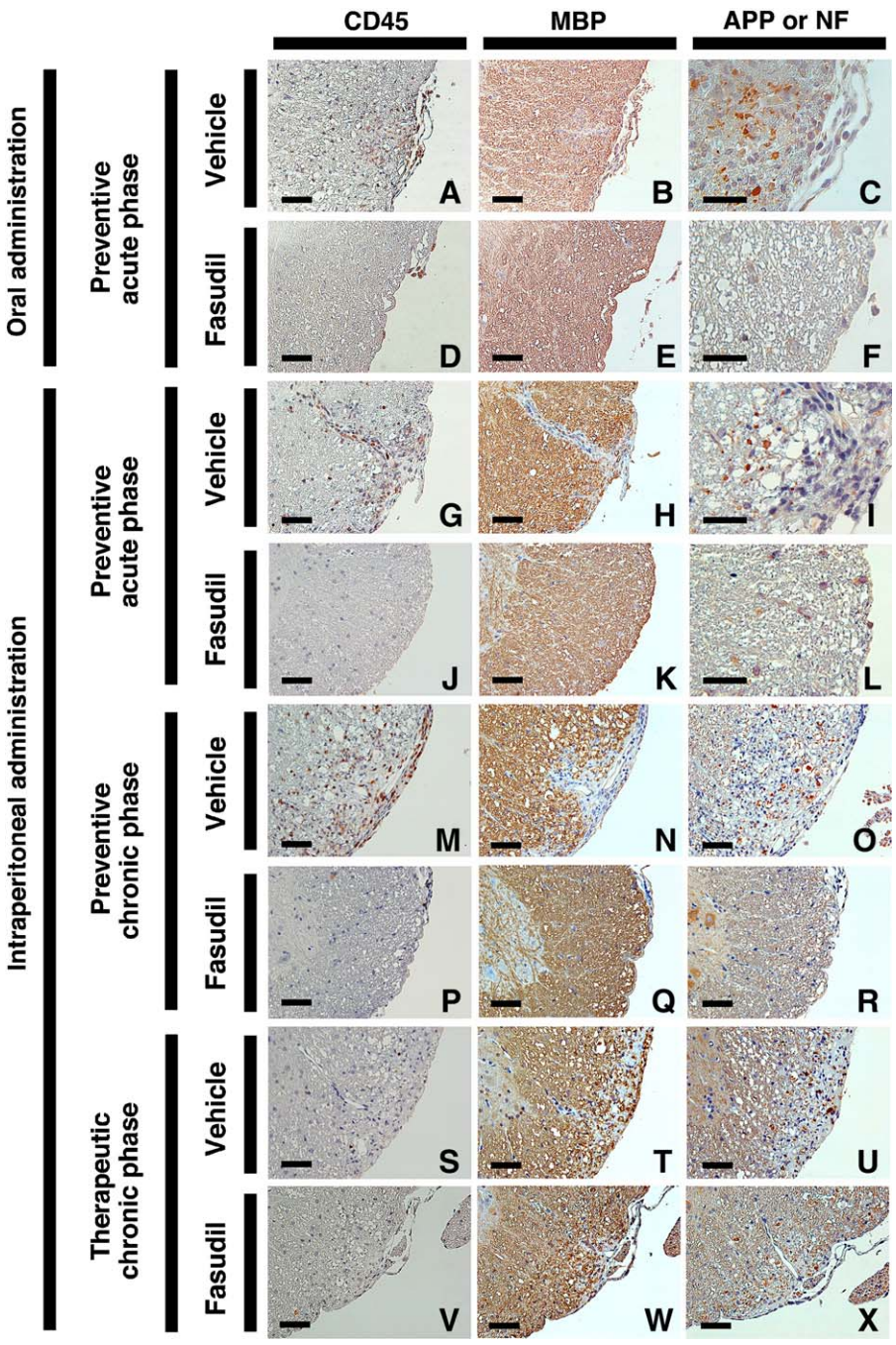

Fig. 3. Decreased inflammation, demyelination and axonal loss in spinal cord of fasudil-treated mice with EAE. Either vehicle or fasudil was given daily until the animals were sacrificed. Spinal cords lesions were assessed by immunohistochemical staining of CD45 (left), MBP (middle), APP (right, C, F, I, L) and NF (right, O, R, U, X). When fasudil administration started before immunization, in the acute phase (spinal cords collected on day 14, A to L), vehicle-treated mice showed leukocyte infiltrations at perivascular areas, parenchyma and meninges (A, G), and demyelination (B, H) and axon transection (C and I) were also evident. Meanwhile, fasudil-treated mice, both with oral (D to F) and intraperitoneal (J to L) administrations, showed a significant reduction of leukocytes infiltration (D and J), demyelination (E and K) and axon transaction (F and L). In the chronic phase (spinal cords collected on day 42, M to R), leukocyte infiltration, demyelination and axonal damage were more distinct in PBS-treated mice (M to O), but these findings were dramatically reduced in fasudil-treated ones (P to R). When fasudil administration started from day 22 after immunization, fasudil-treated mice demonstrated a significant reduction of leukocyte infiltration (V), demyelination (W) and axonal destruction (X) at day 67 compared with those of PBS-treated mice (S to U). The scale bar in A, B, D, E, G, H, J, K, M–X = 50 μm, C, F, I, L = 25 μm.

15 min to enhance the immunoreactivity of CD45, MBP, NF and APP. Subsequently, sections were incubated with primary antibody diluted in 5% non-fat milk in TBST (25 mM Tris–HCl pH 7.6 containing 0.5 M NaCl, 0.05%, NaN_3 and 0.05% Tween 20) for 1 h at room temperature (Kikuchi et al., 1999). As secondary antibody, biotinylated goat anti-rabbit IgG and streptavidin peroxidase or Simple Stain mouse MAX-PO (Rat) (Nichirei, Japan) were used. When mouse primary antibody

Table 2
Fasudil suppresses histological EAE[a]

Treatment	Inflammatory infiltrates[b]	Demyelination (%)[c]	Axonal loss (%)[c]	APP positivity[d]
Intraperiponeal administration before immunization				
Acute phase (day 14)				
Vehicle	1.94±0.16	1.28±0.18	0.66±0.12	16.41±3.01
Fasudil	0.82±0.15[e]	0.38±0.10[e]	0.15±0.08[e]	1.16±0.51[e]
Chronic phase (day 42)				
Vehicle	1.96±0.24	7.96±2.42	13.18±3.83	NA
Fasudil	0.20±0.09[e]	0.45±0.19[e]	0.31±0.14[e]	NA
Oral administration before immunization				
Acute phase (day 14)				
Vehicle	2.37±0.07	2.69±0.30	2.64±0.31	19.76±3.06
Fasudil	1.28±0.11[e]	0.91±0.17[e]	0.71±0.15[e]	5.83±1.81[e]
Intraperiponeal administration from day 22				
Chronic phase (day 67)				
Vehicle	2.63±0.11	8.01±0.91	10.63±1.40	NA
Fasudil	1.24±0.14[e]	2.77±0.43[e]	3.30±1.16[e]	NA

NA: not applicable.
[a] A total of 3–4 mice per group, and 12 spinal cord sections per mouse which covered the whole length of the spinal cord were examined and quantified.
[b] Inflammatory infiltrates: 0, no positive cells visible; 1, a few inflammatory cells; 2, organization of perivascular infiltrates; 3, increasing severity of perivascular cuffing with extension into the adjacent tissue.
[c] Demyelination and axonal loss were quantified as percentages of areas of demyelination and axonal loss against total white matter area examined by MBP and NF immunostaining, respectively.
[d] APP positivity was quantified by counting APP positive axons in a defined quarter of each section and calculated as positive axons per mm^2.
[e] $p<0.01$, significantly different from vehicle-treated control group.

was used, the Histofine mouse staining kit (Nichirei, Japan) was used to block endogenous mouse immunoglobulins in the tissue. The colored reaction product was developed using Simple Stain DAB solution (Nichirei, Japan). The sections were counterstained lightly with hematoxylin. In each group, 12 sections per mouse which covered the whole length of spinal cord were histologically examined and quantified. Inflammatory cell infiltrates were graded by CD45 immunostaining as: 0: no positive cells visible, 1: a few inflammatory cells, 2: organization of perivascular infiltrates, 3: increasing severity of perivascular cuffing with extension into the adjacent tissue. ImageJ 1.34s was used to calculate percentages of demyelinated areas per total white matter area examined by MBP immunostaining, and areas of axonal loss per total white matter area examined by NF immunostaining (Pluchino et al., 2003). Axonal transection was evaluated by APP immunoreactivity and quantified by counting APP positive axons in a defined quarter of each section and calculated as positive axons per mm^2.

2.9. Statistical analysis

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

3. Results

3.1. Fasudil suppresses PLP-induced EAE

Intraperitoneal injection of fasudil significantly reduced incidence of EAE in SJL/J mice immunized with PLP p139-151 ($p<0.05$) (Table 1). All PBS-treated mice developed neurological symptoms with the average onset at day 13.3 and the peak at day 14 while in fasudil-treated mice, only 42.9% of mice developed neurological symptoms with the average onset at day 12.7 and the peak at day 16. The severity of the disease in fasudil-treated mice was significantly reduced at day 19 and day 22–35, as compared with PBS-treated mice ($p<0.05$) (Fig.1A). The oral administration of fasudil was examined next. According to the results of preliminary experiments on the volume of daily water intake of mice, the concentration of fasudil was determined to reach a dosage of 100 mg/kg/day. All control mice developed neurological impairment while only 54.5% of mice orally treated with fasudil developed a comparatively mild EAE (Table 1). The incidence and severity of EAE after day 24 were significantly reduced in the orally fasudil-administered group compared to vehicle controls ($p<0.05$) (Fig. 1B).

3.2. Rho-kinase activity in mice on EAE with or without fasudil

Ezrin/radixin/moesin (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 as a marker of the Rho-kinase activity *in vivo* in the spinal cord, liver and spleen. In the spinal cord (day 14) and spleen (day 10), the ratio of phosphorylated ERM against total ERM in PBS-treated animals showed a major increase compared with normal mice ($p<0.01$), and was inhibited significantly in fasudil-treated animals ($p<0.05$) (Fig. 2). In the liver, 10 days after antigen immunization the ratio of phosphorylated to total ERM in fasudil-treated animals also decreased significantly compared with PBS-treated animals and normal animals ($p<0.05$). These data indicate that Rho-kinase activity is up-regulated in the spinal cord and spleen upon EAE and fasudil markedly suppresses its activity *in vivo*.

3.3. Fasudil decreases infiltration of inflammatory cells into the CNS

Histopathological examination of the preventive study (administration before immunization) revealed moderately damaged lesions in the white matter of the spinal cord in the acute phase of the vehicle group at the peak of disease progression (day 14) (Fig. 3A to C, G to I and Table 2). In this group, CD 45 positive inflammatory cells infiltrated the

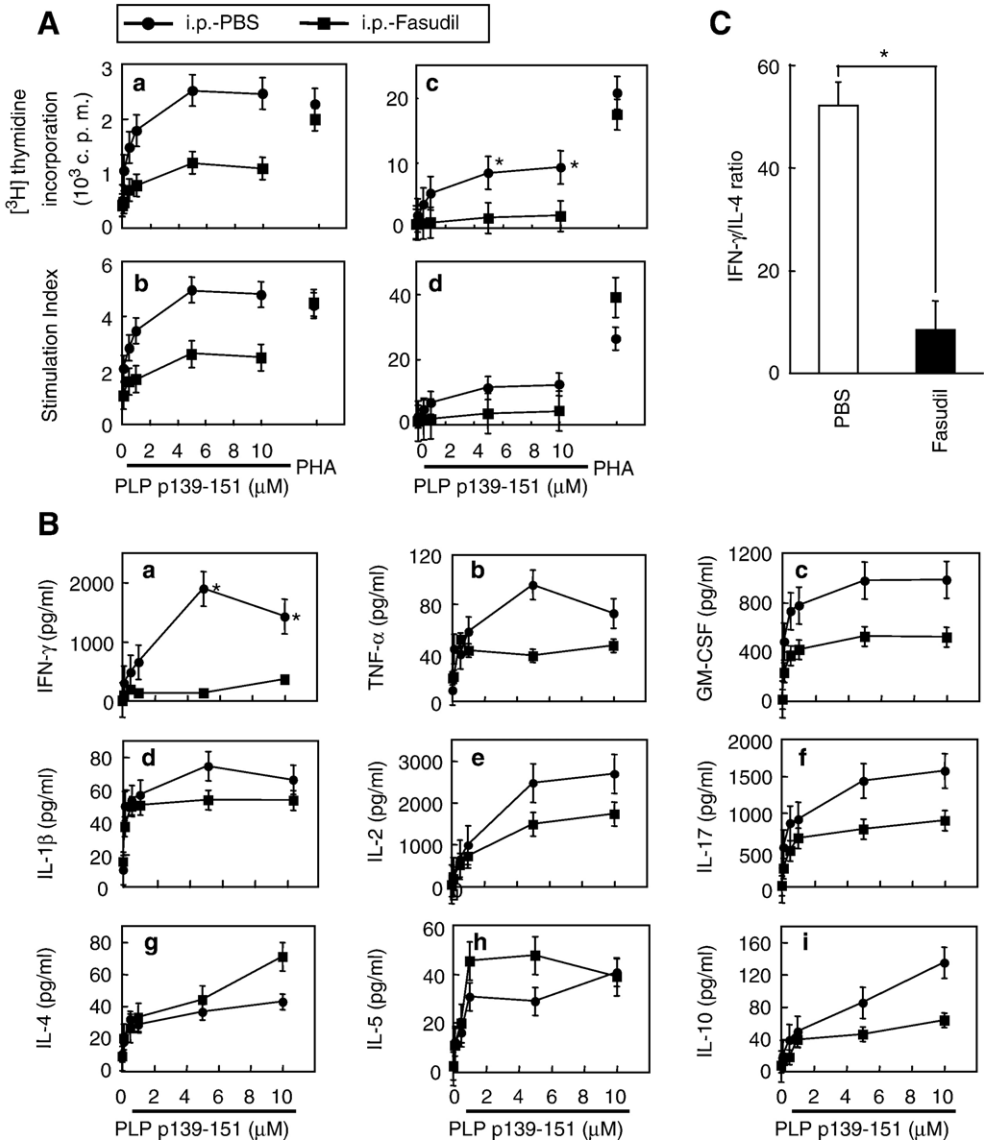

Fig. 4. Fasudil suppressed antigen-dependent proliferation of lymphocytes and induced a Th2 cytokine bias in PLP-induced EAE mice. Splenocytes and LN cells were harvested from fasudil-treated ($n=3$) or PBS-treated ($n=3$) mice at day 10 after immunization. The cells were cultured with PLP p139-151, PHA or medium alone. T cell proliferation was determined by [^{3}H] thymidine incorporation. Cytokines were measured by Bio-Plex Cytokine Assay System and ELISA. (A) PLP p139-151-specific proliferation of splenocytes (a, b) and LN cells (c, d) were markedly reduced in fasudil-treated mice (closed square) compared with PBS-treated mice (closed circle). (B) Cytokine assay of supernatants from splenocyte culture with PLP p139-151. Fasudil treatment was associated with a marked reduction in secretion of IFN-γ (a) ($p<0.05$) and a moderately reduction of IL-17 (f), TNF-α (b), GM-CSF (c), IL-1β (d), IL-10 (i), IL-2 (e) secretion. In contrast, secretion of IL-4 (g) and IL-5 (h) was slightly increased. (C) The IFN-γ/IL-4 ratio in the culture supernatant of splenocytes from PLP-sensitized mice stimulated with PLP p139-151 greatly decreased in fasudil-treated mice compared with PBS-treated mice ($p<0.05$). Data shown are mean±SE of a group ($n=3$).

demyelinated areas (Fig. 3A, G) and numerous amyloid precursor protein (APP)-positive transected axons were visible (Fig. 3C, I) in the lesions. Neurofilament (NF) immunostaining was relatively preserved in this phase (Table 2). In contrast, inflammatory infiltrated cells, demyelinated areas and axonal transactions were dramatically recovered in the fasudil treated groups with both oral (Fig. 3D to F) and intraperitoneal administration (Fig. 3J to L) as compared with the vehicle group (Table 2). In the chronic phase, inflammatory infiltrates (Fig. 3M, P) were significantly reduced in fasudil-treated mice as compared with PBS-treated mice, both myelin basic protein (MBP) (Fig. 3N, Q) and NF (Fig. 3O, R) immunostaining severely decreased in PBS-treated mice but not in fasudil-treated mice (Table 2). These findings indicated that fasudil suppresses inflammatory cell infiltration, demyelination and acute axonal transection in the CNS.

3.4. Fasudil treatment induces a reduction of PLP-specific T cell proliferation and a Th2 cytokine bias

To gain insights into the mechanisms of the inhibitory effects of fasudil, splenocytes and lymph node (LN) cells

were removed from fasudil-treated and PBS-treated mice on day 10, and the cells were restimulated with PLP p139-151 at different concentrations and their cytokine production and antigen-specific proliferation were measured. Fig. 4A shows the results of the proliferation of splenocytes and LN cells, which indicated that treatment of mice with fasudil suppressed the proliferative response to the antigen. Inhibition of proliferation appeared to be dose-related (LN cells: $p<0.05$). Culture supernatants were then examined for cytokines. As shown in Fig. 4B, fasudil treatment was associated with a marked reduction in secretion of IFN-γ ($p<0.05$) and a moderately reduction of IL-17, TNF-α, GM-CSF, IL-1β, IL-10, IL-2 secretions. In contrast, secretion of IL-4 and IL-5 was slightly increased. As a result, the IFN-γ/IL-4 ratio in the supernatant was strongly suppressed in fasudil-treated mice as compared with PBS-treated ones ($p<0.05$) (Fig. 4C).

3.5. Fasudil treats chronic EAE when administered after disease onset

The therapeutic potential of fasudil was assessed by intraperitoneal treatment of PLP-induced EAE mice daily with 50 mg/kg fasudil from day 22 after immunization when all mice were in their first recovery phase. As shown in Fig. 1C, PBS-treated mice developed a more severe relapse than fasudil-treated mice. In fasudil-treated mice, clinical symptoms showed a significant reduction at day 45, 46, and 51 ($p<0.05$). Moreover, in this therapeutic study, histological examination (Fig.3S to X and Table 2) disclosed that demyelination was significantly reduced in the fasudil-treated mice (Fig. 3T, W and Table 2) together with decreased inflammatory cell infiltration (Fig. 3S, V and Table 2) while NF staining was significantly preserved (Fig. 3U, X and Table 2). These data demonstrate that fasudil administrated after the onset of EAE significantly reduces the development and severity of EAE through prevention of leukocyte infiltration into the CNS and rescuing myelin and axons from damage.

4. Discussion

As shown in the present study, the specific Rho-kinase inhibitor fasudil, is protective in acute and chronic EAE induced by PLP p139-151 in mice. The drug was preventive when administered before immunization and therapeutic when administered after the onset of the disease. According to previous studies, the beneficial effects of statins on EAE and MS can partly be explained by inhibition of the isoprenylation of Rho GTPase (Greenwood et al., 2003; Neuhaus et al., 2004), which results in a Th2 shift acting on both T cells and antigen-presenting cells (APC) (Aktas et al., 2003; Nath et al., 2004; Youssef et al., 2002) and an inhibition of T cell migration to the CNS acting on both T cells (Weitz-Schmidt et al., 2001) and brain endothelial cells(Greenwood et al., 2003). In accordance with this, Walters et al. (2002) demonstrated that protein prenyltransferase inhibitors partially suppressed EAE when administered before the onset of disease, whereas no therapeutic effect was found when the administration started after the onset. Flavonoids have also been shown to be protective in EAE by modulating the activity of Rho GTPase (Hendriks et al., 2004). In addition, 17β-estradiol, which is also protective in EAE through inhibition of the production and migration of encephalitogenic T cells and is neuroprotective when initiated before immunization (Offner, 2004), downregulates expression and function of Rho-kinase *in vivo* (Chrissobolis et al., 2004; Hiroki et al., 2005). Collectively, inhibition of the Rho/Rho-kinase system is considered to be protective against EAE. Based on our findings that fasudil markedly reduced relapse and protected mice from development of progressive disability even when initiated after the first episode of EAE, the direct inhibition of Rho-kinase itself appears to be more beneficial in the progression of inflammatory demyelination of the CNS. The effects of Rho-kinase inhibitors have been shown to be more evident in males than in females (Chrissobolis et al., 2004), partly reflecting the suppressive effects of estrogen on Rho-kinase (Hiroki et al., 2005); however, in our study, female SJL/J mice reacted well to fasudil in EAE.

The Rho family proteins of small 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 (Amano et al., 2000), although several other proteins have also been identified as effectors of Rho, including protein kinase N, rhophilin, rhotekin, citron, p140mDia and citron kinase (Hall, 1998; Kaibuchi et al., 1999). 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 (Fukata et al., 2001; Shimokawa and Takeshita, 2005). Direct inhibition of Rho-kinase activity induced suppression of cell proliferation and motility. For immune cells, Rho-kinase inhibitor has been shown to suppress T cell proliferation (Tharaux et al., 2003) and traffic (Bardi et al., 2003) and down-modulate both Th1 and Th2 cytokine production (Aihara et al., 2003). Furthermore, Rho-kinase inhibitor suppressed chemotaxis of neutrophils and eosinophils (Adachi et al., 2001; Alblas et al., 2001; Niggli, 1999). In the present study, specific proliferations to PLP p139-151 of LN cells and splenocytes were significantly suppressed in the fasudil-administered animals and the production of IL-17 and Th1 cytokines were severely inhibited while Th2 cytokines, such as IL-4 and IL-5, were slightly enhanced, resulting in a marked decrease of the IFN-γ/IL-4 ratio. These findings are consistent with the report of another Rho-kinase inhibitor, Y-27632, that strongly suppressed the production of IFN-γ but suppressed only weakly IL-4 and IL-5 in human peripheral blood T cells (Aihara et al., 2003). Therefore, in addition to a direct inhibition on leukocyte proliferation, a pronounced Th2 shift in fasudil-treated animals may well be beneficial for EAE. Yet, the production of the anti-inflammatory cytokine IL-10 was also suppressed in our study. Moreover, increasing evidence suggests that IL-17

producing T cells are a completely separate lineage from Th1 and Th2 cells (Harrington et al., 2005; Park et al., 2005) and play a critical role in the induction of autoimmune diseases such as MS and rheumatoid arthritis (Ishizu et al., 2005; Langrish et al., 2005; Murphy et al., 2003). In this study, the antigen-specific production of IL-17 was markedly suppressed in the fasudil-treated mice. Thus, fasudil's protective effects on EAE may in part be explained by a down-modulation of IL-17 producing encephalitogenic T cells.

Previous studies by Ford et al. (2003) demonstrated that EAE was significantly attenuated in $CD43^{-/-}$ mice due to decreased lymphocyte trafficking to the CNS and affected T cell differentiation and cytokine production, as myelin-specific $CD43^{-/-}$ $CD4^{+}$ T cells exhibited reduced IFN-γ and increased IL-4 production. CD43 has been implicated in the regulation of both T cell homing and activation (Cullinan et al., 2002; Ostberg et al., 1998) and has been shown to move away from the immunological synapse between T cells and APCs, where ERM function is necessary (Allenspach et al., 2001). In fact, 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 states to many transmembrane proteins, such as CD43 (Cullinan et al., 2002). Inhibition of ERM function thus decreases production of IFN-γ and IL-2 (Allenspach et al., 2001). Thus, suppression of ERM by fasudil *in vivo*, as shown in the present study, may also contribute to the amelioration of EAE partly through a down-regulation of Th1 cytokine.

Increasing evidence suggests that Rho-kinase induces retraction of axons while fasudil facilitates axonal growth through inhibition of the kinase (Mueller et al., 2005; Sakisaka et al., 2004). Previous studies by Wolf et al. (2001) demonstrated that Rho-kinase also regulates oligodendrocyte process formation. Expression of dominant negative Rho in primary oligodendrocytes caused a hyperextension of processes, whereas constitutively activated Rho reduced process formation. As fasudil was shown to transfer intrathecally (Hanada et al., 2005), fasudil may offer the possibility of functional recovery of EAE through facilitation of axonal growth and myelination. Since inflammatory stimuli up-regulated Rho-kinase expression (Hiroki et al., 2004) and Rho was shown to be up-regulated in MS plaques (Tajouri et al., 2003), direct inhibition of Rho-kinase is expected to be beneficial through not only a suppression of inflammatory cell infiltration into the CNS but also neuroregeneration. A marked reduction in the clinical disability and preservation of neurites in the chronic EAE model treated after the first attack with fasudil may suggest that this is plausible. As it is possible that HMG-CoA reductase inhibition by statins induces neurite loss and subsequent neuronal death (Schulz et al., 2004) and cholesterol is indispensable for myelination (Saher et al., 2005), Rho-kinase inhibitors may be more favorable than statins for MS.

Thus, our results indicate a beneficial role of selective blockade of Rho-kinase in autoimmune inflammation of the CNS and may provide a rationale for oral use of fasudil, which has been used with minimal side effects in more than 30,000 patients with subarachnoid hemorrhage (Mueller et al., 2005), in the treatment of MS. Although in an animal model of cardiovascular diseases, high dose fasudil (100 mg/kg/day), like in our study, was applied with the drinking water (Higashi et al., 2003; Abe et al., 2004), low dose fasudil (80 mg three times daily) has been reported to be effective and safe in human cardiovascular diseases (Vicari et al., 2005). Further study of low dose fasudil is called for.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Neuroimmunological Disease Research Committee grant from the Ministry of Health, Labour and Welfare of Japan for Research on Brain Science.

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