Evidence for the Role of Peroxisome Proliferator-Activated Receptor-β/δ in the Development of Spinal Cord Injury

Irene Paterniti, Emanuela Esposito, Emanuela Mazzon, Maria Galuppo, Rosanna Di Paola, Placido Bramanti, Amar Kapoor, Christoph Thiemermann, and Salvatore Cuzzocrea

Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, University of Messina, Messina, Italy (I.P., E.E., E.M., M.G.); Instituto di Ricerco e Cura a Carattere Scientifico Centro Neurolesi “Bonino-Pulej,” Messina, Italy (E.E., P.B., S.C., R.D.P.); and Centre for Experimental Medicine, Nephrology, and Critical Care, William Harvey Research Institute, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, London, United Kingdom (A.K., C.T.)

Received January 6, 2010; accepted February 19, 2010

ABSTRACT

Several lines of evidence suggest a biological role for peroxisome proliferator-activated receptor (PPAR)-β/δ in the pathogenesis many diseases. The aim of the present study was to evaluate the contribution of PPAR-β/δ in the secondary damage in experimental spinal cord injury (SCI) in mice. To this purpose, we used 4-[[2-[3-fluoro-4-(trifluoromethyl)phenyl]-4-methyl-5-thiazolyl]methylthio]-2-methylphenoxy]acetic acid (GW0742), a high-affinity PPAR-β/δ agonist. Spinal cord trauma was induced by the application of vascular clips (force of 24 g) to the dura via a four-level T5 to T8 laminectomy. SCI in mice resulted in severe trauma characterized by edema, neutrophil infiltration, production of inflammatory mediators, tissue damage, and apoptosis. GW0742 treatment (0.3 mg kg⁻¹ i.p.) 1 and 6 h after the SCI significantly reduced 1) the degree of spinal cord inflammation and tissue injury (histological score), 2) neutrophil infiltration (myeloperoxidase activity), 3) nitrotyrosine formation, 4) proinflammatory cytokines expression, 5) nuclear factor-κB activation, 6) inducible nitric-oxide synthase expression, and 6) apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labeling staining, FasL, Bax, and Bcl-2 expression). Moreover, GW0742 significantly ameliorated the recovery of limb function (evaluated by motor recovery score). To elucidate whether the protective effects of GW0742 are related to activation of the PPAR-β/δ receptor, we also investigated the effect of PPAR-β/δ antagonist methyl 3-[[2-(methoxy)-4-phenyl]amino]sulfonyl]-2-thiophenecarboxylate (GSK0660) on the protective effects of GW0742. GSK0660 (1 mg/kg i.p. 30 min before treatment with GW0742) significantly blocked the effect of the PPAR-β/δ agonist and thus abolished the protective effect. Our results clearly demonstrate that GW0742 treatment reduces the development of inflammation and tissue injury associated with spinal cord trauma.

Spinal cord injury (SCI) is a highly debilitating pathology. Although innovative medical care has improved patient outcome, advances in pharmacotherapy for the purpose of limiting neuronal injury and promoting regeneration have been limited. The complex pathophysiology of SCI may explain the difficulty in finding a suitable therapy. The primary traumatic mechanical injury to the spinal cord causes the death of many neurons that cannot be recovered and regenerated.

Studies indicate that neurons continue to die for hours after traumatic SCI (Profyris et al., 2004). The events that characterize this successive phase to mechanical injury are called “secondary damage.” The secondary damage is determined by a large number of cellular, molecular, and biochemical cascades. Recent data suggest the presence of a local inflammatory response, which amplifies the secondary damage. The cardinal features of inflammation, namely, infiltration of inflammatory cells (polymorphonuclear neutrophils, macrophages and lymphocytes); release of inflammatory mediators; and activation of endothelial cells leading to increased vascular permeability, edema formation, and tissue destruction have been well and extensively characterized in animal SCI models. The phases that govern the biology of secondary injury after acute SCI are then necrosis, infarction, excito-

ABBREVIATIONS: SCI, spinal cord injury; PPAR, peroxisome proliferator-activated receptor; GW0742, 4-[[2-[3-fluoro-4-(trifluoromethyl)phenyl]-4-methyl-5-thiazolyl]methylthio]-2-methylphenoxy]acetic acid; GSK0660, methyl 3-[[2-(methoxy)-4-phenyl]amino]sulfonyl]-2-thiophenecarboxylate; MPO, myeloperoxidase; TNF, tumor necrosis factor; INOS, inducible nitric-oxide synthase; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; NF-κB, nuclear factor-κB; PMSF, phenylmethylsulfonyl fluoride; MDA, malondialdehyde; BBB, Basso, Beattie, and Bresnahan; IL, interleukin; LPS, lipopolysaccharide.
toxicity, reperfusion injury, apoptosis, axonal disruption, Wallerian degeneration, and glial scar (Profyris et al., 2004).

Moreover, various evidence has suggested that resident microglia and macrophages originating from blood are two key cell types related to the occurrence of neuronal degeneration in central nervous system after traumatic injury. In particular, when SCI occurs, microglia in parenchyma is activated and macrophages in circulation get across the blood-brain barrier to act as intrinsic spinal phagocytes. Therefore, these cells can release various neurotrophic peptides such as brain-derived neurotrophic factor, glial-derived neurotrophic factor (Batchelor et al., 2002), and laminin, which are excellent substrates for growing neuritis. Concomitantly, different proinflammatory mediators, such as proinflammatory cytokines and reactive oxygen species, are also produced (Genovese et al., 2006).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily, which includes the classical steroid, thyroid, and retinoid hormone receptors as well as many orphan receptors. So far, three PPAR isotypes have been identified and are commonly designated PPAR-α, PPAR-β/δ, and PPAR-γ (Cuzzocrea, 2006). Both PPAR-α and PPAR-γ have been well characterized for their roles in lipid and glucose metabolism by using specific marketed drugs such as the thiazolidinediones, ligands of PPAR-γ prescribed for the treatment of type-2 diabetes (Rodriguez-Calvo et al., 2008); and the fibrates, PPAR-α ligands prescribed for their lipid-modulating properties (Ricote et al., 2004). PPARs have been implicated in the pathogenesis several diseases, including diabetes mellitus, obesity, atherosclerosis, neurological diseases, and SCI (Esposito et al., 2006; Di Paola and Cuzzocrea, 2007); therefore, they represent an important pharmacological target.

In contrast, the biological role and function of PPAR-β/δ remain relatively unclear. PPAR-β/δ is ubiquitously expressed in a variety of tissues, including liver, adipose tissue, skeletal muscle, kidney, cerebellum, thalamus, cerebella cortex, and intestine. Physiologically, PPAR-β/δ has been associated with adipocyte precursor cell proliferation (Hansen et al., 2001), oligodendrocyte differentiation (Saluja et al., 2001), and cholesterol homeostasis. The multiplicity of phenotypes induced by PPAR-β/δ gene disruption in the mouse reflects the importance of this nuclear receptor in development, and early functional studies indicate PPAR-β/δ involvement in epidermal differentiation (Kim et al., 2006), maturation, and skin wound healing (Matsura et al., 1999). Evidence has also suggested that activation of PPAR-β/δ promotes fatty acid catabolism in several tissues, such as skeletal muscle and adipose (Tanaka et al., 2003). More recent studies indicate a potential role of PPAR-β/δ in regulating glucose metabolism and insulin sensitivity (Lee et al., 2006). These actions could explain the apparent beneficial effects of synthetic PPAR-β/δ agonists on circulating lipids, insulin resistance, and obesity that have been reported in some animal models (Olive et al., 2001; Lee et al., 2006). Preclinical, in vivo studies using high-affinity PPAR-β/δ agonists have demonstrated efficacy in models of diabetes as well as obesity β-oxidation, suggesting that modulation of the δ isofrom may have a role in treating these diseases as well as metabolic syndrome. The use of selective PPAR-β/δ agonists in preclinical studies suggests that this subtype also possesses anti-inflammatory properties. In vivo data suggest that ligands of the β/δ isofrom have activity many disease models that are partly driven by the inflammatory response (Kilgore and Billin, 2008).

In contrast, the role of the PPAR-β/δ receptor in conditions associated with experimental SCI have not yet been fully investigated. The present study was designed to gain a better understanding of the possible influence of PPAR-β/δ in the modulation of secondary injury in the spinal cord by using the high-affinity PPAR-β/δ agonist GW0742.

Materials and Methods

Animals. Adult male CD1 mice (25–30 g; Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986).

Spinal Cord Injury. Mice were anesthetized using chloral hydrate (400 mg kg⁻¹ i.v.). As described by Rivlin and Tator (1978), an aneurysm clip was used, and SCI was induced by extradural compression of a section of the spinal cord exposed via a four-level T₅ to T₈ laminectomy. With the aneurysm clip applicator oriented in the bilateral direction, an aneurysm clip with a closing force of 24 g was applied extradurally at T₅ to T₈ level. The clip was then rapidly released with the clip applicator, which caused spinal cord compression. In the injured groups, the cord was compressed for 1 min. After surgery, 1.0 ml of saline was administered subeutaneously to replace the blood volume lost during the surgery. During recovery from anesthesia, the mice were placed on a warm heating pad and covered with a warm towel. The mice were singly housed in a temperature-controlled room at 27°C for a survival period of 10 days. Food and water were provided to the mice ad libitum. During this time period, the animals’ bladders were manually voided twice a day until the mice were able to regain normal bladder function. Sham-injured animals were only subjected to laminectomy. Spinal cord tissues were taken at 24 h after trauma. Tissue segments contained the lesion (1 cm on each side of the lesion), according to counts of retrogradely labeled neurons at the origin of distinct descending motor pathways and to morphometric assessments of normal residual tissue at the injury epicenter, as described by Joshi and Fehlings (2002).

Experimental Design. Mice were randomly allocated into the following groups:

1. Sham + vehicle group. Mice were subjected to surgical procedures as detailed above except that the aneurysm clip was not applied. Mice were administered vehicle (10% dimethyl sulfoxide in saline i.p. bolus) (N = 40).
2. Sham + GW0742 group. Identical to sham + vehicle group except for the administration of GW0742 (0.3 mg kg⁻¹ i.p. bolus) 1 and 6 h after surgical procedures (N = 40).
3. Sham + GSK0660 group. Identical to sham + GW0742 group, except for the administration of GSK0660 (1 mg kg⁻¹ i.p. bolus) 30 min before GW0742 (N = 40).
4. SCI + vehicle group. Mice were subjected to SCI and were administered vehicle (10% dimethyl sulfoxide in saline i.p. bolus) 1 and 6 h after SCI (N = 40).
5. SCI + GW0742 group. Mice were subjected to SCI and administered GW0742 (0.3 mg kg⁻¹ i.p. bolus) 1 and 6 h after SCI (N = 40).
6. SCI + GSK0660 group. Identical to the SCI + vehicle group, but GSK0660 was administered (1 mg kg⁻¹ i.p. bolus) 1 and 6 h after SCI (N = 40).
7. SCI + GSK0660 + GW0742 group. Identical to the SCI +
GW0742 group, but GSK0660 was administered (1 mg kg\(^{-1}\) i.p. bolus) 30 min before GW0742.

To investigate the motor score, additional animals were treated with GW0742 1 and 6 h after SCI and daily until day 9 as well as with GSK0660 that was administered 30 min before GW0742. The dose of GW0742 (0.3 mg kg\(^{-1}\)) used here was based on a previous dose-response study in a myocardial ischemia and reperfusion study (Kapoor et al., 2009). GW0742 has an EC\(_{50}\) value of 1 nM compared with 1 and 2 mM for PPAR-\(\alpha\) and PPAR-\(\gamma\), respectively. Ten mice from each group were sacrificed at different time points to collect samples for the evaluation of the parameters as described below.

**Myeloperoxidase Activity.** Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined in the spinal cord tissues as described previously (Mullane, 1989) at 24 h after SCI. At the specified time after SCI, spinal cord tissues were obtained and weighed, and each piece was homogenized in a solution containing 0.5% (w/v) hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer, pH 7.0, and centrifuged for 30 min at 20,000 g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H\(_2\)O\(_2\). The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 \(\mu\)mol of peroxide per min at 37°C and was expressed as units of MPO per milligram of proteins.

**Immunohistochemical Localization of TNF-\(\alpha\), Nitrotyrosine, FasL, iNOS, Bax, and Bel-2.** Twenty-four hours after SCI, nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in the spinal cord sections to determine the localization of “peroxynitrite formation” and/or other nitrogen derivatives produced during SCI. At 24 h after SCI, the tissues were fixed in 10% (v/v) PBS-buffered formaldehyde and 8-mm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation with 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with anti-TNF-\(\alpha\) (1:500 in PBS (v/v); Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-iNOS antibody (1:500 in PBS (v/v); BD Biosciences Transduction Laboratories, Lexington, KY), or anti-nitrotyrosine rabbit polyclonal antibody [1:500 in PBS (v/v); Millipore, Billerica, MA], with anti-FasL antibody [1:500 in PBS (v/v); Abcam Inc., Cambridge, MA], anti-Bax antibody [1:500 in PBS (v/v); Santa Cruz Biotechnology, Inc.], or anti-Bcl-2 polyclonal antibody [1:500 in PBS (v/v); Santa Cruz Biotechnology, Inc.]. Sections were washed with PBS and incubated with secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG or anti-mouse and avidin-biotin peroxidase complex (DBA). To verify the binding specificity for nitrotyrosine, TNF-\(\alpha\), FasL, iNOS, Bax, and Bel-2, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all the experiments carried out.

**Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay.** TUNEL assay was conducted by using a TUNEL detection kit (Apopat, HRP kit; DBA), according to the manufacturer’s instructions. In brief, sections were incubated with 15 \(\mu\)g ml\(^{-1}\) proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated with 3% H\(_2\)O\(_2\) for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase buffer containing deoxynucleotidyl transferase and biotinylated dUTP in terminal deoxynucleotidyltransferase buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine. The number of TUNEL-positive cells/high-power field was counted in 5 to 10 fields for each coded slide.

**Western Blot Analysis for I\(\beta\)-\(\alpha\), NF-\(k\)B, Bax, Bel-2, iNOS, and PPAR-\(\gamma\).** Cytosolic and nuclear extracts were prepared as described previously (Bethea et al., 1998), with slight modifications. In brief, spinal cord tissues from each mouse were suspended in extraction buffer A containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 mM peptatin A, 20 \(\mu\)M leupeptin, and 1 mM sodium orthovanadate; homogenized at the highest setting for 2 min; and centrifuged at 1000 g for 10 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 \(\mu\)M leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation at 15,000 g for 30 min at 4°C, the supernatants containing the nuclear protein were stored at −80°C for further analysis. The levels of I\(\beta\)-\(\alpha\), iNOS, Bax, and Bel-2 were quantified in the cytosolic fraction from spinal cord tissue collected after 24 h after SCI, whereas NF-\(k\)B p65 and PPAR-\(\gamma\) were quantified in the nuclear fraction. The filters were blocked with 1× PBS and 5% (w/v) nonfat dried milk for 40 min at room temperature and subsequently probed with specific antibodies against I\(\beta\)-\(\alpha\) (1:1000; Santa Cruz Biotechnology, Inc.), or phospho-NF-\(k\)B p65 (serine 536) (1:1000; Cell Signaling Technology Inc., Danvers, MA), or Bax (1:500; Santa Cruz Biotechnology, Inc.), or Bel-2 (1:500; Santa Cruz Biotechnology, Inc.), or iNOS (1:1000; Cell Signaling Technology Inc.), or PPAR-\(\gamma\) (1:500; Santa Cruz Biotechnology, Inc.) in 1× PBS, 5% (w/v) nonfat dried milk, and 0.1% Tween 20 at 4°C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000; Jackson ImmunoResearch Laboratories Inc.) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against \(\beta\)-actin or lamin A/C protein (1:10,000; Sigma, Milan, Italy).

**Image Analysis and Quantification of Signals.** Signals were detected with enhanced chemiluminescence detection system reagent according to manufacturer’s instructions (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemical, Rockford, IL). The relative expression of the protein bands of I\(\beta\)-\(\alpha\) (−37 kDa), NF-\(k\)B p65 (65 kDa), Bax (−23 kDa), Bel-2 (−29 kDa), iNOS (−130 kDa), and PPAR-\(\gamma\) (−60 kDa) was quantified by densitometry with ImageQuant TL software (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and standardized to \(\beta\)-actin or lamin levels.

Images of blot signals (8 bit/600 dpi resolution) were imported to analysis software (ImageQuant TL, version 2003). A preparation of commercially available molecular mass markers (Amersham Dual-Vue Western Blotting Markers; GE Healthcare) consisting of proteins of a molecular mass of 15 to 150 kDa was used to define molecular mass positions and as reference concentrations for each molecular mass.

**Light Microscopy.** Spinal cord tissues were taken at 24 h after trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin-embedded and cut into 5-\(\mu\)m-thick sections. Tissue sections (5 \(\mu\)m) were deparaffinized with xylene, stained with hematoxylin and eosin or with silver impregnation for reticulum, and studied using light microscopy (Dialux 22 Leitz microscope; Leitz, Wetzlar, Germany). The segments of each spinal cord were evaluated by an experienced histopathologist. Damaged neurons were counted, and the histopathology changes of the gray matter were scored on a 6-point scale (Sirin et al., 2002): 0, no lesion observed; 1, gray matter contained 1 to 5 eosinophilic neurons; 2, gray matter contained 5 to 10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction (one third to one half of the gray matter area).
matter area); and 6, large infarction (more than half of the gray matter area). The scores from all the sections from each spinal cord were averaged to give a final score for individual mice. All the histological studies were performed in a blinded manner.

**Measurement of Spinal Cord TNF-α and IL-1β Levels.** Portions of spinal cord tissues, collected at 24 h after SCI, were homogenized as described previously in PBS containing 2 mmol l$^{-1}$ PMSF (Sigma), and tissue TNF-α and IL-1β levels were evaluated. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem, San Diego, CA) according to the manufacturer’s instructions. The TNF-α and IL-1β determinations were performed in duplicate serial dilutions.

**Thiobarbituric Acid-Reactant Substances Measurement.** Thiobarbituric acid-reactant substances measurement is considered a good indicator of lipid peroxidation, and was determined as described previously (Ohkawa et al., 1979), in the spinal cord tissue at 24 h after SCI. Thiobarbituric acid-reactant substances were calculated by comparison with optical density of standard solutions of 1,1,3,3-tetramethoxypropan 99% malondialdehyde bis (dimethyl acetel) 99% (MDA) (Sigma). The absorbance of the supernatant was measured by spectrophotometry at 650 nm. MDA quantities were calculated by linear regression analysis of the standard curve. Values were expressed as micromol MDA per milligram of proteins.

**Grading of Motor Disturbance.** The motor function of mice subjected to compression trauma was assessed once a day for 10 days after injury. Recovery from motor disturbance was graded using the modified murine Basso, Beattie, and Bresnahan (BBB) hind limb locomotor rating scale (Joshi and Fehlings, 2002). The following criteria were considered: 0, no hind limb movement; 1, slight (<50% range of motion) movement of one to two joints; 2, extensive (>50% range of motion) movement of one joint and slight movement of one other joint; 3, extensive movement of two joints; 4, slight movement in all three joints; 5, slight movement of two joints and extensive movement of one joint; 6, extensive movement of two joints and slight movement of one joint; 7, extensive movement of all three joints; 8, sweeping without weight support or plantar placement and no weight support; 9, plantar placement with weight support in stance only or dorsal stepping with weight support; 10, occasional (0–50% of the time) weight-supported plantar steps and no coordination (front/hind limb coordination); 11, frequent (50–94% of the time) to consistent (95–100% of the time) weight-supported plantar steps and no coordination; 12, frequent to consistent weight-supported plantar steps and occasional coordination; 13, frequent to consistent weight-supported plantar steps and frequent coordination; 14, consistent weight-supported plantar steps, consistent coordination and predominant paw position is rotated during locomotion (lift off and contact) or frequent plantar stepping, consistent coordination and occasional dorsal stepping; 15, consistent plantar stepping and coordination, no/occasional toe clearance, paw position is parallel at initial contact; 16, consistent plantar stepping and coordination (front/hind limb coordination) and frequent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; 17, consistent plantar stepping and coordination and frequent toe clearance and predominant paw position is parallel at initial contact and lift off; 18, consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and lift off; 19, consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and lift off and trunk instability; and 21, consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off and trunk stability.

**Materials.** All compounds, including GW0742 and GSK0660, were obtained from Sigma-Aldrich. All other chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; J.T. Baker, Deventer, Holland). The nuclear hormone receptors of the NR1C family conform to the British Journal of Pharmacology’s Guide to Receptors and Channels (Alexander et al., 2008).

**Statistical Evaluation.** All values in the figures and text are expressed as mean ± S.E.M. of N observations. For the in vivo studies, N represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analyzed by one-way analysis of variance followed by a Bonferroni post hoc test for multiple comparisons. A p value of <0.05 was considered significant. BBB scale data were analyzed by the Mann-Whitney U test and considered significant when p value was <0.05.

**Results**

GW0742 Reduces the Severity of Spinal Cord Trauma. The severity of the trauma at the level of the perilesional area was assessed by the presence of edema as well as any alteration of the white matter at 24 h after injury. Significant damage to the spinal cord was observed in the spinal cord tissue from SCI mice (Fig. 1B, and see histological score in E) compared with sham-operated mice (Fig. 1A and see histological score in E). Notably, a significant protection against the spinal cord injury was observed in GW0742-treated mice (Fig. 1C and see histological score in E). Co-administration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist (Fig. 1D and see histological score in E). Moreover, reticular and nervous fibers tissues structures were observed by silver impregnation. In sham-operated mice, a normal presence of reticular and nervous fibers was observed (Fig. 1F, particle F1). On the contrary, a significant alteration of reticular and nervous fibers was observed in the spinal cord tissue collected at 24 h after SCI (Fig. 1G). GW0742 treatment significantly reduced the alteration of reticular and nervous fibers associated with SCI (Fig. 1H, particle H1). To evaluate whether histological damage to the spinal cord was associated with a loss of motor function, the modified BBB hind limb locomotor rating scale score was evaluated. Although motor function was only slightly impaired in sham-operated mice, mice subjected to SCI had significant deficits in hind limb movement (Fig. 1I). GW0742 treatment ameliorated the functional deficits induced by SCI (Fig. 1I). Co-administration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist on motor function impairment (Fig. 1I).

Effects of GW0742 on Neutrophil Infiltration. The above-mentioned histological pattern of spinal cord injury appeared to be correlated with the influx of leukocytes into the spinal cord. Therefore, we investigated the effect of GW0742 on the neutrophil infiltration by measuring tissue MPO activity. MPO activity was significantly elevated in the spinal cord at 24 h after injury in mice subjected to SCI compared with sham-operated mice (Fig. 2). Treatment with GW0742 attenuated neutrophil infiltration into the spinal cord at 24 h after injury (Fig. 2). Co-administration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist (data not shown).

GW0742 Modulates Levels of TNF-α and IL-1β after SCI. To test whether GW0742 may modulate the inflammatory process through the regulation of the secretion of proinflammatory cytokines, we analyzed spinal cord tissue levels.
of TNF-α and IL-1β. A substantial increase in TNF-α and IL-1β production was found in spinal cord tissues samples collected from SCI mice 24 h after SCI (Fig. 3, A and B, respectively). Spinal cord levels of TNF-α and IL-1β were significantly attenuated by the intraperitoneal injection of GW0742 (Fig. 3, A and B, respectively). Likewise, at 24 h after SCI, expression of TNF-α in the spinal cord homogenates was determined by the immunohistological staining for TNF-α expression. There was no staining for TNF-α in spinal cord obtained from sham-operated mice (Fig. 3C, see densi-
tometry analysis in F). A substantial increase in TNF-α expression was found in inflammatory cells as well as in the nuclei of Schwann cells in the white and gray matter of the spinal cord tissues collected from SCI mice 24 h after SCI (Fig. 3D, particle D1, and see densitometry analysis in F). Spinal cord expression of TNF-α (Fig. 3E and see densitometry analysis in F) was significantly attenuated in GW0742-treated SCI mice compared with vehicle-treated SCI animals. Coadministration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist (data not shown).

**Effect of GW0742 on IκB-α Levels and NF-κB p65 Activation.** We evaluated IκB-α levels and nuclear NF-κB p65 by Western blot analysis to investigate the cellular mechanisms by which treatment with GW0742 may attenuate the development of SCI. A basal level of IκB-α was detected in the spinal cord from sham-operated animals, whereas IκB-α levels were substantially reduced in SCI mice. GW0742 administration reduced the SCI-induced IκB-α degradation (Fig. 4, A and A1). Moreover, NF-κB p65 levels in the nuclear fractions from spinal cord tissue were also significantly increased at 24 h after SCI compared with the sham-operated mice (Fig. 4, B and B1). GW0742 treatment significantly reduced the levels of NF-κB p65 as shown in Fig. 4, B and B1. Coadministration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist (data not shown).

**GW0742 Modulates Expression of iNOS after SCI.** To determine the role of NO produced during SCI, iNOS expression was evaluated by immunohistochemical analysis of spinal cord tissue. A substantial increase in TNF-α expression was found in inflammatory cells as well as in the nuclei of Schwann cells in the white and gray matter of the spinal cord tissues collected from SCI mice 24 h after SCI (Fig. 3D, particle D1, and see densitometry analysis in F).
mainly localized in various inflammatory cells in the gray matter. GW0742 treatment reduced the degree of positive staining for iNOS in the spinal cord tissues (Fig. 5C and see densitometry analysis in D). Likewise, at 24 h after SCI, the expression of iNOS in the spinal cord homogenates was investigated by Western blot. A significant increase of iNOS (Fig. 5, E and E1) levels was observed in the spinal cord from mice subjected to SCI. On the contrary, GW0742 treatment prevented the SCI-induced iNOS expression (Fig. 5, E and E1). Coadministration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist (data not shown).

Effects of GW0742 on Nitrotyrosine Formation and Lipid Peroxidation after SCI. Spinal cord sections from sham-operated mice did not stain for nitrotyrosine (Fig. 6A and see densitometry analysis in D), whereas spinal cord sections obtained from SCI mice exhibited positive staining for nitrotyrosine (Fig. 6B and see densitometry analysis in D). The positive staining was mainly localized in inflammatory cells as well as in the nuclei of Schwann cells in the white and gray matter of the spinal cord tissues. GW0742 treatment reduced the degree of positive staining for nitrotyrosine (Fig. 6C and see densitometry analysis in D) in the spinal cord. In addition, 24 h after SCI, thiobarbituric acid-reactant substance levels were also measured in the spinal cord tissue as an indicator of lipid peroxidation. A significant increase in thiobarbituric acid-reactant substances (Fig. 6E) was observed in the spinal cord at 24 h after SCI compared with sham-operated mice. Thiobarbituric acid-reactant substances (Fig. 6E) were significantly attenuated by the intraperitoneal injection of GW0742. Coadministration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist (data not shown).

Effects of GW0742 on Apoptosis in Spinal Cord after Injury. Immunohistological staining for FasL in the spinal cord was also determined 24 h after injury. Spinal cord sections from sham-operated mice did not stain for FasL (Fig. 7A and see densitometry analysis in D), whereas spinal cord sections obtained from SCI mice exhibited positive staining for FasL (Fig. 7B and see densitometry analysis in D) mainly localized in inflammatory cells as well as in the nuclei of Schwann cells. GW0742 treatment reduced the degree of positive staining for FasL in the spinal cord (Fig. 7C and see densitometry analysis in D). Coadministration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist (data not shown).

Effects of GW0742 on Apoptosis in Spinal Cord after Injury. To test whether spinal cord damage was associated with cell death by apoptosis, we measured TUNEL-like staining in the perilesional spinal cord tissue. Almost no apoptotic cells were detected in the spinal cord from sham-operated mice (Fig. 8A). At 24 h after the trauma, tissues from SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 8, B and B1). In contrast, tissues obtained from mice treated with GW0742 demonstrated no apoptotic cells or fragments (Fig. 8C). Coadministration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist (data not shown).

Western Blot Analysis and Immunohistochemistry for Bax and Bcl-2. At 24 h after SCI, samples of spinal cord tissue were taken to determine the immunohistological stain-
ing for Bax and Bcl-2. Spinal cord sections from sham-operated mice did not stain for Bax (Supplemental Fig. 1A), whereas spinal cord sections obtained from SCI mice exhibited a positive staining for Bax (Supplemental Fig. 1B). GW0742 treatment reduced the degree of positive staining for Bax in the spinal cord of mice subjected to SCI (Supplemental Fig. 1C). In addition, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Supplemental Fig. 1D), whereas in SCI mice the staining was significantly reduced (Supplemental Fig. 1E). GW0742 treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (Supplemental Fig. 1F).

Moreover, 24 h after SCI, expression of the proapoptotic protein Bax was investigated in spinal cord homogenates by Western blot. Bax levels were appreciably increased in the spinal cord from mice subjected to SCI (Supplemental Fig. 2, A and A1). On the contrary, GW0742 treatment reduced the SCI-induced Bax expression (Supplemental Fig. 2, A and A1). Western blot analysis was also carried out for Bcl-2 expression in homogenates from the spinal cord of each mouse. A basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (Supplemental Fig. 2, B and B1). Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in the spinal cord of SCI mice (Supplemental Fig. 2, B and B1). Treatment of mice with GW0742 significantly blunted the SCI-induced inhibition of antiapoptotic protein expression (Supplemental Fig. 2, B and B1). Coadministration of GSK0660 and GW0742 significantly blocked the effect of the PPAR-β/δ agonist on Bax and Bcl-2 expression (data not shown).

Fig. 5. Effects of GW0742 on iNOS expression and its immunohistochemical localization in spinal cord tissue. Spinal cord sections were processed at 24 h after SCI to determine the immunohistochemical staining for iNOS expression. No evidence of positive staining for iNOS was observed in spinal cord sections obtained from sham-group animals (A). A substantial increase in iNOS expression was found in inflammatory cells, in the nuclei of Schwann cells in the white and gray matter of the spinal cord tissues from SCI mice at 24 h after SCI (B). Spinal cord levels of iNOS were significantly attenuated in GW0742-treated mice compared with SCI animals (C). Densitometry analysis of immunocytochemistry photographs (N = 5 photos from each sample collected from all mice in each experimental group) for iNOS (D) from spinal cord tissues was assessed. In addition, iNOS levels analyzed by Western blot analysis, were significantly increased in the spinal cord from SCI mice (E and E1). On the contrary, GW0742 treatment significantly reduced the SCI-induced expression of iNOS (E and E1). The relative expression of the protein band was standardized for densitometric analysis to β-actin levels and is reported in E1(OD, optical density) and expressed as mean ± S.E.M. from N = 5 to 6 spinal cord for each group. Data are expressed as percentage of total tissue area. The figure is representative of at least three experiments performed on different experimental days. *, p < 0.01 versus sham; ○, p < 0.01 versus SCI vehicle.
Effects of GW0742 on PPAR-β/δ Expression. Western blot analysis shows that PPAR-β/δ was expressed in uninjured spinal cords (Fig. 9). Twenty-four hours after spinal cord injury, PPAR-β/δ protein expression was significantly reduced in spinal cord homogenates. GW0742 treatment significantly prevented the SCI-induced down-regulation of PPAR-β/δ (*, p < 0.01 versus sham; ○, p < 0.01 versus SCI + vehicle).

Discussion

Primary injury to the adult spinal cord is irreversible, whereas much of the damage that occurs in the spinal cord after traumatic injury is due to the secondary injury and in particular to the effects of glutamate excitotoxicity, Ca²⁺ overload, and oxidative stress, three mechanisms that take part in a spiralling interactive cascade ending in neuronal dysfunction and death (Tator and Koyanagi, 1997).

In this report, we demonstrate that GW0742, a high-affinity PPAR-β/δ agonist, exerts beneficial effects in a mouse model of SCI. We demonstrate that SCI resulted in edema and loss of myelin in lateral and dorsal funiculi. This histological damage was associated with the loss of motor function. SCI induced an inflammatory response in the spinal cord, with increased IκB-α degradation; enhanced NF-κB activation; amplified expression of proinflammatory mediators, proinflammatory cytokines, and nitrotyrosine; and increased MPO activity. Our results show that GW0742 reduced the degree of all inflammatory and apoptotic parameters.

The multiplicity of phenotypes induced by PPAR-β/δ gene disruption in the mouse reflects the importance of this nuclear receptor in development, and early functional studies indicate PPAR-β/δ involvement in epidermal differentiation, maturation, and skin wound healing (Matsuura et al., 1999). Evidence has also suggested that activation of PPAR-β/δ promotes fatty acid catabolism in several tissues, such as skeletal muscle and adipose (Tanaka et al., 2003; Yue et al., 2008). More recent studies indicate a potential role of PPAR-
β/δ in regulating glucose metabolism and insulin sensitivity (Lee et al., 2006). These actions could explain the apparently beneficial effects of synthetic PPAR-β/δ agonists on circulating lipids, insulin resistance, and obesity that have been reported in some animal models (Oliver et al., 2001; Lee et al., 2006).

Compared with PPAR-α and PPAR-γ, relatively little is known on the role of PPAR-β/δ in the regulation of inflammatory responses. Welch et al. (2003) demonstrated that a PPAR-β/δ agonist inhibited LPS-inducible genes, such as iNOS and COX-2, in murine peritoneal macrophages. It has also been shown that PPAR-β/δ ligands inhibited LPS-induced TNF-α production from cardiomyocytes (Ding et al., 2006). The present results, showing an attenuation of SCI-induced up-regulation of TNF-α and iNOS by GW0742 are in good agreement with these previous reports showing anti-inflammatory effects of PPAR-β/δ agonist. These effects could be mediated through regulation of the NF-κB pathway, as it had been proposed previously (Kapoor et al., 2009).

Recent studies have suggested one distinct model for corepressor-dependent transrepression by PPAR-β/δ. Evidence has been presented in which PPAR-β/δ controls the inflam-
In the absence of ligand, PPAR-β/δ expression was significantly reduced in spinal cord homogenates. GW0742 treatment significantly prevented the SCI-induced down-regulation of PPAR-β/δ. The results are expressed as mean ± S.E.M. from N = 5 to 6 spinal cord for each group. OD, optical density. *, p < 0.01 versus sham; ○, p < 0.01 versus SCI + vehicle.

Fig. 9. GW0742 reverted SCI-induced down-regulation of PPAR-β/δ expression. Western blot analysis shows that PPAR-β/δ was expressed in uninjured spinal cords. After spinal cord injury, PPAR-β/δ protein expression was significantly reduced in spinal cord homogenates. GW0742 treatment significantly prevented the SCI-induced down-regulation of PPAR-β/δ. The results are expressed as mean ± S.E.M. from N = 5 to 6 spinal cord for each group. OD, optical density. *, p < 0.01 versus sham; ○, p < 0.01 versus SCI + vehicle.

In addition, in the present study we clearly demonstrated that GW0742 treatment fully inhibited the appearance of nitrotyrosine, an indication of “increased nitrosative stress,” staining in the inflamed tissue. Moreover, the reduced neutrophil infiltration into the inflamed tissue may be related to these previously described actions of GW0742. Further study is needed to fully determine the anti-inflammatory and cytoprotective effects of GW0742 observed in the current study.

Several studies suggest that glial cells in neurodegenerative diseases are affected more than neurons by apoptotic cell death (Beattie et al., 2000). Apoptosis is an important mediator of secondary damage after SCI. It incurs its effects through at least two phases: an initial phase, in which apoptosis accompanies necrosis in the degeneration of multiple cell types; and a later phase that is predominantly confined to white matter and involves oligodendrocytes and microglia (Chittenden et al., 1995). Chronically, apoptosis initially occurs 6 h after injury at the lesion center and lasts for several days in association with a steadily increased number of apoptotic cells in this region. An important intracellular signal transduction pathway that leads to apoptosis after SCI involves activation of the caspases, in particular, caspase-3 (Chittenden et al., 1995). In an effort to prevent or diminish levels of apoptosis, we have demonstrated that treatment with GW0742 attenuates the degree of apoptosis in the spinal cord after injury, measured by a TUNEL detection kit.

Recently, it has been demonstrated, in vitro, that the neuroprotective effects of the selective PPAR-β/δ agonists were closely correlated with caspase-3/7 inhibitory activity, suggesting that the PPAR-β/δ agonists possess potent antiapoptotic properties (Iwashita et al., 2007). In addition, it was recently reported that PPAR-β/δ activation stimulates promoter activity of the gene coding the 14-3-3 protein (Liou et al., 2006). Up-regulation of the 14-3-3 protein amplifies phosphorylated Bad binding, sequesters Bad in the cytosol, and results in reduced Bad translocation to the mitochondria, ultimately inhibiting cytochrome c release, caspase-3 activation, and apoptosis (Liou et al., 2006). Furthermore, the 14-3-3 protein is most abundantly expressed in neurons of the central nervous system, and its binding to Bad is known to inhibit the apoptotic process (Berg et al., 2003).

Moreover, various studies have postulated that preserving Bax, a proapoptotic gene, plays an important role in developmental cell death and in central nervous system injury (Nesic-Taylor et al., 2005). Likewise, it has been shown that the administration of Bcl-XL fusion protein (Bcl-XL FP) (Bcl-2 is the most expressed antiapoptotic molecule in adult central nervous system) into injured spinal cords significantly increased neuronal survival, suggesting that SCI-induced changes in Bcl-XL contribute considerably to neuronal death increase in TNF-α and IL-1β in SCI. On the contrary, no significant increase in expression of TNF-α and IL-1β was observed in the spinal cord sections obtained from SCI mice treated with GW0742, suggesting that the PPAR-β/δ pathway may play an important role in the regulation of proinflammatory cytokines. Therefore, the inhibition of the production of TNF-α and IL-1β by GW0742 described in the present study is probably attributed to the inhibitory effect of activated NF-κB. This observation is in agreement with a previous study in which GW0742 treatment reduced the production of proinflammatory cytokines in the bronchoalveolar lavage of LPS-treated mice (Haskova et al., 2008).

We report here that SCI caused a significant reduction of the basal level of IκB-α in the spinal cord tissues at 24 h, whereas IκB-α levels were transcriptionally up-regulated by treatment with GW0742. Moreover, we also demonstrated that GW0742 inhibited NF-κB translocation. Taken together, the balance between proinflammatory and prosurvival roles of NF-κB may depend on the phosphorylation status of p65, and PPAR-β/δ may play a central role in this process (Esposito et al., 2006; Glass and Ogawa, 2006; Straus and Glass, 2007).

NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation. In this regard, it has been well demonstrated that in SCI the expression of proinflammatory cytokines (TNF-α and IL-1β) at the site of injury regulate the cascade of cellular and biochemical events and generation of free radicals that lead to further tissue damage after SCI (Genovese et al., 2006). We have confirmed a significant


Matsushita T, Nakamura Y, Sakanaka M, Araki H, and Matsuura T (2003) Peroxisome proliferator-activated receptor-beta/delta expression is increased in mice treated with GW0742. Furthermore, some studies have also shown that FasL and p75 receptors are expressed on oligodendrocytes, astrocytes, and microglia in the spinal cord after SCI. FasL and p75 colocalize on many TUNEL-positive cells, suggesting that the FasL- and p75-initiated cell death cascades may participate in the demise of some glia after SCI.

Recently, it has been pointed out that FasL signaling plays a central role in SCI (Ackery et al., 2006). We confirm here that SCI leads to a substantial activation of FasL in the spinal cord that probably contributes to the evolution of tissue injury. In the present study, we found that GW0742 treatment leads to a substantial reduction of FasL activation. It is not possible to exclude the antiapoptotic effect observed after GW0742 treatment may be partially dependent on the attenuation of the inflammation-induced damage.

To elucidate whether the anti-inflammatory effect of GW0742 observed here is related to activation of the PPARbeta/delta receptor, we also investigated the effect of the PPARbeta/ delta antagonist GSK0660 on the anti-inflammatory effects of GW0742. We demonstrated in vivo that GSK0660 significantly attenuates the protective effect of GW0742. It is interesting to note that we used a spinal compression model to examine the expression of PPARbeta/ delta in sham and injured spinal cords. A basal level of PPARbeta/ delta expression was detected in spinal cord homogenates from sham-operated mice. After spinal cord injury, PPARbeta/ delta protein expression was significantly reduced, whereas GW0742 treatment significantly prevented the SCI-induced down-regulation of PPARbeta/ delta.

Finally, in this study we demonstrate that GW0742 treatment significantly reduced the SCI-induced spinal cord tissue alteration in addition to improving motor function. The results of the present study enhance our understanding of the role of PPARbeta/ delta in the pathophysiology of spinal cord injury after trauma, implying that a PPARbeta/ delta ligand may be useful in the therapy of spinal cord injury, trauma, and inflammation.

Acknowledgments

We thank Giovanni Pergolizzi and Carmelo La Spada for excellent technical assistance during this study, Caterina Cutrona for secretarial assistance, and Valentina Malvagini for editorial assistance with the manuscript.

References


Address correspondence to: Prof. Salvatore Cuzzocrea, Department of Clinical and Experimental Medicine and Pharmacology, School of Medicine, University of Messina, Torre Biologica-Policlinico Universitario, Via C. Valeria-Gazzi, 98100 Messina, Italy. E-mail: salvator@unime.it