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Protective effects of a peroxisome proliferator-activated receptor-β/δ agonist in experimental autoimmune encephalomyelitis

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Abstract

Agonists of the peroxisome proliferator-activated receptor gamma (PPARγ) exert anti-inflammatory and anti-proliferative effects which led to testing of these drugs in experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis. In contrast, the effect of PPARdelta (PPARδ) agonists in EAE is not yet known. We show that oral administration of the selective PPARδ agonist GW0742 reduced clinical symptoms in *C57BL/6* mice that had been immunized with encephalitogenic myelin oligodendrocyte glycoprotein (MOG) peptide. In contrast to previous results with PPARγ agonists, GW0742 only modestly attenuated clinical symptoms when the drug was provided simultaneously with immunization, but a greater reduction was observed if administered during disease progression. Reduced clinical symptoms were accompanied by a reduction in the appearance of new cortical lesions, however cerebellar lesion load was not reduced. Treatment of T-cells with GW0742 either in vivo or in vitro did not reduce IFNγ production; however GW0742 reduced astroglial and microglial inflammatory activation and IL-1β levels in EAE brain. RTPCR analysis showed that GW0742 increased expression of some myelin genes. These data demonstrate that PPARδ agonists, like other PPAR ligands, can exert protective actions in an autoimmune model of demyelinating disease.

Keywords: Myelin; Multiple Sclerosis; Inflammation; Oligodendrocyte; Stem cells

1. Introduction

Peroxisome Proliferator-Activated Receptors (PPARs) are nuclear hormone receptors. In response to ligand binding, PPARs undergo conformational changes leading to heterodimerization with the retinoic acid receptor (RXRα), binding to specific peroxisome proliferator response elements (PPREs) in promoter regions of target genes and modulation of transcriptional events (Willson et al., 2000). There are 3 major PPAR isoforms; α, β/δ, and γ, each with distinct tissue and cellular distributions, different regulation of expression, and distinct agonist binding properties (Kliewer et al., 2001). PPARs were originally shown to regulate lipid and glucose metabolism, and agonists of PPARγ are effective for the treatment of type 2 diabetes. In the recent years, additional effects of PPAR activation have been described, most notably their ability to decrease inflammatory gene expression (Jiang et al., 1998; Henson, 2003; Clark et al., 2000; Storer et al., 2005; Ricote et al., 1999) and to inhibit T-cell proliferation (Padilla et al., 2002; Schmidt et al., 2004; Tautenhahn et al., 2003; Harris and Phipps, 2000). These properties led to examining the effects of PPAR agonists on clinical progression in

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experimental autoimmune encephalomyelitis (EAE) (Storch et al., 1998; Johns et al., 1995), a T-cell mediated, autoimmune disease involving inflammatory activation of brain glial cells (astrocytes and microglial cells) and commonly used as a model for multiple sclerosis (MS). Several studies have demonstrated efficacy of orally administered PPARγ agonists to reduce the incidence and severity of clinical, histological, and biochemical symptoms in chronic and relapsing EAE induced by immunization with an encephalitogenic MOG peptide (Niino et al., 2001; Feinstein et al., 2002); by immunization with myelin basic protein (MBP) peptide or by injection of MBP peptide activated T-cells (Diab et al., 2002); and by immunization with whole mouse spinal cord homogenate (Natarajan and Bright, 2002). More recently gemfibrozil and fenofibrate, PPARα agonists, were shown to reduce clinical and histological disease in MBP induced EAE (Lovett-Racke et al., 2004). A physiological role for PPARs in restricting inflammatory brain disease is suggested by findings that mice heterozygous for PPARγ show increased EAE disease severity (Bright et al., 2003). While the exact mechanisms are not yet clear, anti-inflammatory effects on T-cells and glial cells, together with induction of T-cell apoptosis, likely contribute to the therapeutic actions of PPARα and PPARγ drugs.

In contrast to PPARα and PPARγ, the consequences of PPARδ activation are not as well known (Michalik et al., 2003; Petrashevskaya and Schwarz, 2005). PPARδ is expressed throughout the body in most tissues, is the predominant PPAR isotype in brain (Braissant and Wahli, 1998), and is expressed in spinal cord (Benani et al., 2003). Several PPARδ activators have been identified, including fatty acids such as bromopalmitate (Brun et al., 1996), bezafibrate (Krey et al., 1997; Peters et al., 2003), the prostanoid prostacyclin PGI2 (Kliewer et al., 1997; Gupta et al., 2000), and several high affinity synthetic drugs including GW0742 (Sznaidman et al., 2003; Oliver, Jr. et al., 2001). The exact functions of PPARδ are not yet well understood, but it is likely to play a role in cell proliferation (Kim et al., 2005), differentiation, survival, lipid metabolism, and development (Michalik et al., 2003).

A role for PPARδ in brain was first suggested by the findings that PPARδ is highly expressed in the developing neural tube (Braissant and Wahli, 1998) as well as in adult brain (Braissant et al., 1996). Functionally, PPARδ agonists have been shown to regulate brain lipid metabolism (Basu-Modak et al., 1999; Rosenberger et al., 2002). Although PPARδ is expressed in many neural cell types (Cristiano et al., 2001; Cimini et al., 2005), studies from the Skoff laboratory (Granneman et al., 1998) showed that PPARδ is the major PPAR isotype expressed in optic nerve oligodendrocytes (OLGs) and sciatic nerve Schwann cells. In primary cultures from neonatal mice, PPARδ was found highly expressed in OLGs but not in astrocytes (Granneman et al., 1998). PPARδ was most highly expressed in immature OLGs, suggesting a role in OLG development; and treatment of cultures containing immature OLGs with selective PPARδ agonists increased OLG maturation, although without strong effects on OLG proliferation. A subsequent study (Saluja et al., 2001) confirmed that PPARδ, but not PPARγ selective agonists increased OLG differentiation, and MBP and PLP protein and mRNA levels. Interestingly, while the total number of OLGs was increased by PPARδ agonists, there was no increase in BrdU staining, suggesting a positive effect on OLG survival. Furthermore, analysis of brains from PPARδ null mice (Peters et al., 2000) revealed smaller overall size compared to wild type mice, and reductions in the levels of myelination in some parts of the brain. Collectively, these observations demonstrate that PPARδ has a functional role in neuronal tissue that likely involves the induction of differentiation, but the mechanisms underlying these changes are unclear.

Since PPAR agonists can prevent EAE and PPARδ is highly expressed in nervous tissue, we tested the hypothesis that a selective PPARδ agonist (GW0742) could influence the development of EAE. Our data show that oral treatment with GW0742 can reduce clinical signs in mice exhibiting signs of disease, that this decrease could be due to a reduction in glial inflammation, and which may lead to changes in myelin gene expression.

2. Methods

2.1. Animals

Female *C57BL/6* mice, aged 6–8 weeks, were from Charles River Breeding (Cambridge, MA). Mice were maintained in a controlled 12 : 12 h light/dark environment and provided food ad libitum. All experiments were approved by the local IACUC committee.

2.2. Cell culture and activation

Enriched cultures of primary rat astrocytes were prepared using standard procedures as previously described (Dello Russo et al., 2003), and at confluency were trypsinized and replated before using. The cultures are approximately 95% astrocytes by staining for the astrocyte specific protein GFAP, and the remaining 5% of cells consist mainly of adherent microglia. Enriched microglial cultures are prepared by shaking off adhering cells from astrocytes, and replating into 96 well plates. Cells are activated to express nitric oxide synthase type 2 (NOS2) with bacterial endotoxin lipopolysaccharide (LPS; 1 μg/ml for astrocytes; 100 ng/ml for microglia) in media containing 1% FCS. Nitrite production is measured in the media by use of the Griess reagent (Green et al., 1982). IL-1β levels are measured by specific ELISA (Quantikine M, R&D Systems, Minneapolis, MN).

2.3. Induction of EAE

EAE was actively induced in 6–8 week old female *C57BL/6* mice using synthetic myelin oligodendrocyte glycoprotein peptide 35–55 (MOG_{35-55}) as described (Feinstein et al., 2002). The MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Dr. Suzanna J. Horvath at the Biopolymer Synthesis Center, California Institute of Technology, Pasadena, CA, and purified to >98% purity by reverse phase HPLC analysis. Mice were injected subcutaneously (two 100 μl injections into adjacent areas in one hind limb) with an emulsion of 300 μg MOG_{35-55} dissolved in 100 μl PBS, mixed with 100 μl CFA containing 500 μg of Mycobacterium tuberculosis (Difco, Detroit, MI). Immediately after MOG_{35-55} injection, the animals received an i.p. injection of pertussis toxin (PT, 200 ng in 200 μl PBS). Two days later the mice received a second PT injection, and 1 week later they received a booster injection of MOG_{35-55}.

2.4. Clinical assessment of EAE

Clinical signs were scored on a 5 point scale: grade 0, no clinical signs; 1, limp tail and/or impaired righting; 2; paresis of one hind limb; 3; paresis of two hind limbs; 4; moribund; 5, death. Scoring was done at the same time each day by a blinded investigator. For calculations of disease incidence, scores of 1.0 or greater were counted.

2.5. Treatment with PPARδ agonist

The selective PPARδ agonist 4-[2-(3-Fluoro-4-trifluoromethylphenyl)-4-methylthiazol-5-methylsulfanyl]-2-methylphenoxy-acetic acid (GW0742) was synthesized at GlaxoSmithKline as previously described (Sznaidman et al., 2003). Chow containing 100 ppm GW0742 was prepared by Research Diets (Nutley, NJ) by mixing 100 mg of drug with 1 kg of Purina mouse chow 5001. Mice were provided free access to chow, and on average consumed 2 g per mouse per day, giving an average daily dose of 10 mg/kg GW0742. This dose is similar to that previously used by our group and others for studies of other PPAR agonists in EAE models (Feinstein et al., 2002). Stock solutions of 10 mM GW0742 were prepared in 100% DMSO and diluted in saline for further use.

2.6. mRNA analysis.

Total cytoplasmic RNA was prepared from frontal cortices of mice using TRIZOL reagent (Invitrogen); aliquots were converted to cDNA using random hexamer primers, and mRNA levels estimated by real time PCR. For amplification of NOS2, we used forward primer 5′-CTGCATGGAACAGTATAAGGCAAAC-3′ and reverse primer 5′-CAGACAGTTTCTGGTCGATGTCATGA-3′ which yield a 230 bp product; for MBP, we used forward primer 5′-CACAGAGACACGGGCATCC-3′ and reverse primer 5′-CTGGGTTTTCATCTTGGGTCC-3′ which yield a 160 bp product; for PLP we used forward primer 5′-GGCGACTACAAGACCACCATC-3′ and reverse primer 5′-AGCAGGCAAACACCAGGAG-3′ which yield a 205 bp product; and for glyceraldehyde 3-phosphate dehydrogenase (GDH) we used forward primer 5′-GCCAAGTATGATGACATCAAGAAG-3′ and reverse primer 5′-TCCAGGGGTTTCTTACTCCTTGGA-3′ which yield a 264 bp product. Cycling conditions were 35 cycles of denaturation at 94 °C for 10 s; annealing at 61 °C for 15 s; and extension at 72 °C for 20 s; followed by 2 min at 72 °C, in the presence of SYBR Green (1:10,000 dilution of stock solution from Molecular Probes) carried out in a 20 μl reaction in a Corbett Rotor-Gene (Corbett Research). Relative mRNA concentrations were calculated from the take-off point of reactions using the software included in the unit. At the end of real time PCR, correct product production was confirmed by melting point analysis and electrophoresis through 2% agarose gels containing 0.1 μg/ml ethidium bromide.

2.7. T-cell isolation and IFNγ assay

Splenic T-cells were isolated from MOG_{35-55} immunized EAE mice 4 days after the booster MOG injection (before appearance of clinical signs). After lysis of red blood cells, splenocytes were plated into 24 well plates at a density of 2×10^5 cells per well in 400 μl RPMI media, and incubated with immunogen (0 or 25 μg/ml MOG_{35-55} peptide) or with 0.5 μg/ml concanavalin A (ConA). In some experiments cells were co-incubated with varying concentrations of GW0742 (0 to 10 μM) or the equivalent amount of vehicle (DMSO). After 2 days incubation, aliquots of the media were assayed for levels of IFNγ by ELISA following recommended procedures (Quantikine M, R&D Systems, Minneapolis, MN). Each sample was assayed in triplicate, and the increase in IFNγ levels due to the presence of MOG peptide or ConA was determined.

2.8. Tissue preparation and sectioning

Mouse brains were perfused with saline and post-fixed in 4% paraformaldehyde or placed in 10% formaldehyde, 10% acetic acid, 80% methanol fixative (FAM) overnight. Dehydration of hemispheres was through a series of 80%, 95% ethanol 1 h each followed by 100% ethanol overnight. Two 100% xylene washes were done for 1 h each and then 1 h in 60 °C Paraplast Plus (Tyco/Healthcare, Mansfield, MA). After a change of Paraplast Plus, tissue was placed in a 60° vacuum oven for 2 h prior to placing in molds to cool and solidify. Tissue was cut 8 μm thick. Sagittal sections (8 or 10 μM) were cut beginning from midline, deparaffinized, and stained with H&E. At least 5 serial sections from each animal were counted for presence of infiltrating cells and lesions.

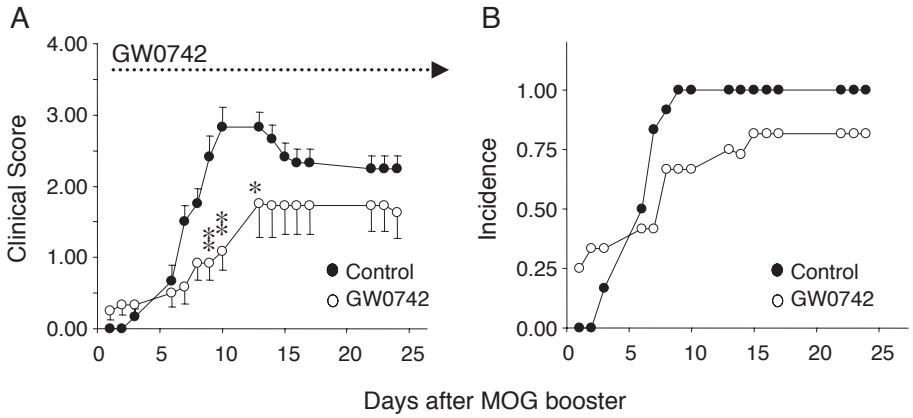

Fig. 1. Effect of GW0742 on initial development of clinical symptoms. *C57BL/6* mice were immunized with MOG_{35-55} peptide, and provided diet containing 0 ($n=12$, filled circles) or 100 ppm GW0742 ($n=12$, open circles) beginning 3 days before the first immunization. Clinical signs were monitored to 25 days after the booster MOG immunization. (A) Clinical scores. Data are mean ± s.e.m. of average daily clinical scores. There was a significant effect of drug treatment on clinical scores ($P=0.012$; 2-way ANOVA). *, $P<0.05$; **, $P<0.01$; Bonferonni post hoc test. (B) Daily average incidence.

2.9. Immunohistochemistry

Sagittal mouse brain sections mounted on slides were washed for 5 min with PBS and blocked with 5% normal donkey serum in PBS at room temperature for 30 min. Primary antibodies were diluted in 1% normal donkey serum : rabbit anti-MBP 1 : 300 (Zymed Laboratories, San Francisco, CA) incubated 1 h at 37 °C; goat anti-IL-1β 1 : 50 (R&D Systems Inc., Minneapolis, MN), incubated 4 °C overnight. Sections were washed 3 times with PBS. Secondary antibodies (Jackson ImmunoResearch Inc., West Grove, PA) were donkey anti-rabbit RRX conjugated and donkey anti-goat FITC conjugated. All secondary antibodies were incubated for 1 h at 37 °C and diluted 1 : 200 in PBS with 1% normal donkey serum. Slides were washed 3 times for 5 min with PBS and post-fixed in 3.7% formaldehyde in PBS for 20 min. Autofluorescence was quenched with 50 mM NH_4Cl in PBS for 15 min. DAPI staining of nuclei, 400 ng/ml in PBS, was done after one wash with PBS and followed with 3 PBS washes. Vectashield mounting fluid (Vector Laboratories Inc., Burlingame, CA) was used. Images were obtained on a Zeiss Axioplan2 fluorescence microscope equipped with an Axiocam MRM digital camera and Axiovision 4.3 imaging software.

3. Results

3.1. Effects of GW0742 on clinical progression in MOG-induced EAE

In a first set of studies, we tested if the selective PPARδ agonist GW0742 afforded protection against the development of clinical signs in EAE when treatment was initiated prior to disease onset. *C57BL/6* mice were provided chow containing 100 ppm GW0742 beginning 3 days before immunization with MOG_{35-55}, and treatment continued until the end of the experiment (Fig. 1). GW0742 did not delay disease onset (6.3 ± 0.5 days after booster MOG, $n=12$ for the control group versus 6.4 ± 1.6 days, $n=10$ for the GW0742 treated group). However, there was a significant effect of drug treatment on average clinical score: the maximum average score in the control group reached 2.83 (at day 10) versus 1.75 (at day 13) for the treated group. However, by day 25 this difference was

Table 1
Effect of GW0742 on clinical scores

Expt	Start	Treat	Ill at start	Score	# recovered	Improve day	End	PD	Full recovery	Ill at end	End score
1	36	Control	7/7	2.29	1	11	58	23	0	7/7	2.14
		GW0742	8/8	2.25	8	17 ± 1.1	58	23	2	6/8	0.88
2	24	Control	4/4	2.25	0	None	46	23	0	4/4	2.25
		GW0742	4/4	2.75	3	13.3 ± 6.0	46	23	0	4/4	2.00
3	29	Control	9/9	1.89	0	None	52	23	0	9/9	2.11
		GW0742	9/9	2.2	5	10.4 ± 5.6	52	23	2	7/9	1.67
4	16	Control	5/6	1.67	0	None	42	26	0	6/6	2.50
		GW0742	5/5	2	4	17.3 ± 4.6	42	26	2	3/5	1.00
All	26.3	Control	25/26	2.0 ± 0.3	1	11	49.5 ± 7	23.8 ± 1.5	0	26/26	2.3 ± 0.2
		GW0742	26/26	2.3 ± 0.3	20	15.1 ± 4.9	49.5 ± 7	23.8 ± 1.5	6	20/26	1.4 ± 0.5

Start is number of days after MOG booster that treatment (drug or control) was started; End is number of days after MOG booster that study ended; PD (post-drug start) is number of days of drug treatment.

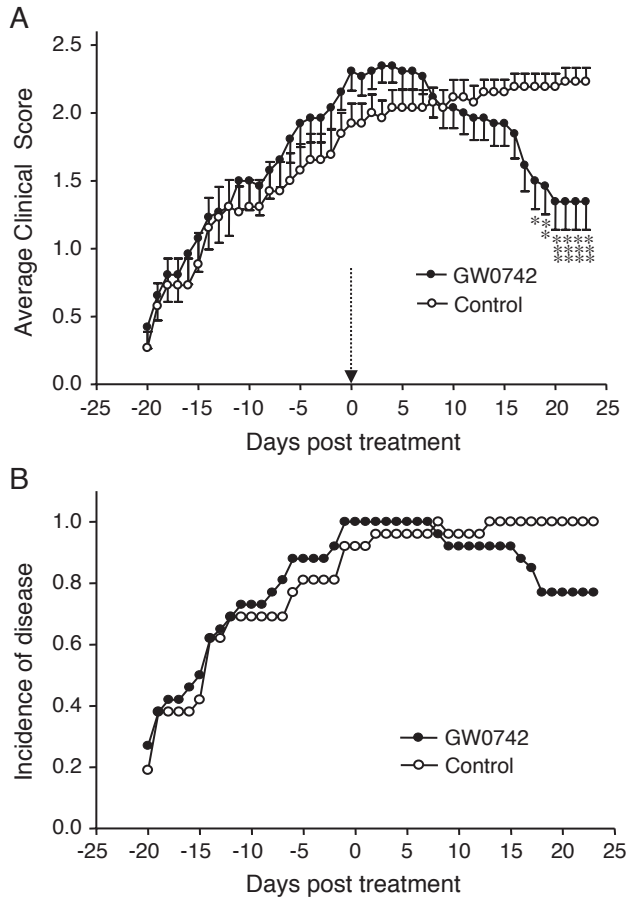

Fig. 2. Effect of GW0742 on clinical symptoms in disease-established mice. *C57BL/6* mice were immunized with MOG_{35-55} peptide, given a booster immunization 7 days later, and at or near the peak of clinical symptoms, were provided either control diet ($n=26$, open circles) or diet containing 100 ppm GW0742 ($n=26$, filled circles). (A) Daily average clinical scores; data represent the mean ± s.d. of all scores from 4 different experiments; (B) daily average incidence. The *x*-axis shows days relative to start of drug (or control) treatment, to allow better comparison of the 4 individual experiments which are also summarized in Table 1. *, $P<0.05$; **, $P<0.005$; and ***, $P<0.0005$ 2-way ANOVA, Bonferroni post hoc analysis.

reduced (2.25 in the controls versus 1.64 in the treated group). Disease incidence was similar, reaching 100% (12/12) in the control group and 82% (10/12) in the treated group. These results indicate that GW0742 exerts some effect on the early events leading to initial appearance of clinical signs in MOG-EAE.

We next tested if GW0742 treatment could provide clinical benefit if administered at a later time during disease progression. Mice were immunized with MOG_{35-55} peptide, allowed to reach moderate clinical severity (scores of 2 to 3), and at that time (between 2 and 5 weeks depending on the experiment) were provided with 0 or 100 ppm GW0742 in the chow. The data from 4 independent studies are summarized in Table 1 and Fig. 2. The average clinical scores at the time of treatment were well matched between the 4 experimental and control groups (2.0 ± 0.3 for all control groups; 2.3 ± 0.3 for all GW0742 groups). In the GW0742 treated group, 20/26 mice showed some signs of clinical recovery, and on average this occurred 15.1 ± 5 days after treatment was started. This decrease was in part due to the reduced incidence of disease, which decreased from 100% at the start of treatment (26/26) to 77% (20/26) at the end (Fig. 2B). In contrast, in the vehicle treated group, only 1 animal showed any clinical recovery (a 1 point decrease from score 3 to score 2) during the treatment period, and that occurred at 11 days after treatment began. Overall, after 23 days of treatment with GW0742, there was a significant decrease in the average clinical score (from 2.3 ± 0.3 to 1.4 ± 0.5) while the average clinical scores of the vehicle treated group increased during this same time (from 2.0 ± 0.3 to 2.3 ± 0.2). These results suggest that treatment with GW0742 beginning near the peak of clinical disease can improve clinical recovery.

3.2. Effects of GW0742 on T-cell activation and CNS infiltration

The effect of GW0742 on T-cell function was assessed by measuring IFNγ production in isolated splenocytes (Fig. 3). Incubation with GW0742 (up to 10 μM) did not significantly reduce ConA dependent IFNγ production from splenic-T cells isolated from naive mice (Fig. 3A). Likewise,

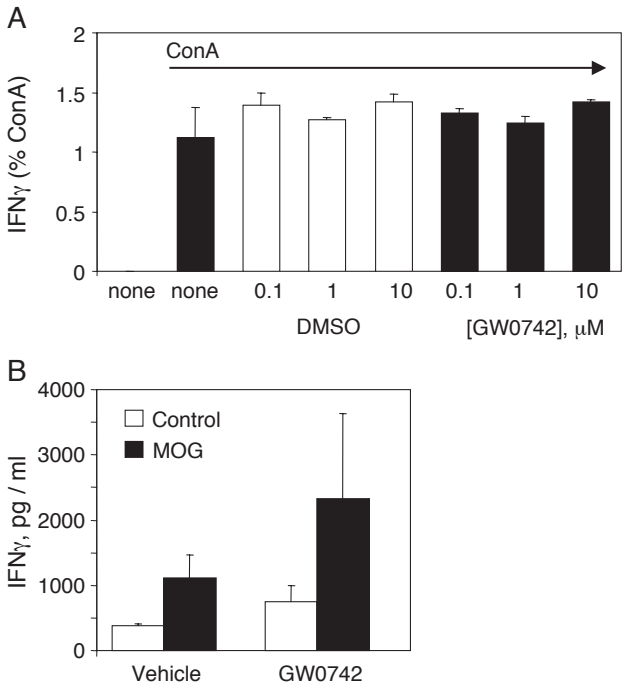

Fig. 3. Effect of GW0742 on T-cell activation. The effects of GW0742 on T-cell function were assessed by measurement of ex vivo IFNγ production. (B) Splenic T-cells were prepared from EAE mice treated with control or GW0742 chow for 2 weeks, incubated ex vivo with 0 (open bars) or 25 μg/ml (filled bars) MOG_{35-55} peptide, and IFNγ levels measured 24 h later by ELISA. Data represent the mean ± s.d. of T-cells prepared from $n=3$ mice in each group. (A) Splenic T-cells from control mice were activated with concanavlin A (0.5 μg/ml), in the presence of indicated amounts of GW0742 or the equivalent amount of vehicle (DMSO). IFNγ levels were determined 24 h by ELISA.

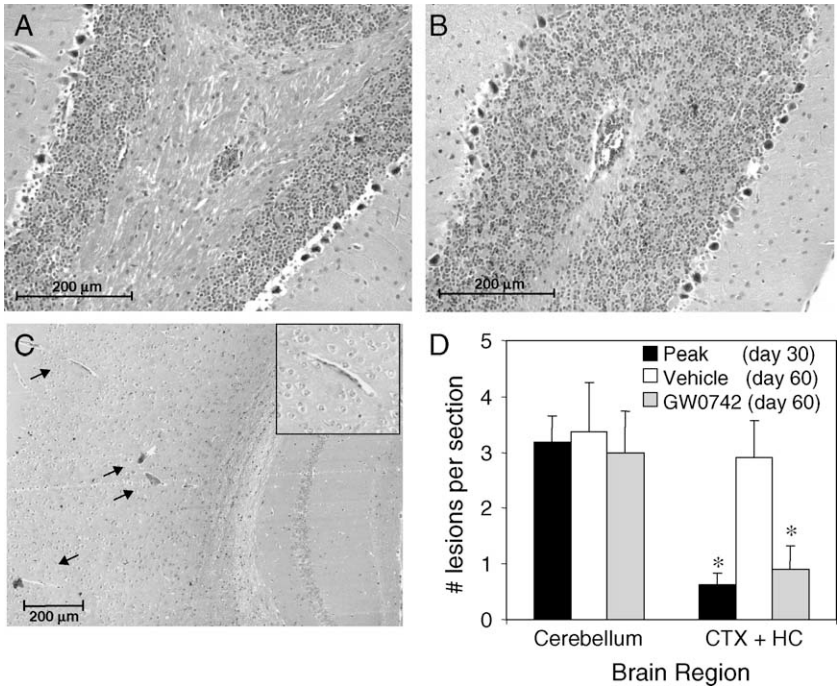

Fig. 4. Effect of GW0742 on CNS lesions. Mice were immunized to develop EAE, and treated at disease peak with control or GW0742 diet (these mice came from experiment 4 as described in Table 1). Brains samples ($n=3$ per group) were prepared at disease peak or after 30 days treatment, and stained with H&E to localize lesions. (A) Typical cerebellar lesion showing infiltrating lymphocytes; (B) more developed cerebellar lesion showing loss of lymphocytes; (C) small cortical lesions showing disarrayed vasculature and single layer of infiltrating cells (insert); (D) quantitative analysis of lesion numbers counted in cerebellum and in other (cortical plus hippocampal) regions. The number of lesions was counted in 4–6 serial sagittal sections taken from each animal. *, $P<0.05$ versus vehicle treatment, *T*-test. Magnification is 125× for panels (A) and (B); 60× panel (C); and 135× insert in panel (C).

MOG_{35-55} peptide dependent IFNγ production was similar in splenocytes from control or vehicle treated EAE mice isolated at an early time point in clinical progression (at which point IFNγ production is high) (Fig. 3B). Assays of splenic cells isolated after 30 days treatment showed only low levels of IFNγ production, and hence no conclusions about effects of drug treatment on those cells could be drawn (data not shown).

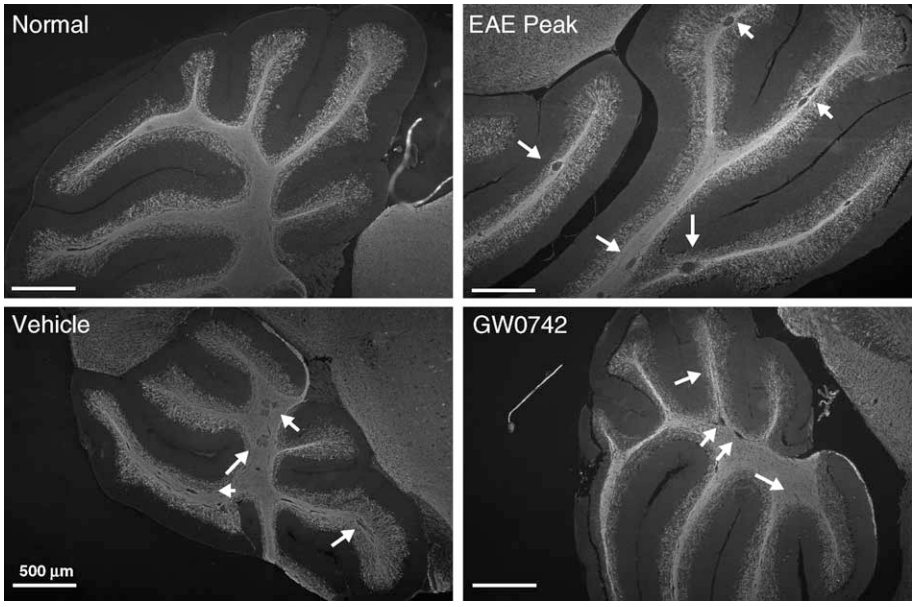

Fig. 5. Effect of GW0742 on myelin staining. Brain sections were prepared from the same mice as described in Fig. 4, and were stained for MBP. Areas of demyelination (marked by arrows) are apparent at peak, and were present after vehicle or GW0742 treatment. Similar results were obtained in 3–4 animals from each group. In the absence of primary antibody, no staining was observed (not shown). The bar shown in each panel is 500 μM. Magnification is 30× for all panels.

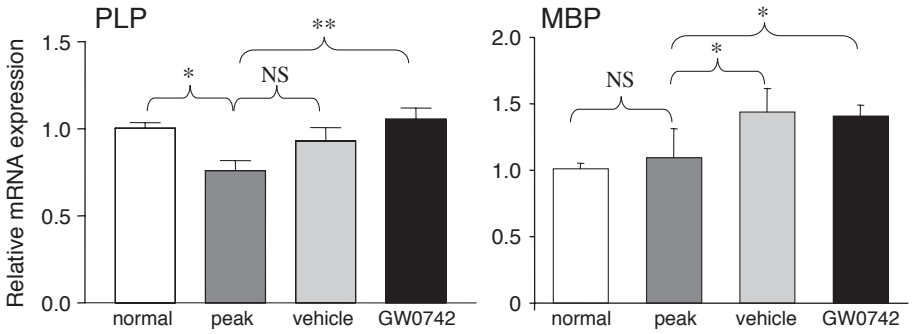

Fig. 6. RTPCR of control and GW0742 treated EAE mice. RNA samples were prepared from individual mouse brain frontal cortices either at the peak of clinical disease ($n=4$ mice); at 1 month after treatment with GW0742 ($n=3$ mice) or vehicle ($n=3$ mice) chow; or from normal (non-EAE) mice ($n=3$). Levels of PLP and MBP mRNAs were determined by real time PCR, and normalized by measurements of GDH mRNA levels. The data is the mean ± s.d. of mRNA levels normalized to normal (non-EAE) values. *, $P<0.05$; **, $P<0.005$, *T*-test.

Histological analysis at the peak of clinical disease revealed numerous infiltrates throughout the brain with the largest number in cerebellum (Fig. 4). Lesions were of two types: 1) blood vessels filled with H&E positively stained lymphocytes (Fig. 4A) and 2) areas devoid of stained cells (Fig. 4B). Counting of serial sagittal sections revealed that the number of lesions in cerebellum was similar at disease peak and after 30 days treatment with either vehicle or GW0742 (Fig. 4D). However, the number of lesions detected outside of cerebellum (primarily in frontal cortex and hippocampal areas; and which in general were of smaller size than those seen in cerebellum) increased significantly at day 65 versus day 35; however this increase was lessened in the GW0742 treated mice. This suggests that during the course of MOG-EAE, infiltrate localization normally begins in cerebellar regions, eventually occurs in non-cerebellar sites, and that GW0742 reduces this latter infiltration.

3.3. Effects of GW0742 on myelin gene expression

The extent of demyelination was assessed by staining for MBP (Fig. 5). At disease peak, loss of myelin in cerebellar white matter was extensive compared to non-EAE mice. After 30 days treatment, areas of demyelination were still apparent in both vehicle and GW0742 treated mice, with similar numbers of lesions observed. Myelin loss was detected in other brain regions including hippocampus and entorhinal cortex, however no significant changes were observed following vehicle or GW0742 treatment (not shown). Quantitative PCR analysis (Fig. 6) of myelin gene expression revealed that PLP mRNA levels were decreased at disease peak versus control (non-EAE) mice, and that treatment with GW0742, but not vehicle, significantly increased PLP mRNA levels (versus peak levels) measured 30 days later. In contrast, while MBP mRNA levels were also decreased at peak versus non-EAE, there was no significant effect of drug treatment on MBP mRNA levels.

3.4. Effects of GW0742 on glial activation

Primary enriched cultures of rat astrocytes or microglia were incubated for 24 h with LPS to induce NOS2 expression (Fig. 7), in the presence or absence of GW0742 or the equivalent amount of vehicle (DMSO). NOS2 activity (assessed by measurement of nitrites in the culture media) was significantly increased by LPS in both cell types, and was dose-dependently reduced by co-incubation with GW0742, although higher efficacy was

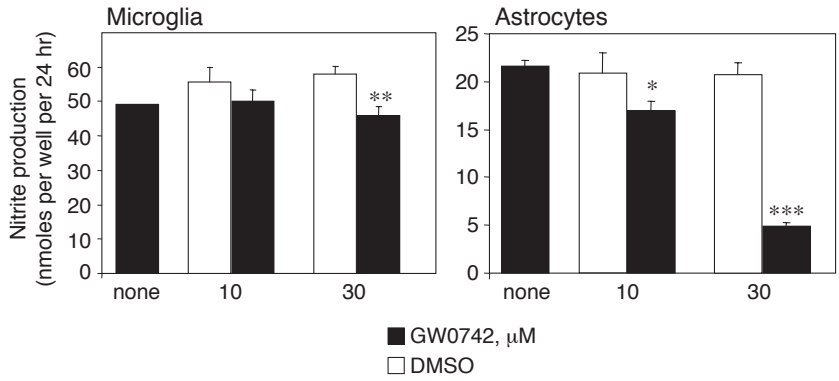

Fig. 7. Effect of GW0742 on glial cell activation in vitro. Primary enriched cultures of rat microglia (left) and astrocytes (right) were activated with LPS (100 ng/ml for microglia; 1 μg/ml for astrocytes) to induce NOS2 expression and NO production, in the presence of the indicated concentrations of GW0742 (filled bars) or the equivalent amount of vehicle (DMSO; open bars). After 24 h, NO production was assessed indirectly by measurement of nitrite levels in the culture media using Griess reagent. The data is mean ± s.d. of $n=3$ to 6 replicates. *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$ versus vehicle controls, *T*-test.

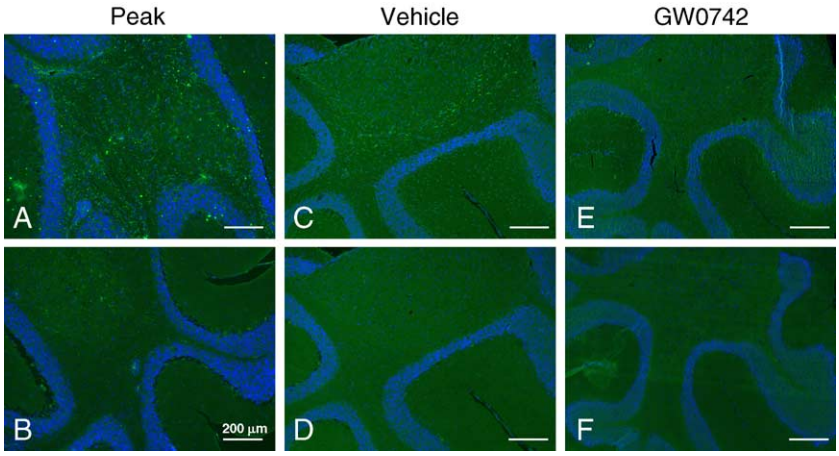

Fig. 8. Effect of GW0742 on glial cell activation in vivo. Cerebellar sections prepared from the same mice as described in Fig. 5 were stained with antibody for IL-1β. (A, B) Peak of disease; (C, D) Vehicle treatment; (E, F) GW0742 treatment. Bottom panels show staining in serial sections in the absence of primary antibody. Similar results were obtained in 3–4 animals from each group. The bar shown in each panel is 200 μM. Magnification is 50× for all panels.

observed in astrocytes (75% inhibition achieved at 30 μM) than in microglial cells (23% inhibition at 30 μM). Consistent with these in vitro observations, treatment of EAE mice with GW0742 also reduced glial activation in vivo, as assessed by immunostaining for IL-1β in cerebellum (Fig. 8).

4. Discussion

The current study demonstrates that an agonist of the PPARδ can exert protective effects in EAE, as has previously been demonstrated for agonists of the PPARα and PPARγ subtypes (Lovett-Racke et al., 2004; Niino et al., 2001; Diab et al., 2002; Natarajan and Bright, 2002; Feinstein et al., 2002; Bright et al., 2003). In contrast to those studies, administration of GW0742 starting at the early stages of disease had a modest effect on the initial appearance or severity of clinical symptoms. However, clinical improvement was reproducibly observed when the drug was provided to mice exhibiting signs of clinical disease. Clinical improvement was observed starting approximately 2 weeks after the drug was provided, which also contrasts to previous studies using PPARγ agonists in which improvements in treated mice was seen after a few days treatment (Feinstein et al., 2002). This delayed recovery time due to GW0742 suggests a different mode of action compared to PPARγ agonists, whose more rapid effects may involve reducing acute inflammatory mediators or edema.

Analysis of T-cell function was done ex vivo using splenic T-cells robustly stimulated with ConA. Under these conditions, co-treatment with GW0742 (up to 10 μM) did not significantly affect IFNγ production. Analysis of T-cell function from vehicle or GW0742 treated mice was also done using cells prepared from early treated animals, and consistent with the lack of robust effect at early times, we saw no difference in T-cell IFNγ production. Our initial studies using T-cells isolated from mice after 30 days of treatment (approximately 60 days after immunization) showed only low levels of IFNγ production, and therefore comparisons between vehicle versus drug treated mice were precluded. These results contrast with findings that agonists of the PPARγ significantly reduce T-cell activation (Clark et al., 2000; Marx et al., 2000; Diab et al., 2002; Natarajan and Bright, 2002; Schmidt et al., 2004) and proliferation (Harris and Phipps, 2000, 2001; Tautenhahn et al., 2003), again pointing to a distinct mechanism of action of PPARδ in amelioration of EAE symptoms.

However, effects of GW0742 treatment on T-cell function at later times are suggested by examination of H&E staining of brains taken approximately after 30 days of vehicle or drug treatment. These results show that whereas GW0742 did not reduce the number of cerebellar infiltrates, the appearance of smaller lesions in other brain regions (primarily hippocampus and cortex) was reduced. The exact nature of these smaller lesions remains to be clarified. A single layer of infiltrating cells lining a small blood vessel was often but not always observed, and in some cases, distended vessel structures were observed in the absence of infiltrating cells. Nevertheless, chronic treatment with GW0742 reduced these alterations, suggesting that inhibition of a specific set of lymphocytes that infiltrate brain at later, but not early, disease stages. In lieu of direct effects upon T-cell function, reduced CNS infiltration could be due to reduced chemokine or adhesion molecule production from parenchymal or endothelial cells. Together, these findings suggest that PPARδ agonists do not prevent T-cell activation, but can reduce late stage migration into brain.

The lack of effect of GW0742 on cerebellar infiltrates is consistent with the findings that the number of cerebellar lesions as observed by areas of reduced MBP staining was also not affected by treatment with this drug. Cerebellar lesions were evident at the peak of clinical disease, and despite decreased clinical scores, the number of cerebellar lesions remained similar after drug treatment. We did not

observe significant lesions (assessed by MBP staining) in other regions of the brain.

Despite the lack of a clear effect on T-cell function, our results show that GW0742 reduces glial cell inflammatory activation. Both astrocytes and microglial cells have been shown to be sensitive to suppressive effects of PPARα and PPARγ agonists (Kielian and Drew, 2003; Storer et al., 2005; Jiang et al., 1998; Chawla et al., 2001; Welch et al., 2003). However, the effects of PPARδ agonists on these cells have not been reported. Our results demonstrate that a highly selective PPARδ agonist can also reduce astrocyte as well as microglial inflammatory activation, since both IL-1β and NOS2 activity were significantly reduced. There are few published studies concerning anti-inflammatory effects of PPARδ drugs, however studies using PPARγ deficient macrophages suggest that at higher concentrations, PPARγ agonists may exert their well-known anti-inflammatory effects through PPARδ activation (Welch et al., 2003). Results from the present studies therefore suggest that PPARδ could also be a potential target for inhibiting brain glial activation.

Previous studies have suggested a role for PPARδ activation in OLG maturation or survival. Initial studies from the Skoff lab (Granneman et al., 1998) showed that PPARδ mRNA was expressed abundantly in relatively pure OLG cultures, as well as in developing optic nerve, suggesting a localization to OLGs undergoing maturation. Treatment of primary OLG cultures with bromopalmitate, a 16-carbon chain fatty acid that binds to PPARδ, increased the number of OLGs having membrane sheets. Similarly, 24 h treatment with a selective PPARδ agonist (or a mixed δ/γ agonist), increased the number of OLGs with processes and large membrane sheets about 2–3-fold; as well as MBP and PLP mRNA levels (Saluja et al., 2001). These agonists also increased OLG cell number, without an increase in BrdU staining, suggesting an effect on OLG survival. In our studies, analysis of mRNA levels after GW0742 treatment did not reveal a significant increase in MBP mRNA levels compared to vehicle treated mice; however we did observe a small, but significantly higher level of PLP mRNA due to drug treatment. Further studies to determine if GW0742 increases myelin gene expression, or alters myelin mRNA degradation or turnover are in progress.

The possible effects of GW0742 on myelin gene expression are consistent with reports showing that PPARδ agonists modulate lipid metabolism. PPARδ is a very low density lipoprotein (VLDL) receptor in macrophages (Chawla et al., 2003). VLDL triglycerides activate PPARδ, which induces transcription of various genes including adipose differentiation relation protein which is involved in triglyceride accumulation and short term lipid storage. Activation of lipid accumulation could conceivably influence OLG maturation. PPARδ agonists have also been shown to increase expression of ABCA1, a member of the ATP-binding cassette protein family, which can increase cholesterol efflux (Oliver, Jr. et al., 2001). ABC proteins are expressed in OLGs and implicated in OLG maturation and myelination (Tanaka et al., 2003; Zhou et al., 2002).

To our knowledge, the current findings represent the first study to examine the potential use of PPARδ agonists for treatment of a neurological disease. The use of PPARα and PPARγ drugs has been reviewed several times (Storer et al., 2005; Pershadsingh, 2004; Feinstein, 2003; Kielian and Drew, 2003), and clinical trials of some PPARγ agonists are in progress for MS and AD. The question arises as to what advantage PPARδ agonists offer versus treatment with PPARγ (or PPARα) ligands. While this remains to be determined, the possibility that these drugs can provide both anti-inflammatory as well as proliferative or maturation effects on neural cells and thereby influence remyelination warrants further studies of this class of drugs.

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