Journal of Neuroimmunology 256 (2013) 13–18

Contents lists available at SciVerse ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Fingolimod phosphate promotes the neuroprotective effects of microglia

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ARTICLE INFO

Article history:
Received 11 September 2012
Received in revised form 19 November 2012
Accepted 11 December 2012

Keywords:
Fingolimod
Microglia
Pro-inflammatory cytokine
Neurotrophic factor
Neuroprotection

ABSTRACT

Fingolimod phosphate (FTY720) is a sphingosine 1-phosphate (S1P) receptor agonist that is being used as a new oral drug for multiple sclerosis. FTY720 prevents lymphocytes from moving out of the lymphoid organs and inhibits autoreactive lymphocytes from infiltrating the central nervous system. Whether FTY720 directly affects microglia—the innate immune cells of the central nervous system—is unclear. Here we show that FTY720 binds $S1P_1$ receptors to downregulate activated microglial production of such pro-inflammatory cytokines as tumor necrosis factor-α, interleukin-1β, and interleukin-6. FTY720 also upregulates microglial production of brain-derived neurotrophic factor and glial cell-derived neurotrophic factor. These results suggested that FTY720 directly promotes the neuroprotective effects of microglia. Therefore, FTY720 may be a potent therapeutic agent for not only multiple sclerosis but also other neurologic diseases associated with microglial activation.

1. Introduction

Although the precise pathophysiologic mechanisms underlying multiple sclerosis (MS) are unknown, the disorder has been characterized as an autoimmune disease caused by autoreactive lymphocytes that specifically attack myelin antigens and induce demyelination and axonal injury (Glass et al., 2010). The immunomodulator fingolimod is a new oral medicine to treat relapsing-remitting MS. One clinical trial showed that oral fingolimod is more effective than the current interferon beta-1α therapies for patients with MS (Cohen et al., 2010). Fingolimod is phosphorylated by sphingosine kinase, resulting in a sphingoshine-1-phosphate (S1P) receptor agonist (Foster et al., 2007). There are five S1P receptor subtypes: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$. The expression levels of these receptors differ among various lymphocyte populations. T cells and B cells mainly express $S1P_1$ and $S1P_4$, whereas natural killer cells express $S1P_5$ (Foster et al., 2007; Chi, 2011). After binding $S1P_1$, FTY720 suppresses lymphocyte egress from lymphoid tissues, which prevents lymphocytes from invading the central nervous system (CNS) (Matloubian et al., 2004).

Microglia are resident immune cells in the CNS, where they play important roles in both innate and adaptive immunity (Kreutzberg, 1996; Town et al., 2005). Some of these monocyte-lineage cells are derived from the yolk sac (Ginhoux et al., 2010). Microglia are potent antigen-presenting cells in the CNS that function through major histocompatibility complex class II molecules to interact with invasive T helper (Th) cells and induce the differentiation of Th (Almolda et al., 2011). They present antigen through MHC class II molecule to invaded T helper cells and induce differentiation of Th. In addition, microglia can damage oligodendrocytes and neurons by producing glutamate and such pro-inflammatory cytokines as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 (Block et al., 2007; Takeuchi, 2010). Our previous studies demonstrated that activated microglia release high levels of glutamate, leading to neuronal damage (Takeuchi et al., 2005; Takeuchi et al., 2006a). Microglia can also release neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) (Mizuno et al., 2004; Liang et al., 2010), which support neural survival, growth, and plasticity. In MS, microglia in demyelinating lesions are thought to be involved in disease progression (Sriram, 2011). Therefore, inhibiting neurotoxic microglial activation may be a useful therapeutic strategy for MS.

Fingolimod can pass through blood–brain-barrier, and effects on CNS cells have been recently reported (Brinkmann, 2009; Chun and Hartung, 2010; Cohen and Chun, 2011). A recent report documented that fingolimod did not affect microglial production of IL-10 and IL-12 (Durafourt et al., 2011), however, the effects of fingolimod on microglia are still unclear. In the present study, we examined S1P receptor expression in microglia, and the potential effects of fingolimod phosphate (FTY720) on microglia. FTY720 inhibited microglial production of pro-inflammatory cytokines via $S1P_1$ and enhanced the expression of neurotrophic factors, suggesting that FTY720 exerts favorable effects in the CNS.

2. Materials and methods

2.1. Reagents

The phosphorylated form of fingolimod (FTY720) was kindly provided by Tanabe Mitsubishi Pharma (Osaka, Japan). Lipopolysaccharide (LPS) was purchased from Sigma. S1P receptor antagonist used in this study included W146 ($S1P_1$ antagonist, Cayman Chemical, USA),

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http://dx.doi.org/10.1016/j.jneuroim.2012.12.005

suramin ($S1P_3$/$S1P_5$ antagonist, Sigma, USA), and CYM50358 ($S1P_4$ antagonists, Calbiochem, USA).

2.2. Cells

All protocols for animal experiments were approved by the Animal Experimental Committee of Nagoya University. Spleens were collected from C57BL/6 mice under deep anesthesia after peritoneal injections of pentobarbital. Mouse primary microglia were isolated from primary mixed glial cell cultures prepared from newborn C57BL/6 mice on day 14 in vitro using the "shaking off" method as described previously (Suzumura et al., 1987). The purity of the culture was 97–100% as determined by immunostaining for the Fc receptors. Cells were maintained in DMEM supplemented with 10% fetal calf serum, 5 μg/ml of bovine insulin, and 0.2% glucose. To activate microglia, cells were treated with 1 μg/ml LPS. Cells were treated with 1–100 nM FTY720 from 1 h before stimulation with LPS. To block FTY720 binding to each S1P receptor subtype, microglia were treated with selective antagonists for $S1P_1$ (250 nM of W146 (Sanna et al., 2006)), $S1P_3$/$S1P_5$ (10 μM of suramin (Yoshino et al., 2011)), or $S1P_4$ (100 nM of CYM50358 (Guerrero et al., 2011)) from 1 h before FTY720 treatment as described previously. Assays were carried out in three independent trials.

2.3. Reverse transcription (RT)-PCR

Microglia were collected 12 h after stimulation with LPS. Total RNA was extracted from microglia or spleen using an RNeasy Mini Kit (Qiagen, USA). A first-strand cDNA library was obtained using SuperScript II and oligo$(dT)_{12-18}$ (Invitrogen, USA). RT-PCRs were performed to amplify transcripts encoding mouse $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$, TNF-α, IL-1β, IL-6, BDNF, GDNF, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using 0.1 μg of first-strand cDNA, Blend Taq polymerase (Toyobo, Japan), and specific oligonucleotide primers, which are described below.

$S1P_1$ forward: 5′-GACCATGGCATTAAACTGACTT-3′
$S1P_1$ reverse: 5′-TGTAGTTTCATCTTCAGCATGG-3′
$S1P_2$ forward: 5′-AAGTTCCACTCAGCAATGTACC-3′
$S1P_2$ reverse: 5′-TAGATGACAGGATTGAGCAGTG-3′
$S1P_3$ forward: 5′-TCTTCTTGGTCACCTGTAGCTT-3′
$S1P_3$ reverse: 5′-TCGATGAGGAAGAGGATAAAAA-3′
$S1P_4$ forward: 5′-GTGTACTACTGCCTGCTGAAC-3′
$S1P_4$ reverse: 5′-GATGAGAGGATTAATGGCTGAG-3′
$S1P_5$ forward: 5′-TATGTGCTCTTCTGCGTGCT-3′
$S1P_5$ reverse: 5′-TAGAGCTGCGATCCAAGGTT-3′
TNF-α forward: 5′-ATGAGCACAGAAAGCATGATCCGC-3′
TNF-α reverse: 5′-CCAAAGTAGACCTGCCCGGACTC-3′
IL-1β forward: 5′-ATGGCAACTGTTCCTGAACTCAACT-3′
IL-1β reverse: 5′-CAGGACAGGTATAGATTCTTTCCTTT-3′
IL-6 forward: 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′
IL-6 reverse: 5′-CACTAGGTTTGCCGAGTAGATCTC-3′
BDNF forward: 5′-AGCCTCCTCTGCTCTTTCTG-3′
BDNF reverse: 5′-TTGTCTATGCCCCTGCAGCC-3′
GDNF forward: 5′-TATCCTGACCAGTTTGATGA-3′
GDNF reverse: 5′-TCTAAAAACGACAGGTCGTC-3′
GAPDH forward: 5′-ACTCACGGCAAATTCAACG-3′
GAPDH reverse: 5′-CCCTGTTGCTGTAGCCGTA-3′

Assays were carried out in three independent trials.

2.4. Flow cytometry

Microglia were collected 24 h after stimulation with LPS. Cells were fixed with 4% paraformaldehyde and stained with anti-$S1P_1$,

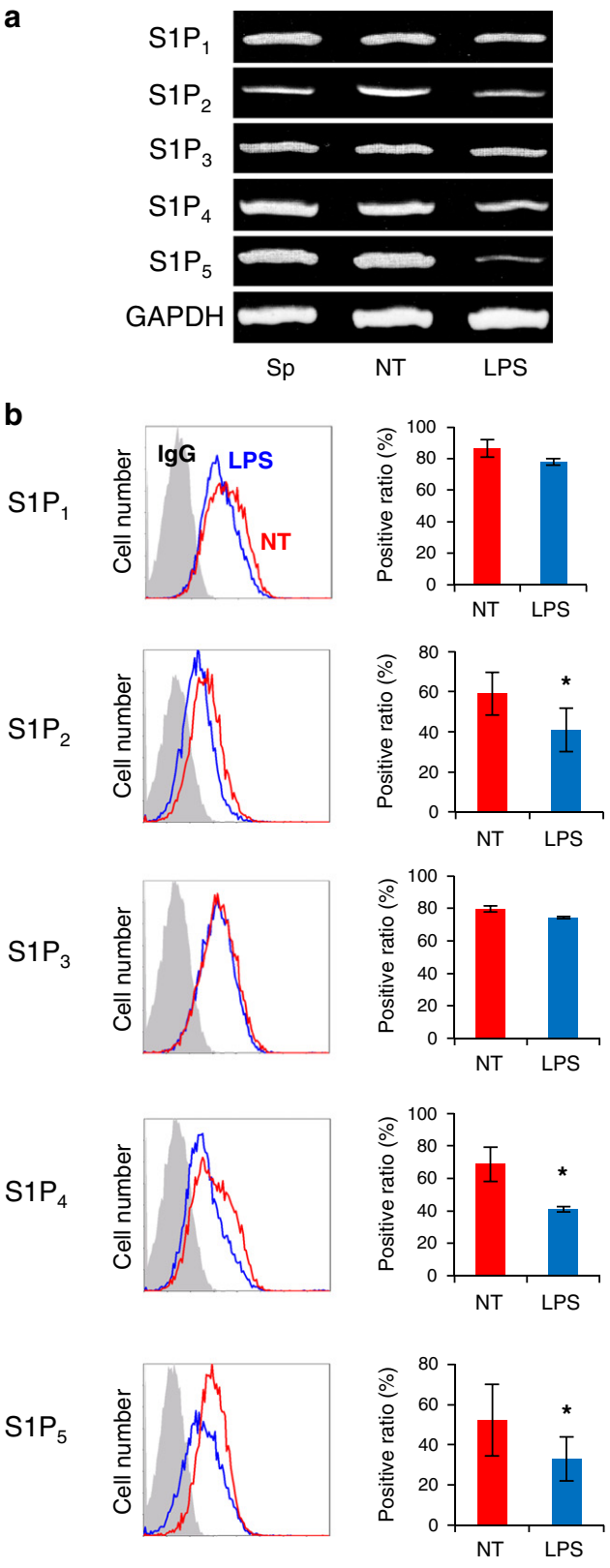

Fig. 1. Microglia express all S1P receptor subtypes. (a) Representative RT-PCR data reflecting S1P receptor subtype expression. Assays were carried out in three independent trials. Sp, control spleen tissue; NT, untreated microglia; LPS, LPS-treated microglia. (b) Flow cytometric analysis of S1P receptor subtype expression. Left panel, representative image of flow cytometric analysis; right panel, quantitative data of flow cytometric analysis. IgG, immunoglobulin G isotype; NT, untreated microglia; LPS, LPS-treated microglia. Microglia expressed all S1P receptor subtypes, and LPS downregulated expression levels of $S1P_2$, $S1P_4$, and $S1P_5$. Assays were carried out in three independent trials. The values shown are means ± SEM. *, $P<0.05$ versus NT samples.

anti-$S1P_2$, anti-$S1P_3$, anti-$S1P_4$, and anti-$S1P_5$ rabbit polyclonal antibodies (Cayman Chemical). Then, samples were stained with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G (IgG; Invitrogen). Samples were examined with a Cytomics FC500 system and analyzed with CXP Software version 2.0 (Beckman Coulter, USA). Assays were carried out in three independent trials.

2.5. Enzyme-linked immunosorbent assays (ELISAs)

Microglia were collected 24 h after stimulation with LPS. Microglial production of IL-1β, IL-6 and TNF-α was assessed with specific ELISA kits (IL-1β, R&D Systems, USA; IL-6 and TNF-α, BD Bioscience, USA) according to the manufacturers' protocols as described previously (Mizuno et al., 2004). Assays were carried out in three independent trials.

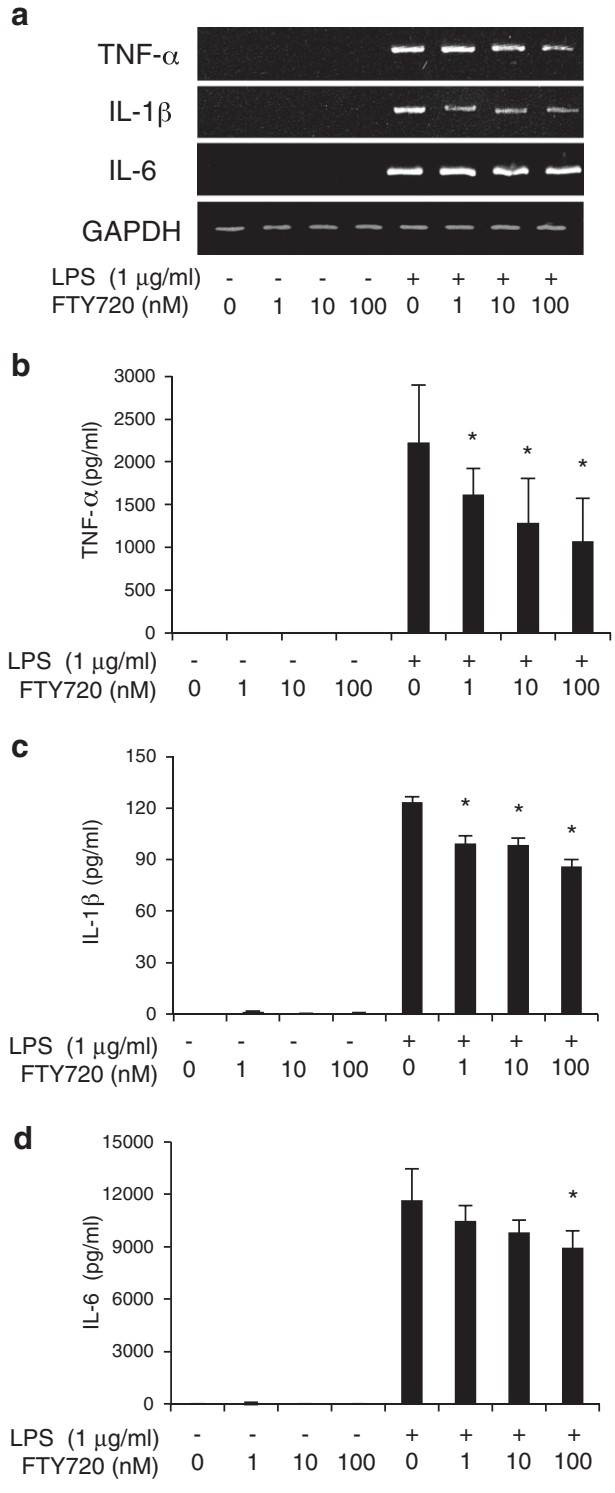

Fig. 2. FTY720 downregulates microglial production of pro-inflammatory cytokines. (a) Representative RT-PCR data reflecting microglial expression of IL-1β, IL-6, and TNF-α. Assays were carried out in three independent trials. (b) ELISA data representing microglial TNF-α production. (c) ELISA data representing microglial IL-1β production. (d) ELISA data representing microglial IL-6 production. FTY720 dose-dependently decreased microglial production of IL-1β, IL-6, and TNF-α. Assays were carried out in three independent trials. The values shown are means ± SEM. *, $P<0.05$ versus LPS-treated microglia.

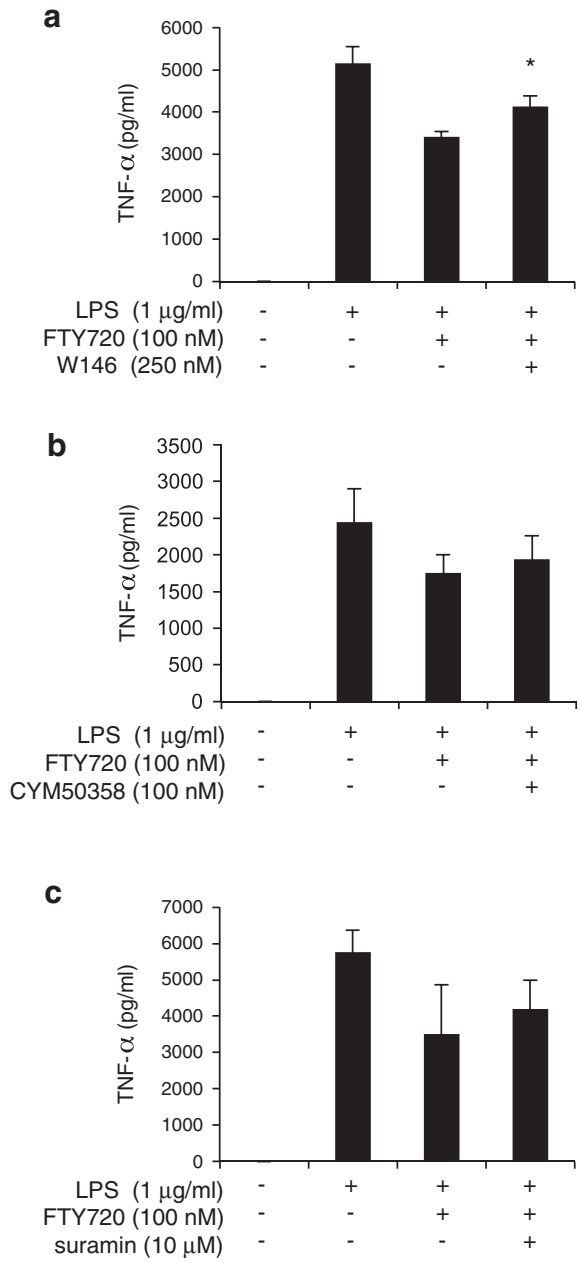

Fig. 3. FTY720 suppresses microglial TNF-α production via $S1P_1$ binding. (a–c) ELISA data representing TNF-α production in microglia treated with the $S1P_1$ antagonist W146 (a), the $S1P_3$/$S1P_5$ antagonist suramin (b), and the $S1P_4$ antagonist CYM50358 (c). Blocking FTY720 binding to $S1P_1$ significantly reduced the suppressive effect of FTY720 on microglial TNF-α production. Assays were carried out in three independent trials. The values shown are means ± SEM. *, $P<0.05$ versus LPS-treated microglia; †, $P<0.05$ versus LPS- and FTY720-treated microglia.

2.6. Western blotting

Microglia were collected 48 h after stimulation with LPS. Cells were lysed in TNE buffer (50 mM Tris–HCl at pH7.5, 150 mM NaCl, 1% Nonidet P-40, and 2 mM EDTA) with a protease inhibitor cocktail (Complete Mini EDTA-free, Roche, Switzerland). Twenty micrograms of protein from total cell lysates was separated on a 4–20% Tris–glycine SDS-polyacrylamide gradient gel and transferred to a Hybond-P polyvinylidene fluoride membrane (Bio-Rad, USA) as described previously (Takeuchi et al., 2002). Membranes were blocked using 1% skim milk in Tris-buffered saline solution containing 0.05% Tween 20. Blots were incubated with rabbit polyclonal anti-mouse BDNF antibodies (sc-546; Santa Cruz Biotechnology, USA), rabbit polyclonal anti-mouse GDNF antibodies (sc-328; Santa Cruz), or mouse monoclonal anti-β-actin antibodies (AC-15; Sigma) overnight at 4 °C. Then, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (GE healthcare, USA) for 1 h at room temperature. The signals were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, USA). The intensity of the bands was calculated using a CS Analyzer 1.0 (Atto Corp, Japan). Assays were carried out in three independent trials.

2.7. Statistical analysis

Statistical significance was assessed using Student's *t*-tests and one-way analysis of variance (ANOVA) in PRISM version 5.0 (GraphPad Software, USA). $P<0.05$ was defined as significant.

3. Results

3.1. Microglia express all S1P receptor subtypes

First, we assessed the expression profiles of S1P receptors in mouse microglia. As shown in Fig. 1, microglia expressed all S1P receptor subtypes, with the highest expression levels observed for $S1P_1$. Stimulation with LPS significantly reduced microglial expression levels of $S1P_2$, $S1P_4$, and $S1P_5$. Thus, S1P receptor expression in microglia may decrease under pathologic, inflammatory conditions.

3.2. FTY720 binds $S1P_1$ to reduce microglial production of pro-inflammatory cytokines

To examine how FTY720 affects neurotoxic effects of microglia, we evaluated the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. LPS enhanced the microglial production of the pro-inflammatory cytokines. These effects were reduced by FTY720 in a dose-dependent manner (Fig. 2a and b). FTY720 binds all S1P receptor subtypes except $S1P_2$ (Brinkmann et al., 2002; Mandala et al., 2002). We then blocked FTY720 binding to each S1P receptor subtype using selective antagonists for $S1P_1$ (W146), $S1P_3$/$S1P_5$ (suramin), and $S1P_4$ (CYM50358). W146 significantly reduced the suppressive effects of FTY720 on TNF-α production by activated microglia (Fig. 3a), whereas treatment with suramin or CYM50358 did not produce significant changes (Fig. 3b and c). These results suggested that FTY720 inhibits microglial production of pro-inflammatory cytokines via $S1P_1$, which is similar to the effects observed in lymphocytes.

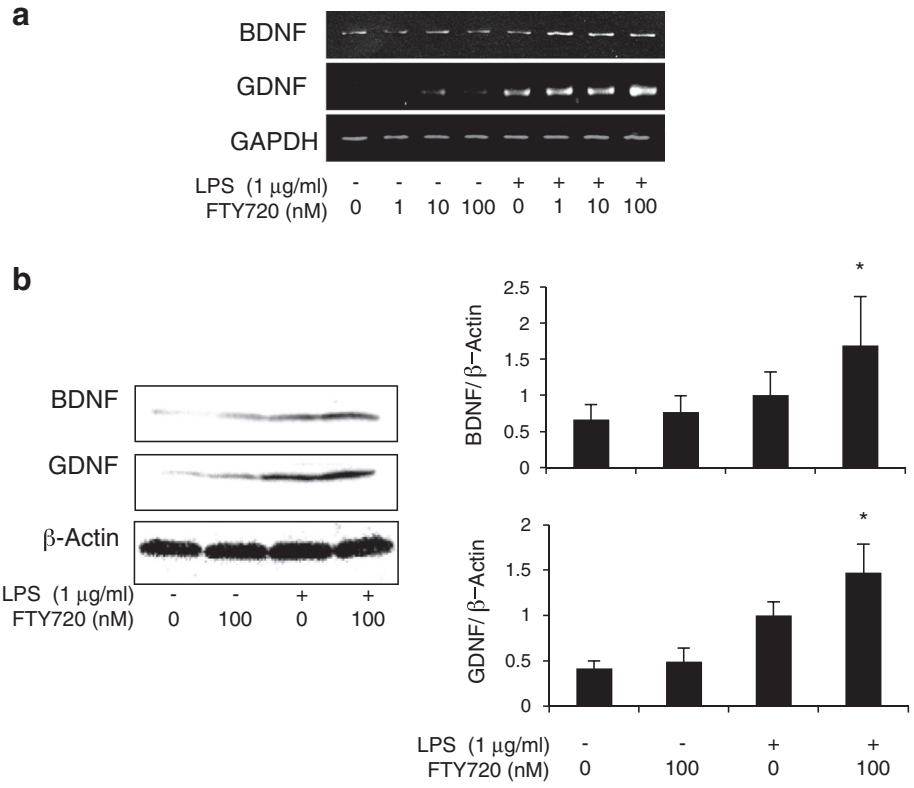

Fig. 4. FTY720 upregulates microglial production of BDNF and GDNF. (a) Representative RT-PCR data reflecting microglial expression of BDNF and GDNF. Assays were carried out in three independent trials. (b) Western blotting analysis of microglia production of BDNF and GDNF. Left, a representative Western blot; right, relative protein expression levels of BDNF and GDNF normalized to β-actin expression levels. FTY720 treatment dose-dependently enhanced microglial production of BDNF and GDNF. Assays were carried out in three independent trials. The values shown are means ± SEM. *, $P<0.05$ versus LPS-treated microglia.

3.3. FTY720 increases microglial production of neurotrophic factors

Activated microglia also produce neurotrophic factors (Mizuno et al., 2004; Liang et al., 2010). We evaluated the effects of FTY720 on microglial expression of BDNF and GDNF. Stimulation with LPS increased BDNF and GDNF levels in microglia. FTY720 enhanced these effects in a dose-dependent manner (Fig. 4a and b). Taken together, our data suggested that FTY720 exerts neuroprotective effects by both downregulating pro-inflammatory cytokine levels and increasing the expression of neurotrophic factors in microglia.

4. Discussion

In this study, we revealed that FTY720 reduces proinflammatory cytokine production and increases neurotrophic factor expression in microglia. Recent reports showed that FTY720 suppressed neuronal damage and microglial activation in rodent disease models such as epilepsy (Gao et al., 2012), spinal cord injury (Norimatsu et al., 2012), and cerebral ischemia (Wei et al., 2011). However, its mechanism has not been elucidated. Our findings provide a possible mechanism on neuroprotective effects of FTY720 via microglia. The effective concentrations of FTY720 in this study are higher (~100-fold) than that observed in the cerebrospinal fluid of patients treated with 0.3 mg/kg of FTY720 (Foster et al., 2007). However, since we used a high concentration of LPS to maximize microglial activation, it might be necessary to use higher concentrations of FTY720. Our data also showed that LPS-stimulation reduced microglial expression of $S1P_2$, $S1P_4$, and $S1P_5$. A recent study reported that the receptor for fractalkine, a negative regulator of microglial activation, was downregulated by LPS-stimulation in microglia from aged mice, suggesting as a mechanism of microglial hyperactivation in aging (Wynne et al., 2010). Therefore, the downregulation of these S1PRs may also contribute to the persistence of microglial activation during inflammatory condition, although the precise mechanism awaits further elucidation.

FTY720 binds $S1P_1$ to suppress the migration of lymphocytes from lymphoid tissues (Matloubian et al., 2004). Signaling pathways downstream of $S1P_1$ modulate a variety of transcription factors, including upregulation of nuclear factor-κB (NF-κB) activity and downregulation of histone deacetylase (HDAC) activity (Lee et al., 2010; Maceyka et al., 2012). Activation of NF-κB induces the expressions of pro-inflammatory cytokines (Newton and Dixit, 2012), whereas inhibition of HDACs suppresses NF-κB activation resulting in downregulation of pro-inflammatory cytokines (Furumai et al., 2011) and promotes the expressions of neurotrophic factors (Wu et al., 2008a). Our data suggested that FTY720 may act as a HDAC inhibitor rather than a NF-κB activator to exert the effects in microglia.

Activated microglia can be neurotoxic or neuroprotective depending on the conditions, including the nature of any stimuli, degree of stimulation, spatiotemporal distribution, and age of the subject (Jimenez et al., 2008; Wu et al., 2008b; Nakanishi and Wu, 2009; Sawada, 2009). The precise mechanisms that translate microglial activation into neurotoxic or neuroprotective outcomes are still unknown, however. Microglial activation is thought to contribute to the progression of various neurologic disorders, such as MS, spinal cord injury, stroke, amyotrophic lateral sclerosis, and Alzheimer's disease (Yrjanheikki et al., 1998; Wu et al., 2002; Zhu et al., 2002; Stirling et al., 2004; Boillee et al., 2006; Seabrook et al., 2006; Sriram, 2011). Activated microglia may also drive neuroinflammation via positive feedback mechanisms (Takeuchi et al., 2006b). Thus, suppressing the neurotoxic effects and/or enhancing the neuroprotective effects of microglia may provide effective therapeutic strategies against a variety of neurologic disorders. Our results suggest that FTY720 may accomplish these goals. In MS, FTY720 may reduce neuroinflammation by inhibiting lymphocyte from invading the CNS and enhancing the neuroprotective effects of microglia. Of note, FTY720 can penetrate the blood–brain-barrier. We, therefore, believe that FTY720 is a promising therapeutic agent for MS and other neurologic diseases associated with microglia activation, such as spinal cord injury, stroke, and various neurodegenerative disorders.

Acknowledgments

This work was supported in part by the Advanced Research for Medical Products Mining Program of the National Institute of Biomedical Innovation (NIBIO); a Grant-in-Aid for the global COE program from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and grants from the Ministry of Health, Labour and Welfare of Japan.

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