

Effects of Palmitoylethanolamide on Signaling Pathways Implicated in the Development of Spinal Cord Injury

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ABSTRACT

Activation of peroxisome proliferator-activated receptor (PPAR)- α , a member of the nuclear receptor superfamily, modulates inflammation and tissue injury events associated with spinal cord trauma in mice. Palmitoylethanolamide (PEA), the naturally occurring amide of palmitic acid and ethanolamine, reduces pain and inflammation through a mechanism dependent on PPAR- α activation. The aim of the present study was to evaluate the effect of the PEA on secondary damage induced by experimental spinal cord injury (SCI) in mice. SCI was induced by application of vascular clips to the dura mater via a four-level T₅-T₈ laminectomy. This resulted in severe trauma characterized by edema, neutrophil infiltration, and production of inflammatory mediators, tissue dam-

age, and apoptosis. Repeated PEA administration (10 mg/kg i.p.; 30 min before and 1 and 6 h after SCI) significantly reduced: 1) the degree of spinal cord inflammation and tissue injury, 2) neutrophil infiltration, 3) nitrotyrosine formation, 4) proinflammatory cytokine expression, 5) nuclear transcription factor activation- κ B activation, 6) inducible nitric-oxide synthase expression, and 6) apoptosis. Moreover, PEA treatment significantly ameliorated the recovery of motor limb function. Together, the results indicate that PEA reduces inflammation and tissue injury associated with SCI and suggest a regulatory role for endogenous PPAR- α signaling in the inflammatory response associated with spinal cord trauma.

Spinal cord injury (SCI) is a highly debilitating pathology (Maegle et al., 2005). 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 a number of neurons that cannot be recovered and regenerated. Studies indicate that neurons continue to die for hours following 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. A large body of

recent data suggests the presence of a local inflammatory response, which amplifies the secondary damage (Blight, 1992).

Moreover, evidence has suggested that resident microglia and macrophages originating from blood are two key cell types related to the occurrence of neuronal degeneration in the central nervous system after traumatic injury. In particular, when SCI occurs, microglia in parenchyma is activated, and macrophages in circulation cross the blood-brain barrier (BBB) to act as intrinsic spinal phagocytes. Therefore, these cells can release various neurotrophic peptides such as brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, and laminin, which are excellent substrates for neurite outgrowth.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (Evans, 1988). The PPARs are ligand-dependent transcription factors that regulate target gene expression by binding as heterodimers with

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ABBREVIATIONS: SCI, spinal cord injury; BBB, blood-brain barrier; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PEA, palmitoylethanolamide; iNOS, inducible nitric-oxide synthase; NF- κ B, nuclear transcription factor activation- κ B; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; MPO, myeloperoxidase activity; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TNF, tumor necrosis factor; IL, interleukin; MDA, malondialdehyde bis (dimethyl acetal); PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP-ribose).

retinoid X receptors (RXRs) to specific peroxisome proliferator response elements in enhancer sites of regulated genes of DNA. The PPAR subfamily comprises three members, PPAR- α , PPAR- β , and PPAR- γ (Murphy and Holder, 2000). RXRs are also members of the nuclear hormone receptor superfamily that are activated by binding of 9-cis retinoic acid (Desvergne and Wahli, 1999). In the absence of a ligand, high-affinity complexes are formed between the PPAR-RXR heterodimer and nuclear receptor corepressor proteins, preventing transcriptional activation by sequestration of the nuclear receptor heterodimer from the promoter. Binding of a ligand to the heterodimer results in the release of the corepressor from the complex, which in turn results in the binding of the activated heterodimer to the response element in the promoter region of the relevant target genes, resulting in either the activation or suppression of a specific gene (Desvergne and Wahli, 1999).

In rats, PPAR- α is most highly expressed in brown adipose tissue, followed by liver, kidney, heart, and skeletal muscle (Wayman et al., 2001). Recently, the presence of PPAR- α in discrete areas of brain and spinal cord has been suggested (Moreno et al., 2004), although their role remains unknown. PPAR- α is a receptor for a diverse set of fatty acid derivatives, including oleoylethanolamide, which binds to the purified ligand-binding domain of PPAR- α with a K_d of 40 nM and activates it with an EC_{50} of 120 nM (Astarita et al., 2006), and palmitoylethanolamide (PEA), a compound whose profound anti-inflammatory effects are mediated by PPAR- α (Lo Verme et al., 2005). Various lines of evidence suggest that the activation of PPAR- α by synthetic agonists causes marked anti-inflammatory effects in experimental models (Cuzzocrea et al., 2004). Indeed, we have recently demonstrated using PPAR- α knockout mice that endogenous PPAR- α activity reduces the degree of development of inflammation and tissue injury events associated with spinal cord trauma in mice, suggesting the existence of an intrinsic anti-inflammatory mechanism mediated by PPAR- α .

PEA was identified more than 5 decades ago (Long and Martin, 1956) and was shown to reduce allergic reactions and inflammation in animals (Perlík et al., 1971) along with influenza symptoms in humans (Kahlich et al., 1979). However, interest in this compound faded until the discovery that one of its structural analogs, anandamide (arachidonylethanolamide), serves as an endogenous ligand for cannabinoid receptors, the molecular target of Δ^9 -tetrahydrocannabinol in marijuana (Devane et al., 1992). Since this finding, PEA has been shown to inhibit peripheral inflammation and mast cell degranulation (Berdyshev et al., 1998), as well as to exert neuroprotective (Lambert et al., 2001) and antinociceptive (Calignano et al., 1998) effects in rats and mice. These actions are mediated by PPAR- α activation and are accompanied by a decrease in nitric oxide production (Ross et al., 2000), neutrophil influx (Farquhar-Smith et al., 2002), and expression of proinflammatory proteins such as inducible nitric-oxide synthase (iNOS) and cyclooxygenase-2 (Costa et al., 2002).

Based on this evidence, in the present study, we investigated the effect of PEA in the secondary damage resulting from experimental SCI in mice. In particular, we examined the following endpoints of the inflammatory response: 1) histological damage, 2) motor recovery, 3) neutrophil infiltration, 4) NF- κ B expression, 5) nitrotyrosine formation and

iNOS expression, 6) proinflammatory cytokine production, 7) apoptosis as terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, and 8) Fas-ligand, Bax, and Bcl-2 expression.

Materials and Methods

Animals. Male adult 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 EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

SCI. Mice were anesthetized using chloral hydrate (400 mg/kg body weight). We used the clip compression model described by Rivlin and Tator (1978) and produced SCI by extradural compression of a section of the SC exposed via a four-level T₅-T₈ laminectomy, in which the prominent spinous process of T₅ was used as a surgical guide. A six-level laminectomy was chosen to expedite timely harvest and to obtain enough SC tissue for biochemical examination. With the aneurysm clip applicator oriented in the bilateral direction, an aneurysm clip with a closing force of 24 g was applied extradurally at the T₅-T₈ level. The clip was then rapidly released with the clip applicator, which caused SC compression. In the injured groups, the cord was compressed for 1 min. After surgery, 1.0 ml of saline was administered s.c. 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.

Experimental Design. Mice were randomized into four groups ($n = 40$ animals/group). Sham animals were subjected to the surgical procedure, with the exception that the aneurysm clip was not applied and treated i.p. with vehicle or PEA (10 mg/kg) 30 min before and 1 and 6 h after surgical procedure. The remaining mice were subjected to SCI (as described above) and treated with an i.p. bolus of vehicle (saline) or PEA (10 mg/kg) 30 min before and 1 and 6 h (pretreatment) or at 6 and 12 h (post-treatment) after SCI. The doses of PEA (10 mg/kg) used here were based on previous in vivo study (Lo Verme et al., 2005). To investigate the motor score, in other set of experiments, the animals were treated with PEA 30 min before and 1 and 6 h (pretreatment) or at 6 and 12 h (post-treatment) after SCI and daily until day 9. 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 PMN accumulation, was determined in the spinal cord tissues as described previously (Mullane, 1989) at 24 h after SCI. At the specified time following SCI, spinal cord tissues were obtained and weighed, and each piece was homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer, pH 7, and centrifuged for 30 min at 20,000g 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 H₂O₂. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μ mol of peroxide/min at 37°C and was expressed as units of MPO per milligram of proteins.

Immunohistochemical Localization of PAR, Nitrotyrosine, FAS-Ligand, iNOS, Bax, and Bcl-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 the 24 h after SCI, the tissues were fixed in 10% (w/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% (w/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 for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with anti-PAR [Trevigen, 1:500 in PBS (v/v)], anti-iNOS antibody [BD Biosciences Transduction Laboratories, Lexington, KY; 1:500 in PBS (v/v)], and anti-nitrotyrosine rabbit polyclonal antibody [Millipore, Billerica, MA; 1:500 in PBS (v/v)], with anti-FAS-ligand antibody (Abcam; 1:500 in PBS, v/v), anti-Bax antibody [Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:500 in PBS (v/v)], or with anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, Inc.; 1:500 in PBS, v/v). Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA). To verify the binding specificity for nitrotyrosine, PAR, iNOS, FAS-L, Bax, and Bcl-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 of the experiments carried out. Immunocytochemistry photographs ($n = 5$ photos from each samples collected from all rats in each experimental group) were assessed by densitometry as described previously (Shea, 1994; Cuzzocrea et al., 2001) by using Optilab Graftek software on a Macintosh personal computer.

TUNEL Assay. TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Apotag, HRP kit; DBA). In brief, sections were incubated with 15 μ g/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyl transferase buffer containing deoxynucleotidyl transferase and biotinylated dUTP in terminal deoxynucleotidyl transferase 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 κ B- α , Phospho-NF- κ B p65 (Ser536), NF- κ B p65, Bax, Bcl-2, and PPAR- α . 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 μ M pepstatin A, 20 μ M leupeptin, and 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1000g 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 μ M of leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation for 30 min at 15,000g at 4°C, the supernatants containing the nuclear protein were stored at -80 for further analysis. The levels of I κ B- α , phospho-NF- κ B p65 (Ser536), Bax, and Bcl-2 were quantified in cytosolic fraction from spinal cord tissue collected 24 h after SCI, whereas PPAR- α and NF- κ B p65 levels were quantified in nuclear fraction. The filters were blocked with 1 \times PBS and 5% (w/v) nonfat dried milk for 40 min at room temperature and subsequently probed with specific Abs, I κ B- α (1:1000; Santa Cruz Biotechnology, Inc.), phospho-NF- κ B p65 (Ser536) (1:1000; Cell Signaling Technology Inc., Danvers, MA), anti-Bax (Santa Cruz Biotechnology, Inc.; 1:500), anti-Bcl-2 (Santa Cruz Biotechnology, Inc.; 1:500), anti-PPAR- α , (1:1000; Santa Cruz Biotech-

nology, Inc.), or anti-NF- κ B p65 (1:1000; Santa Cruz Biotechnology, Inc.) in 1 \times 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., West Grove, PA) for 1 h at room temperature.

To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against α -tubulin protein (1:10,000; Sigma-Aldrich, Milan, Italy). The relative expression of the protein bands of I κ B- α (~37 kDa), phospho-NF- κ B (75 kDa), NF- κ B p65 (65 kDa), Bax (~23 kDa), Bcl-2 (~29 kDa), and PPAR- α (~55 kDa) was quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (GS-700; Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst; IBM, White Plains, NY) and standardized for densitometric analysis to α -tubulin levels.

Light Microscopy. Spinal cord tissues were taken at 24 h following trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5- μ m-thick sections. Tissue sections (5- μ m thickness) were deparaffinized with xylene, stained with hematoxylin/eosin, and studied using light microscopy (Dialux 22 Leitz).

The segments of each spinal cord were evaluated by an experienced histopathologist. Damaged neurons were counted, and the histopathologic changes of the gray matter were scored on a six-point scale (Sirin et al., 2002): 0, no lesion observed; 1, gray matter contained one to five eosinophilic neurons; 2, gray matter contained five 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); 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 of the histological studies were performed in a blinded fashion.

Measurement of Spinal Cord TNF- α and IL-1 β Levels. Portions of spinal cord tissues, collected at 24 h after SCI, were homog-

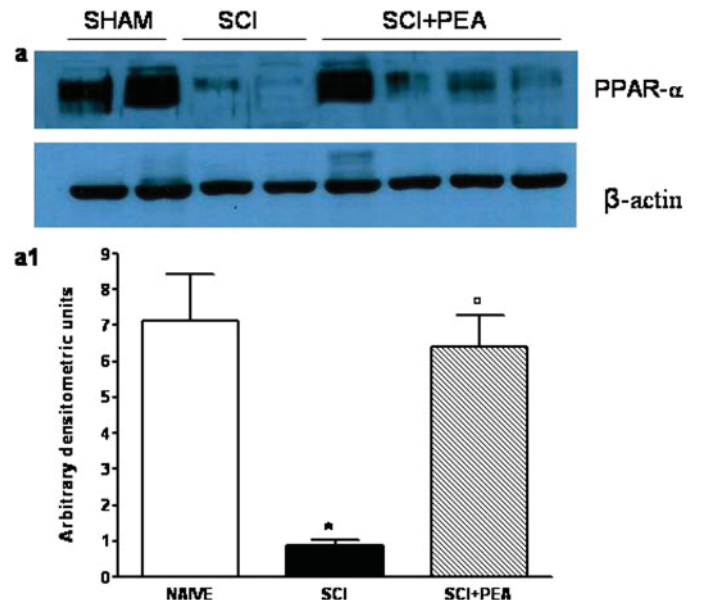


Fig. 1. PPAR- α expression in spinal cord tissues. A basal level of PPAR- α was detected in the spinal cord tissues from sham-operated mice, whereas in SCI-operated mice, the PPAR- α levels were substantially reduced (a and a1). Significant reduced levels of PPAR- α degradation were observed in the spinal cord tissues collected from SCI-operated mice, which have been treated with PEA (a and a1). The relative expression of the protein bands was standardized for densitometric analysis to β -actin levels; reported in a1 are expressed as mean \pm S.E.M. from $n = 5$ to 6 spinal cords for each group. *, $p < 0.01$ versus sham; ○, $p < 0.01$ versus SCI + vehicle.

enized as previously described in PBS containing 2 mM PMSF (Sigma-Aldrich), and tissue TNF- α and IL-1 β levels were evaluated. The assay was carried out by using a colorimetric, commercial kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. All TNF- α and IL-1 β determinations were performed in duplicate serial dilutions.

Thiobarbituric Acid-Reactant Substance Measurement. Thiobarbituric acid-reactant substances measurement, which is considered a good indicator of lipid peroxidation, 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 OD₅₅₀ of standard solutions of 99% 1,1,3,3-tetraethoxypropan and 99% malondialdehyde bis (dimethyl acetal) (MDA) (Sigma-Aldrich). 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 micromolar 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 (Basso et al., 1995) hind limb locomotor rating scale (Joshi and Fehlings, 2002a,b). 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 are 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 rotated at lift off; 19, consistent plantar stepping and coordination and consistent toe clearance, and predominant paw position is parallel at initial contact and lift off; 20, consistent plantar stepping, coordinated gait, 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 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; Baxter, Italy, UK).

Statistical Evaluation. All values in the figures and text are expressed as mean \pm S.E.M. of *n* observations. For the in vivo

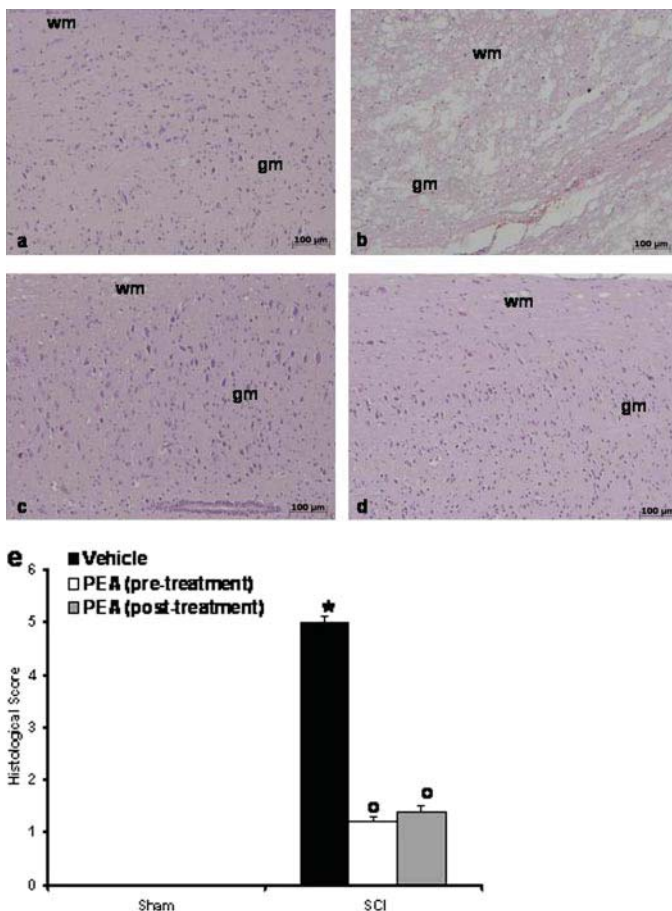


Fig. 2. Effect of PEA treatment on histological alterations of the spinal cord tissue 24 h after injury. No histological alterations were observed in the spinal cord tissues collected from sham-operated mice (a). A significant damage to the spinal cord, from SCI-operated mice at the perilesional area, was assessed by the presence of edema as well as alteration of the white matter 24 h after injury (b). It is noteworthy that a significant protection from the SCI was observed in the tissue collected from PEA (c). The histological score (d) was made by an independent observer. wm, white matter; gm, gray matter. This figure is representative of at least three experiments performed on different experimental days. Values shown are mean \pm S.E.M. of 10 mice for each group. *, $p < 0.01$ versus SCI.

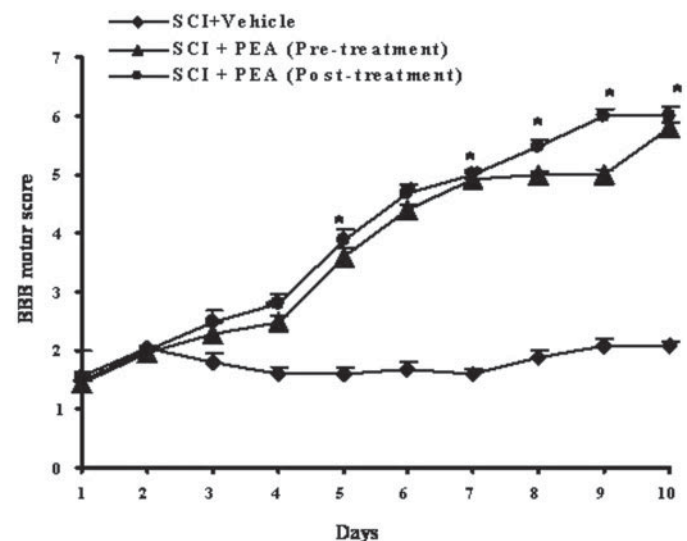


Fig. 3. Effect of PEA on hind limb motor disturbance after spinal cord injury. The degree of motor disturbance was assessed every day until 10 days after SCI by Basso, Beattie, and Bresnahan criteria. Treatment with PEA reduces the motor disturbance after SCI. Values shown are mean \pm S.E.M. of 10 mice for each group. ^o, $p < 0.01$ versus SCI.

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 (histological or immunohistochemistry coloration) performed on different experimental days on the tissue sections collected from all the animals in each group; 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 less than 0.05 was considered significant. BBB scale data were analyzed by the Mann-Whitney test and considered significant when the *p* value was <0.05.

Results

Effects of PEA Treatment on PPAR- α Expression in Spinal Cord Tissue. Previous studies have demonstrated

an important role for PPAR α in SCI (Genovese et al., 2005) and have suggested that the ability of PEA to reduce pain and inflammation is dependent on activation of PPAR- α . Thus, we evaluated PPAR α expression in the nuclear fractions from spinal cord tissue by Western blot analysis. A basal level of PPAR- α was detected in spinal cord tissue from sham-operated mice, whereas PPAR- α levels were substantially reduced in SCI-operated mice (Fig. 1, a and a1). The effect of SCI was significantly reduced by PEA administration (Fig. 1, a and a1).

PEA Reduces the Severity of Spinal Cord Trauma.

The severity of trauma in the perilesional area, assessed by presence of edema and white matter alteration (Fig. 2b and see histological score in d), was evaluated 24 h after injury. Significant damage was observed in spinal cord tissue from

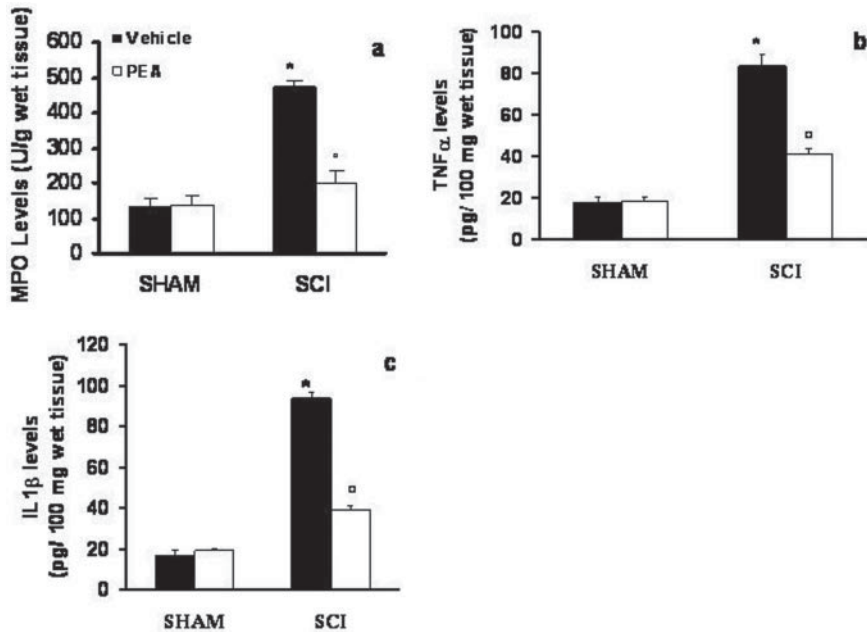


Fig. 4. Effects of PEA on MPO activity and spinal cord TNF- α and IL-1 β levels. After the injury, MPO activity in spinal cord from SCI mice was significantly increased at 24 h after the damage in comparison with sham groups (A). In addition, a substantial increase in TNF- α (B) and IL-1 β (C) production was found in spinal cord tissues from SCI mice 24 h after SCI. Treatment with PEA significantly attenuated neutrophil infiltration as well as TNF- α and IL-1 β levels into the spinal cord. Data are mean \pm S.E.M. of 10 mice for each group. *, *p* < 0.01 versus sham; \circ , *p* < 0.01 versus SCI + vehicle.

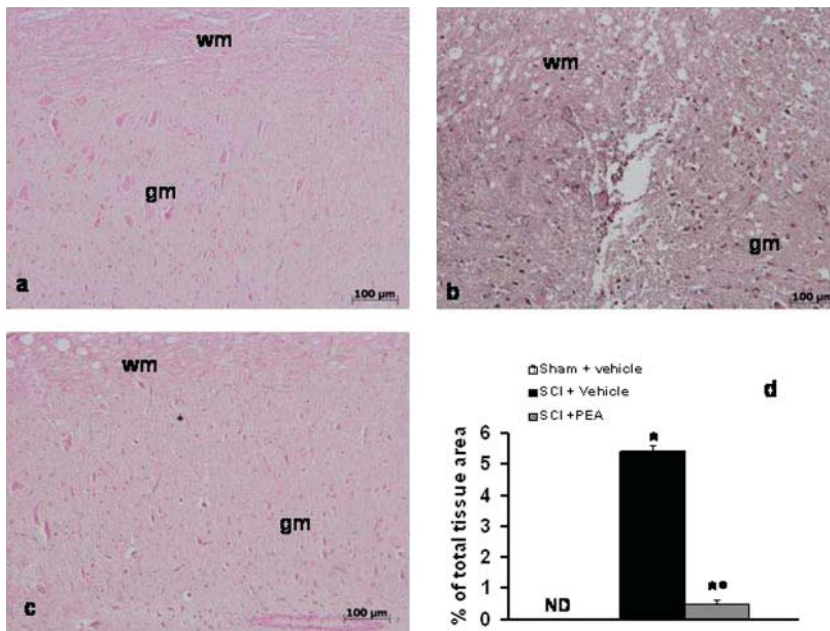


Fig. 5. Effects of PEA on iNOS expression. Spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for iNOS expression. No positive staining for iNOS was observed in the spinal cord tissues from sham-operated mice (a). A substantial increase in iNOS expression was found in inflammatory cells in nuclei of Schwann cells in wm and gm of the spinal cord tissues from SCI mice at the 24th h after SCI (b). Spinal cord levels of iNOS were significantly attenuated in PEA-treated SCI mice (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. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days. *, *p* < 0.01 versus sham; \circ , *p* < 0.01 versus SCI + vehicle.

SCI mice, compared with sham-operated mice (Fig. 2a). It is noteworthy that significant protection against injury was observed in PEA (pretreatment)-treated mice (Fig. 2c and see histological score 6) as well as in PEA (post-treatment)-

treated mice (Fig. 2d and see histological score e). To evaluate whether the observed histological damage was associated with loss of motor function, we utilized the modified BBB hind limb motor rating scale score. Although motor function

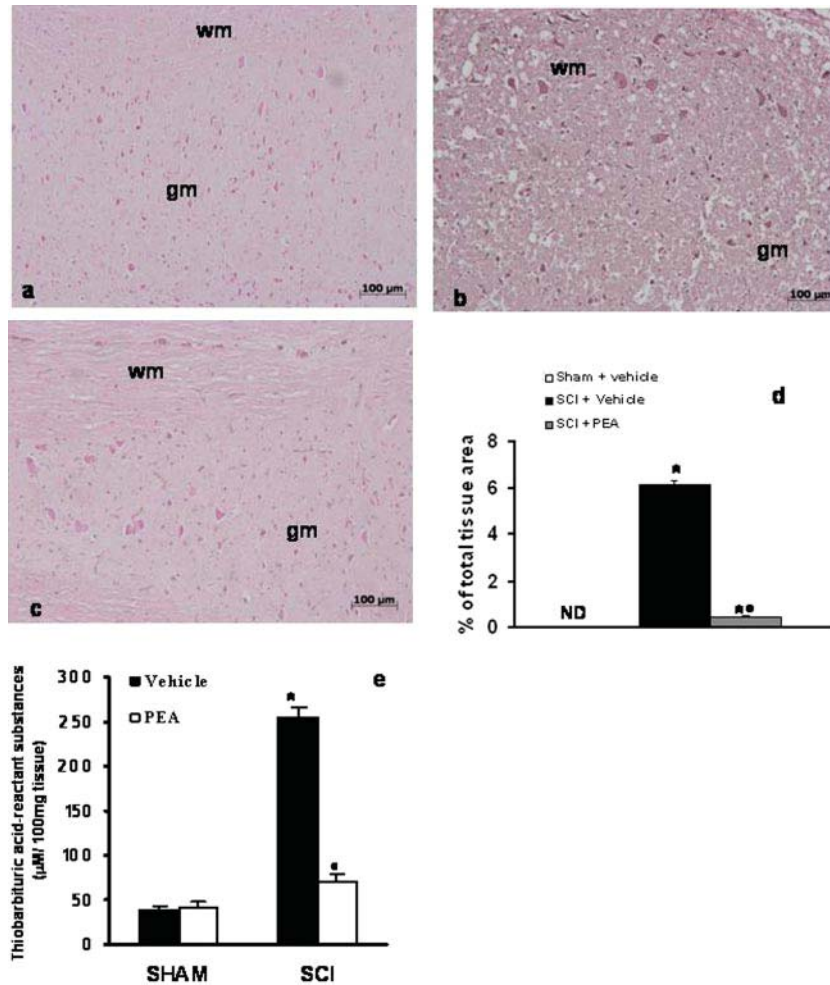


Fig. 6. Effects of PEA on nitrotyrosine formation and lipid peroxidation. No positive staining for nitrotyrosine was observed in the spinal cord tissues from sham-operated mice (a). Sections obtained from vehicle-treated animals after SCI demonstrate positive staining for nitrotyrosine (b) mainly localized in inflammatory cells, in nuclei of Schwann cells in the white and gray matter. PEA treatment reduced the degree of positive staining for nitrotyrosine (c) in the spinal cord. Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for nitrotyrosine (d) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. In addition, a significant increase in thiobarbituric acid-reactant substances (e) were observed in the spinal cord collected at 24 h from mice subjected to SCI compared with sham-operated mice. Thiobarbituric acid-reactant substances were significantly attenuated by PEA treatment (e). Figures are representative of at least three experiments performed on different experimental days. Data are mean \pm S.E.M. of 10 mice for each group. *, $p < 0.01$ versus sham; \circ , $p < 0.01$ versus SCI + vehicle.

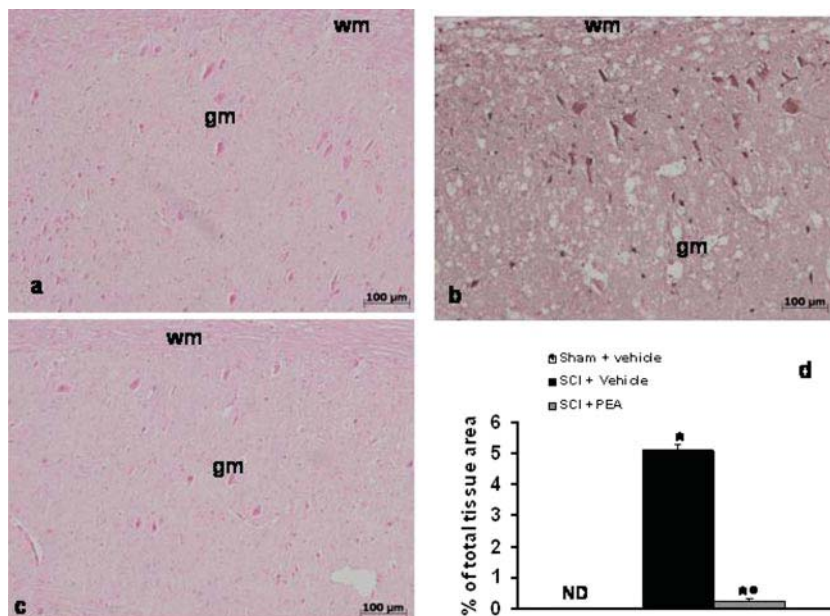


Fig. 7. Effects of PEA on PAR activation. Spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for PAR, product of PARP activation. No positive staining for PAR observed in the spinal cord tissues from sham-operated mice (a). A substantial increase in PAR formation was found in inflammatory cells, in nuclei of Schwann cells in wm and gm of the spinal cord tissues from SCI mice at 24th h after SCI (b). Spinal cord levels of PAR were significantly attenuated in PEA-treated SCI mice (c). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for PAR (d) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days. *, $p < 0.01$ versus sham; \circ , $p < 0.01$ versus SCI + vehicle.

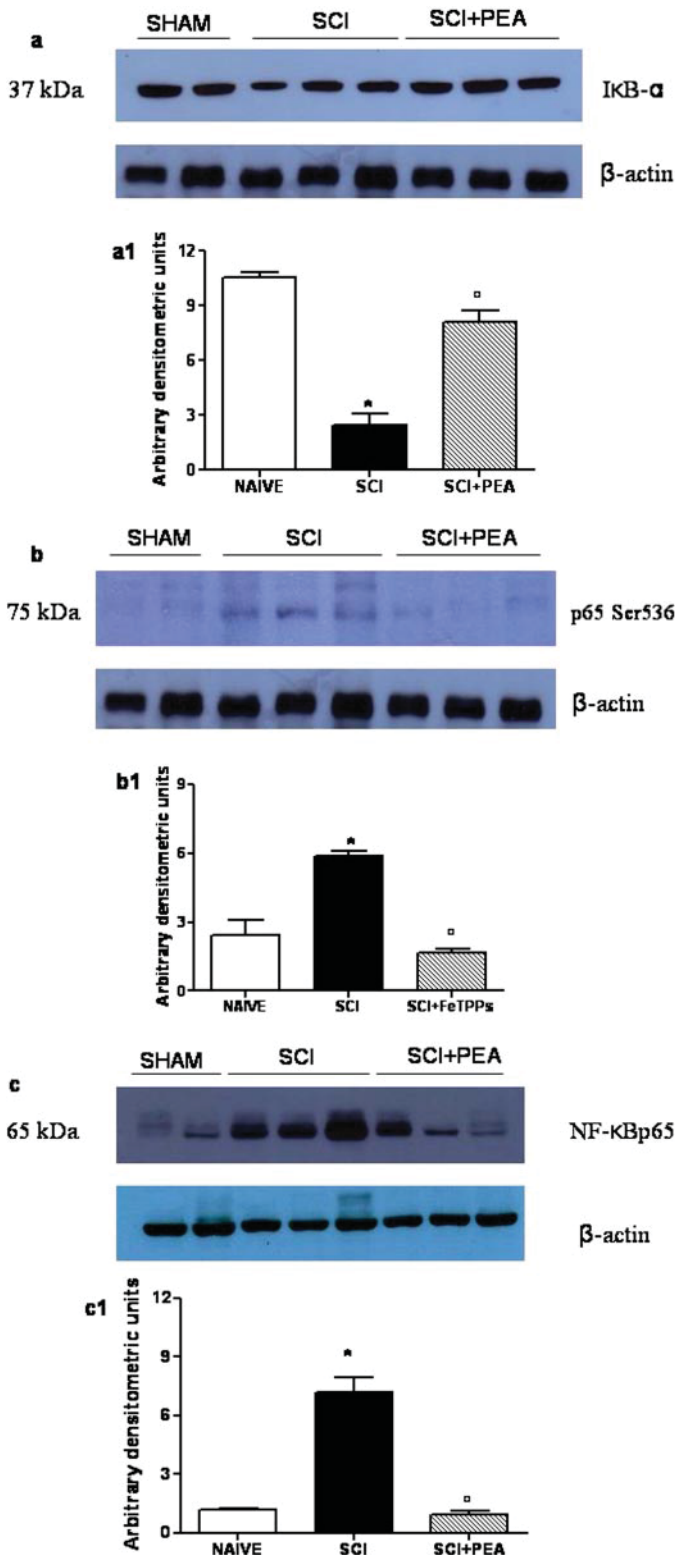


Fig. 8. Effects of PEA treatment on IκB-α degradation, phosphorylation of Ser536 on the NF-κB subunit p65, and total NF-κB p65. By Western blot analysis, a basal level of IκB-α was detected in the spinal cord from sham-operated animals, whereas IκB-α levels were substantially reduced in SCI mice. PEA treatment prevented the SCI-induced IκB-α degradation (a). In addition, SCI caused a significant increase in the phosphorylation of Ser536 at 24 h (b) and in nuclear NF-κB p65 compared with the sham-operated mice (c). PEA treatment significantly reduced the phosphorylation of p65 on Ser536 (b) and NF-κB p65 levels as shown in c. β-Actin was used as an internal control. A representative blot of lysates obtained from each group is shown, and densitometric analysis of all

was only slightly impaired in sham-operated mice (data not shown), mice subjected to SCI displayed significant deficits in hind limb movement (Fig. 3). PEA pre- or post-treatment significantly ameliorated the functional deficits induced by SCI (Fig. 3). Please note that no significant difference was found in the ability to reduce spinal cord injury by PEA administered as pre- or post-treatment (Figs. 2 and 3).

Effects of PEA on Neutrophil Infiltration. The histological pattern of spinal cord injury described above appeared to be correlated with leukocyte influx into the spinal cord. Therefore, we investigated the effect of PEA on neutrophil infiltration by measuring tissue MPO activity. In mice subjected to SCI, MPO activity was significantly elevated in spinal cord tissue 24 h after injury, compared with sham-operated mice (Fig. 4a). PEA administration markedly attenuated this response (Fig. 4a).

Effects of PEA on TNF-α and IL-1β Expression. To test whether PEA modulates the inflammatory process through regulation of proinflammatory cytokine secretion, the spinal cord levels of TNF-α and IL-1β were evaluated 24 h after injury. 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. 4, b and c, respectively). Spinal cord levels of TNF-α and IL-1β were significantly reduced by PEA (Fig. 4, b and c, respectively).

Effects of PEA on iNOS Expression. Spinal cord sections from sham-operated mice did not stain for iNOS (Fig. 5a), whereas spinal cord sections obtained from SCI mice at 24 h after injury exhibited positive staining for this protein (Fig. 5 and see densitometry analysis d). The staining was mainly localized in inflammatory cells and in nuclei of Schwann cells in the white and gray matter of the cord. PEA treatment strongly reduced the degree of positive staining for iNOS in spinal cord (Fig. 5c and see densitometry analysis d).

Effects of PEA on Nitrotyrosine Formation, Lipid Peroxidation, and PAR Formation. Spinal cord sections from sham-operated mice did not stain for nitrotyrosine (Fig. 6a), whereas those obtained from SCI mice at 24 h after injury exhibited positive staining for nitrotyrosine (Fig. 6b and see densitometry analysis d). Positive staining was mainly localized in inflammatory cells and in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues. PEA treatment reduced the degree of positive staining for nitrotyrosine (Fig. 6c and see densitometry analysis d). In addition, 24 h after SCI, levels of thiobarbituric acid-reactant substances were also measured in the spinal cord tissue as an indicator of lipid peroxidation. A significant increase in thiobarbituric acid-reactant substances (Fig. 6e) were observed in spinal cord collected 24 h after SCI, but not after sham surgery. Thiobarbituric acid-reactant substances (Fig. 6e) were significantly reduced by PEA. The effect of PEA treatment on nitrotyrosine formation as well as on thiobarbituric acid-reactant substances is likely to be related to reduced inflammatory cell infiltration. Infiltration of leukocytes into the white matter has been suggested to contribute significantly to the SCI releasing free oxygen and nitrogen radicals and favoring PARP activation (Cuzzocrea et al.,

animals is reported ($n = 5$ rats from each group). The relative expression of the protein bands from three separated experiments was standardized by densitometric analysis to α-tubulin levels and reported in a1, b1, and c1. *, $p < 0.01$ versus sham; ○, $p < 0.01$ versus SCI.

2006). In our study, immunohistochemistry for PAR, as an indicator of *in vivo* PARP activation related to DNA damage, revealed the occurrence of positive staining for PAR localized in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues collected at 24 h after SCI (Fig. 7a and see densitometry analysis d). PEA treatment reduced the degree of positive staining for PAR (Fig. 7c and see densitometry analysis d) in the spinal cord. No positive staining for PAR was found in the spinal cord tissues from sham-operated mice (Fig. 7a).

Effect of PEA on Activation of the NF- κ B Pathway. We evaluated in the spinal cord tissues collected at 24 h after injury, I κ B- α degradation, phosphorylation of Ser536 on the NF- κ B subunit p65, and nuclear NF- κ B p65 by Western blot analysis to investigate the cellular mechanisms by which treatment with PEA may attenuate the development of SCI. A basal level of I κ B- α was detected in the spinal cord from sham-operated animals, whereas I κ B- α levels were substantially reduced in SCI mice (Fig. 8, a and a1). PEA administration prevented the SCI-induced I κ B- α degradation (Fig. 8, a and a1). In addition, SCI caused a significant increase in the phosphorylation of Ser536 at 24 h (Fig. 8, b and b1). Treatment with PEA significantly reduced phosphorylation of p65 on Ser536 (Fig. 8, b and b1). Moreover, NF- κ B p65 levels in the nuclear fractions from spinal cord tissue were significantly increased 24 h after SCI, compared with the sham-operated mice (Fig. 8, c and c1), and were significantly reduced by PEA treatment (Fig. 8, c and c1).

Effects of PEA on FAS Ligand Expression and Apoptosis. Immunohistological staining for FAS Ligand in the spinal cord was also determined. Spinal cord sections from sham-operated mice did not stain for FAS ligand (Fig. 9a), whereas spinal cord sections obtained from SCI mice at 24 h after injury exhibited positive staining for FAS Ligand (Fig. 9b and see densitometry analysis d) mainly localized in inflammatory cells and in nuclei of Schwann cells. PEA treatment reduced the degree of positive staining for FAS ligand in the spinal cord (Fig. 9c and see densitometry analysis d). Moreover, to test whether spinal cord damage was associated

to apoptotic cell death, we measured TUNEL-like staining in perilesional spinal cord tissue at 24 h after injury. Almost no apoptotic cells were detected in the spinal cord from sham-operated mice (Fig. 10a). Twenty-four hours after the trauma, tissues from SCI mice demonstrated a marked appearance of apoptotic cells and intercellular apoptotic fragments (Fig. 10b). In contrast, few or no apoptotic cells and fragments were noted in tissues obtained from mice treated with PEA (Fig. 10c).

Western Blot Analysis and Immunohistochemistry for Bax and Bcl-2. Twenty-four hours after SCI, the appearance of the proapoptotic protein, Bax, in spinal cord homogenates was investigated by Western blot analysis. Bax levels were appreciably increased in the spinal cord from mice subjected to SCI (Fig. 11, a and a1). PEA treatment prevented SCI-induced Bax expression (Fig. 11, a and a1). Bcl-2 expression in spinal cord homogenates was also measured by Western blot. A basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (Fig. 11, b and b1). Twenty-four hours after SCI, the Bcl-2 expression was significantly reduced in spinal cord from SCI mice (Fig. 11, b and b1). Treatment of mice with PEA significantly blunted the SCI-induced inhibition of antiapoptotic protein expression (Fig. 11, b and b1).

Moreover, samples of spinal cord tissue were taken at 24 h after SCI to confirm the PEA effect by immunohistological staining for Bax and Bcl-2. Spinal cord sections from sham-operated mice did not stain for Bax (Fig. 12a), whereas spinal cord sections obtained from SCI mice exhibited a positive staining for Bax (Fig. 12b and see densitometry analysis d). PEA treatment reduced the degree of positive staining for Bax in the spinal cord of mice subjected to SCI (Fig. 12c and see densitometry analysis d). In addition, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Fig. 12e), whereas in SCI mice, the staining significantly reduced (Fig. 12f and see densitometry analysis h). PEA treatment significantly attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (Fig. 12g and see densitometry analysis h).

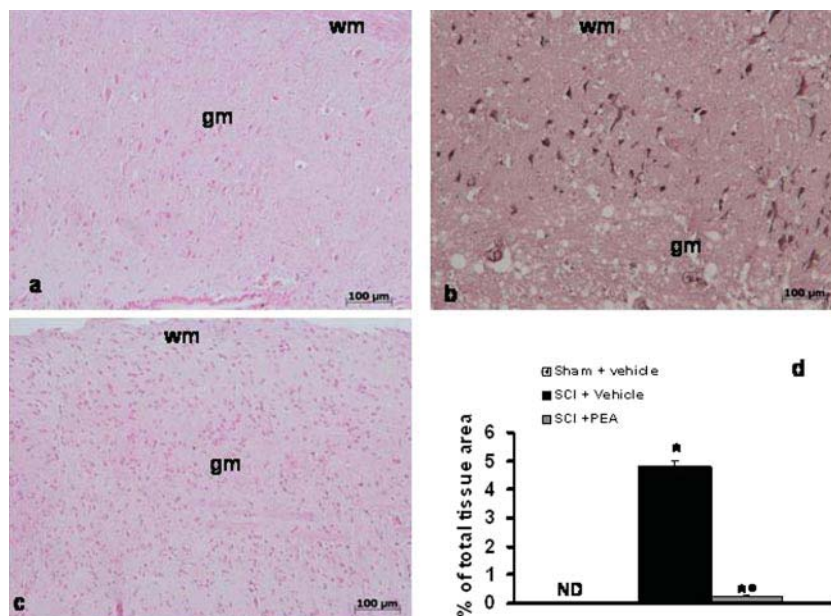


Fig. 9. Effects of PEA on FAS ligand expression. Spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for FAS ligand. No positive staining for FAS ligand was observed in the spinal cord tissues from sham-operated mice (a). A substantial increase in FAS ligand expression was found in inflammatory cells, in nuclei of Schwann cells in wm and gm of the spinal cord tissues from SCI mice at 24th h after SCI (b). Spinal cord levels of FAS ligand were significantly attenuated in PEA-treated SCI mice (c). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for FAS ligand (d) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days. *, $p < 0.01$ versus sham; \circ , $p < 0.01$ versus SCI + vehicle.

Discussion

Primary injury to the adult spinal cord is irreversible, whereas secondary degeneration is delayed and therefore amenable to intervention. Accordingly, several studies have shown that therapies targeting various factors involved in the secondary degeneration cascade lead to tissue sparing and improved behavioral outcomes in spinal cord-injured animals (Bao et al., 2003; Cuzzocrea et al., 2006; Glaser et al., 2006). Much of the damage that occurs in the spinal cord following traumatic injury is due to the secondary effects of glutamate excitotoxicity, Ca^{2+} overload, and oxidative stress, three mechanisms that take part in a spiraling interactive cascade ending in neuronal dysfunction and death (Anderson and Hall, 1993).

In this study, we demonstrate that PEA treatment exerts beneficial effects in a mice model of spinal cord injury. We demonstrate here that SCI induced by the application of vascular clips to the dura via a four-level T_5 - T_8 laminectomy resulted in edema and loss of myelin in lateral and dorsal funiculi. This histological damage was associated to the loss of motor function. SCI induced an inflammatory response in the spinal cord, characterized by activation of NF- κ B pathway increasing I κ B- α degradation and enhancing NF- κ B activation, as well as amplifying the expression of proinflammatory mediators, proinflammatory cytokines and nitrotyrosine, and increased MPO activity. Our results show that PEA reduced: 1) the degree of spinal cord damage, 2) neutrophil infiltration, 3) NF- κ B activation, 4) I κ B- α degradation, 5) nitrotyrosine formation, 6) proinflammatory cytokines production, 7) apoptosis as TUNEL staining, and 8) Bax and Bcl-2 expression. All of these findings support that PEA exerts potent anti-inflammatory effects.

Recent evidence suggests that the activation of NF- κ B may also be under the control of oxidant/antioxidant balance (Haddad, 2002). Moreover, various experimental evidence has clearly suggested that NF- κ B plays a central role in the regulation of many genes responsible for the generation of

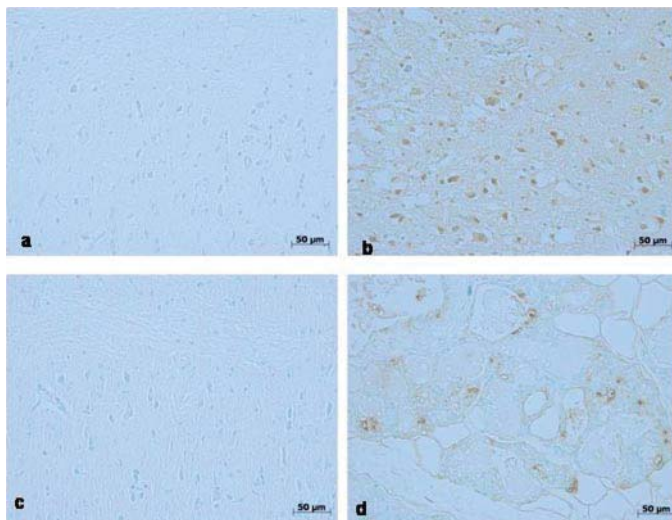


Fig. 10. Effects of PEA on TUNEL-like staining in the perilesional spinal cord tissue. In sham animals, no apoptotic cells were observed (a). In contrast, at 24 h after the trauma, SCI mice demonstrated a marked appearance of dark-brown apoptotic cells and intercellular apoptotic fragments (b). In contrast, tissues obtained from mice treated with PEA demonstrated no apoptotic cells or fragments (c). d, positive KIT control section. Figure is representative of at least three experiments performed on different experimental days.

mediators or proteins in secondary inflammation associated with SCI (La Rosa et al., 2004). NF- κ B is normally sequestered in the cytoplasm, bound to regulatory proteins I κ Bs. In response to a wide range of stimuli, including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, I κ B is phosphorylated by the enzyme I κ B kinase (Bowie and O'Neill, 2000). The net result is the release of the NF- κ B dimer, which is then free to translocate into the nucleus. The mechanisms by which PEA suppress NF- κ B activation in inflammation are not known. We report here that SCI caused a significant increase in the phosphorylation of Ser536 on p65 in the spinal cord tissues at 24 h, whereas PEA treatment significantly reduced this phosphorylation. Moreover, we also demonstrate that the PEA inhibited I κ B- α degradation as well as the NF- κ B translocation. Moreover,

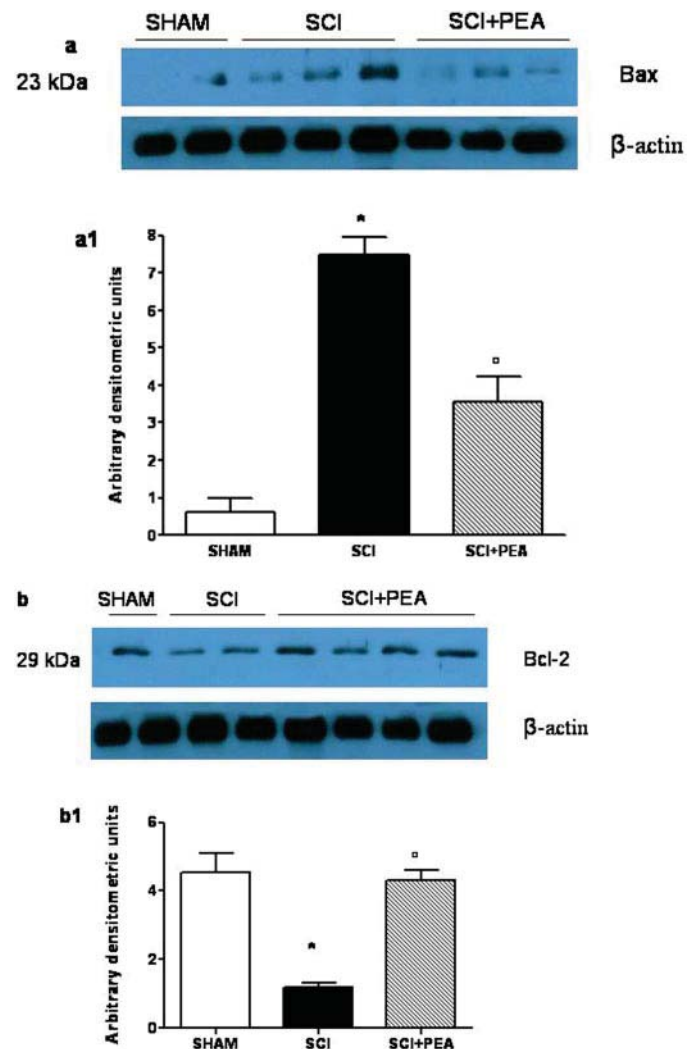


Fig. 11. Western blot analysis for Bax and Bcl-2. By Western blot analysis, Bax levels were appreciably increased in the spinal cord from SCI mice (a and a1). On the contrary, PEA treatment prevented the SCI-induced Bax expression (a and a1). Moreover, a basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (b and b1). Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cord from SCI mice (b and b1). PEA significantly reduced the SCI-induced inhibition of Bcl-2 expression (b and b1). The relative expression of the protein bands was standardized by densitometric analysis to α -tubulin levels, and reported in a1 and b1 are mean \pm S.E.M. from $n = 5$ to 6 spinal cord for each group. *, $p < 0.01$ versus sham; \circ , $p < 0.01$ versus SCI + vehicle.

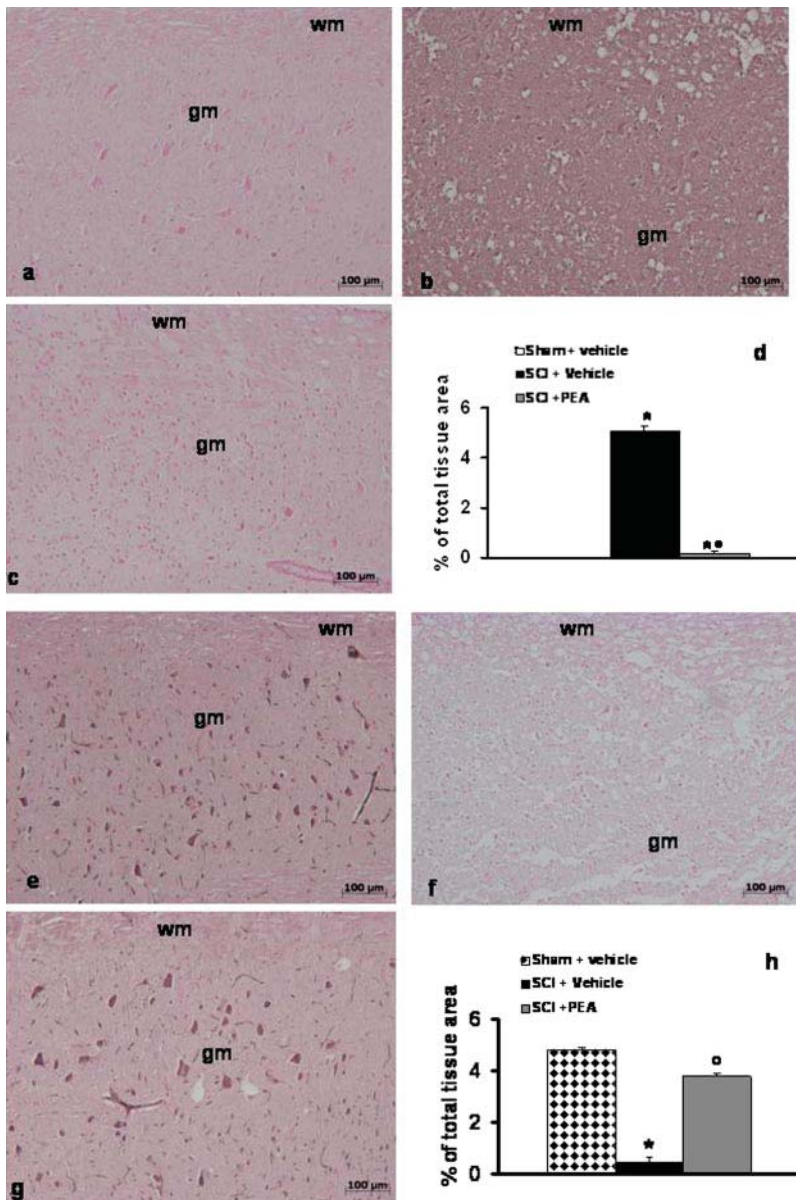


Fig. 12. Immunohistochemical expression of Bax and Bcl-2. No positive staining for Bax was observed in the spinal cord tissues from sham-operated mice (a). SCI caused, at 24 h, an increase in Bax expression (b). PEA treatment reduced the degree of positive staining for Bax in the spinal cord (c). On the contrary, positive staining for Bcl-2 was observed in the spinal cord tissues from sham-operated mice (e), whereas the staining was significantly reduced in SCI mice (f). PEA treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (g). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for Bax (d) and for Bcl-2 (h) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days. *, $p < 0.01$ versus sham; ○, $p < 0.01$ versus SCI + vehicle.

we have also demonstrated in the present study that PEA treatment is able to prevent the PPAR- α degradation in the spinal cord at 24 h after injury.

PPAR- α activation leads to a reduction in the formation of nuclear CCAAT/enhancer-binding protein b/p50-NF- κ B complexes and thereby reduces CRP promoter activation. Moreover, PPAR- α increases I κ B α expression, thus preventing nuclear p50/p65 NF- κ B translocation and arresting their nuclear transcriptional activity. Moreover, chronic treatment with fibrates decreases hepatic CCAAT/enhancer-binding protein b and p50-NF- κ B protein expression in mice in a PPAR- α -dependent manner (Bregman et al., 1998). Thus, the effect of PEA treatment on NF- κ B activation may be related to activation of PPAR- α , resulting in an up-regulation of PPAR- α expression.

NF- κ B plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation. These include the genes for TNF- α , IL-1 β , iNOS, and cyclooxygenase-2, to name but a few (Verma,

2004). In this regard, it has been well demonstrated that in SCI the expression of proinflammatory cytokines (TNF- α and IL-1 β) at the site of injury regulates the precise cellular events after SCI (Genovese et al., 2006). We have clearly confirmed a significant increase in TNF- α and IL-1 β in SCI. On the contrary, no significant expression of TNF- α and IL-1 β was observed in the spinal cord sections obtained from SCI-operated mice that received PEA suggesting that this natural fatty acid amide is also able to regulate the release of proinflammatory cytokines. This observation is in agreement with a previous study in which it has been demonstrated that PPAR- α expression was less expressed in the gray matter, whereas a high expression was observed in some cells in the white matter, especially in astrocytes (Benani et al., 2003). The presence of PPAR- α in astrocytes suggested to these authors that this isoform modulates central inflammation, possibly by regulation of cytokine production by astrocytes. Moreover, we have also reported that the endogenous PPAR- α ligand reduces (among other effects) the biosynthe-

sis and/or the effects of the proinflammatory cytokine TNF- α in an experimental model of spinal cord injury (Genovese et al., 2005).

Several studies suggest that glial cells in neurodegenerative diseases (i.e., Alzheimer's disease) are affected more than neurons by apoptotic cell death (Beattie et al., 2000). Apoptosis is an important mediator of secondary damage after SCI (Beattie et al., 2002). 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, which is predominantly confined to white matter and involves oligodendrocytes and microglia (Chittenden et al., 1995). Chronologically, apoptosis initially occurs 6 h postinjury at the lesion center and lasts for several days associated with the steadily increased number of apoptotic cells in this region. In an effort to prevent or diminish levels of apoptosis, we have demonstrated that the treatment with PEA attenuates the degree of apoptosis, measured by the TUNEL detection kit, in the spinal cord after the damage. Moreover, various studies have postulated that preserving Bax, a proapoptotic gene, plays an important role in developmental cell death (Bar-Peled et al., 1999) and in central nervous system injury (Nesic-Taylor et al., 2005). Similarly, it has been shown that the administration of the Bcl-xL fusion protein (Bcl-xL FP) (Bcl-2 is the most expressed anti-apoptotic molecule in the 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 (Casha et al., 2001). Based on this evidence, we have identified in SCI proapoptotic transcriptional changes, including up-regulation of proapoptotic Bax and down-regulation of antiapoptotic Bcl-2, by immunohistochemical staining. We report in the present study that the PEA treatment significantly reduced the apoptotic cell death after SCI. In particular, we demonstrated that the treatment with PEA reduced Bax expression, whereas on the contrary, Bcl-2 expressed much more in mice treated with PEA. A number of studies have linked apoptosis to thoracic SCI. Furthermore, some authors have also shown that Fas and p75 receptors are expressed on oligodendrocytes, astrocytes, and microglia in the spinal cord following SCI. Fas and p75 colocalize on many TUNEL-positive cells, suggesting that the Fas- and p75-initiated cell death cascades may participate in the demise of some glia following SCI.

Therefore, FasL plays a central role in apoptosis induced by a variety of chemical and physical insults (Dosreis et al., 2004). 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 tissues, which likely contributes in different capacities to the evolution of tissue injury. In the present study, we found that PEA treatment led to a substantial reduction of FasL activation. However, it is not possible to exclude that antiapoptotic effect observed after PEA treatment may be partially dependent on the attenuation of the inflammatory-induced damage.

On the other hand, in our opinion, the observed effects of PEA treatment on apoptosis are at least partially dependent on the activation of PPAR- α . In fact, it has been demonstrated that PPAR- α suppresses the apoptosis of hepatocytes (Roberts et al., 1998). In addition, Inoue et al. (2003) have

clearly demonstrated that apoptosis in human umbilical vein endothelial cells was prevented by transfection with the gene for the human full-length PPAR- α or acyl-coenzyme A synthetase into human umbilical vein endothelial cells. Therefore, we have recently shown that exogenous PPAR- α agonists inhibit apoptotic cell death in spinal cord tissues in wild-type mice subjected to SCI (Genovese et al., 2005).

Finally, in this study, we demonstrate that PEA treatment significantly reduced the SCI-induced spinal cord tissue alteration as well as improved the motor function. Together, our results enhance our understanding of the mechanism related to the anti-inflammatory property of the PEA and suggest that PEA and other PPAR- α agonists may be useful in the treatment of spinal cord injury.

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