Effects of palmitoylethanolamide on signaling pathways implicated in the development of spinal cord injury

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Nonstandard Abbreviations used: MPO, myeloperoxidase activity; NF-κB, nuclear transcription factor activation-κB; TNF-α, tumor necrosis factor-α; SCI, spinal cord injury; TUNEL, Terminal Deoxynucleotidyltransferase-Mediated UTP End Labelling Assay; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; SDS, dodecyl sulphate; H&E, Haematoxylin/Eosin; BBB, Basso, Beattie, and Bresnahan; ONOO\(^-\), peroxynitrite;

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Abstract

Activation of peroxisome proliferator-activated receptor-α (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 damage, 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) pro-inflammatory cytokine expression; (5) NF-κB activation; (6) inducible nitric-oxide synthase (iNOS) 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.
Introduction

Spinal cord injury (SCI) is a highly debilitating pathology (Maegele 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 CNS 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 (BDNF), glial cell line-derived neurotrophic factor (GDNF) 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 retinoid X receptors (RXRs) to specific peroxisome proliferator response elements (PPREs) 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 co-repressor 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 co-repressor 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 alpha 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 oleoyl ethanolamide – which binds to the purified ligand-binding domain of PPAR-α with a K_D of 40 nM and activates it with a median effective concentration (EC50) of 120 nM (Astarita et al., 2006) – and palmitoyl ethanolamide (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 knock-out 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 five decades ago (Long and Martin, 1956) and was shown to reduce allergic reactions and inflammation in animals (Perlik et al., 1971) along with influenza symptoms in humans (Kahlich et al., 1979). Interest in this compound faded, however, until the discovery that one of its structural analogs, anandamide (arachidonoyl ethanolamide), serves as an endogenous ligand for cannabinoid receptors, the molecular target of 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 (COX-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 spinal cord injury (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-κB expression, (5) nitrotyrosine formation and iNOS expression, (6) pro-inflammatory cytokines production, (7) apoptosis as TUNEL staining, (8) Fas-ligand, Bax and Bcl-2 expression.
Methods

Animals. Male adult CD1 mice (25-30g, 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 anaesthetized using chloral hydrate (400 mg/kg body weight). We used the clip compression model described by Rivlin and Tator (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 T-5 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 T5-T-8 level. The clip was then rapidly released with the clip applicator, which caused SC compression. In the injured groups, the cord was compressed for 1min. Following surgery, 1.0 cc of saline was administered subcutaneously in order 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 4 groups (N= 40 animals/group). Sham animals were subjected to the surgical procedure except that the aneurysm clip was not applied and treated intraperitoneally (i.p.) with vehicle or PEA (10 mg/kg) 30 min before and 1h and 6h 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 1h and 6h (pretreatment) or at 6h and 12h (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 1h and 6h (pretreatment) or at 6h and 12h (post treatment) after SCI and daily until day 9. Ten mice from each group were sacrificed at different time points in order to collect samples for the evaluation of the parameters as described below.

Myeloperoxidase activity. Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined in the spinal cord tissues as previously described (Mullane, 1989) at 24 hours after SCI. At the specified time following SCI, spinal cord tissues were obtained and weighed and each piece 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 20,000 x 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 H2O2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per min at 37°C and was expressed as units of MPO/mg of proteins.

Immunohistochemical localization of PAR, nitrotyrosine, FAS-ligand, 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 the 24h 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. Non-specific 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), respectively. Sections were incubated overnight with anti-PAR (Trevigen, 1:500 in
PBS, v/v), anti-iNOS antibody (BD Transduction Laboratories, 1:500 in PBS, v/v), anti-
nitrotyrosine rabbit polyclonal antibody (Upstate, 1:500 in PBS, v/v), with anti-FAS-ligand
antibody (Abcam, 1:500 in PBS, v/v), anti-Bax antibody (Santa Cruz Biotechnology, 1:500 in PBS,
v/v) or with anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, 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 Lab. Inc., 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 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 previously
described (Shea, 1994; Cuzzocrea et al., 2001) by using Optilab Graftek software on a Macintosh
personal computer.

**Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay.**

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer’s
instruction (Apotag, HRP kit DBA, Milan, Italy). Briefly, sections were incubated with 15 µg/ml
proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase
was inactivated by 3% H₂O₂ for 5 min at room temperature and then washed with PBS. Sections
were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl
transferase and biotinylated dUTP in TdT 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 IκB-α, phospho-NF-κB p65 (serine 536), NF-κB p65, Bax, Bcl-2, PPAR-α.**

Cytosolic and nuclear extracts were prepared as previously described (Bethea et al., 1998) with slight modifications. Briefly, spinal cord tissues from each mouse were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 µM pepstatin A, 20 µM leupeptin, 1mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1,000 x g for 10 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended 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 µm leupeptin, 0,2 mM sodium orthovanadate. After centrifugation 30 min at 15,000 x g at 4°C, the supernatants containing the nuclear protein were stored at -80 for further analysis. The levels of IκB-α, phospho-NF-κB p65 (serine 536), Bax, and Bcl-2 were quantified in cytosolic fraction from spinal cord tissue collected after 24 hours after SCI, while PPAR-α and NF-κB p65 levels were quantified in nuclear fraction. The filters were blocked with 1x PBS, 5 % (w/v) non fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific Abs IκB-α (Santa Cruz Biotechnology, 1:1000), or phospho-NF-κB p65 (serine 536) (Cell Signaling, 1:1000), or anti-Bax (Santa Cruz Biotechnology, 1:500), or anti-Bcl-2 (Santa Cruz Biotechnology, 1:500), or anti-PPAR-α, (Santa Cruz Biotechnology, 1:1000) or anti- NF-κB p65 (Santa Cruz Biotechnology, 1:1000) in 1x PBS, 5 % w/v non fat dried milk, 0.1 % Tween-20 (PMT) at 4°C, overnight. Membranes were incubated with peroxidase-
conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, 1:2000) 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 Corp.). The relative expression of the protein bands of IκB-α (~37 kDa), phospho NF-κB (75 kDa), NF-κB p65 (65kDa), Bax (~23 kDa), Bcl-2 (~29 kDa), PPAR-α (~55 kDa), was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM), 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-µm-thick sections. Tissue sections (thickness 5 µm) were deparaffinized with xylene, stained with Haematoxylin/Eosin (H&E) 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 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); 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 fashion.
Measurement of spinal cord TNF-α and IL-1β levels. Portions of spinal cord tissues, collected at 24 hours after SCI, were homogenized as previously described in PBS containing 2 mmol/L of phenyl-methyl sulfonyl fluoride (PMSF, Sigma Chemical Co.) and tissue TNF-α and IL-1β levels were evaluated. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation, USA) according to the manufacturer instructions. All TNF-α and IL-1β determinations were performed in duplicate serial dilutions.

Thiobarbituric acid-reactant substances measurement. Thiobarbituric acid-reactant substances measurement, which is considered a good indicator of lipid peroxidation, was determined, as previously described (Ohkawa et al., 1979) in the spinal cord tissue at 24 hours after SCI. Thiobarbituric acid-reactant substances were calculated by comparison with OD_{650} of standard solutions of 1,1,3,3-tetramethoxypropan 99% malondialdehyde bis (dymethyl acetal) 99% (MDA) (Sigma, Milan). 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 µM MDA/mg 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, 2002b; Joshi and Fehlings, 2002a). The following criteria were considered: 0=No hind limb movement; 1=Slight (<50% range of motion) movement of 1-2 joints; 2=Extensive (>50% range of motion) movement of 1 joint and slight movement of one other joint; 3=Extensive movement of 2 joints; 4=Slight movement in all 3 joints; 5=Slight movement of 2 joints and extensive movement of 1 joint; 6=Extensive movement of 2 joints and slight movement of 1 joint; 7=Extensive movement of all 3 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 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 and trunk instability; 20=Consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off and trunk instability; 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 Company Ltd. (Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).
**Statistical evaluation.** All values in the figures and text are expressed as mean ± standard error of the mean (SEM) 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 (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 ANOVA 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 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 PPARα 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 PPARα 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 (Figure 1, panels a and a1). The effect of SCI was significantly reduced by PEA administration (Figure 1, panels 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 (Figure 2b and see histological score d), was evaluated 24 h after injury. Significant damage was observed in spinal cord tissue from SCI mice, when compared to sham-operated mice (Figure 2a). Notably, significant protection against injury was observed in PEA (pretreatment) treated mice (Figure 2c see histological score 6) as well as in PEA (post treatment) treated mice (Figure 2d see histological score e). To evaluate if the observed histological damage was associated with loss of motor function, we utilized the modified BBB hind limb motor rating scale score. While motor function was only slightly impaired in sham-operated mice (data not shown), mice subjected to SCI displayed significant deficits in hind limb movement (Figure 3). PEA pre or post treatment significantly ameliorated the functional deficits induced by SCI (Figure 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 (Figures 2-3).

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 to sham-operated mice (Figure 4a). PEA administration markedly attenuated this response (Figure 4a).

**Effects of PEA on TNF-α and IL-1β expression.** To test whether PEA modulates the inflammatory process through regulation of pro-inflammatory 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 (Figure 4bc respectively). Spinal cord levels of TNF-α and IL-1β were significantly reduced by PEA (Figure 4bc, respectively).

**Effects of PEA on iNOS expression.** Spinal cord sections from sham-operated mice did not stain for iNOS (Figure 5a), whereas spinal cord sections obtained from SCI mice at 24 h after injury exhibited positive staining for this protein (Figure 5b, 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 (Figure 5c, 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 (Figure 6a), whereas those obtained from SCI mice at 24 h after injury exhibited positive staining for nitrotyrosine (Figure 6b, 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 (Figure 6c and see densitometry analysis d). In addition, 24 hours 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 (Figure 6e) were observed in spinal cord collected 24 h after SCI, but not after sham surgery. Thiobarbituric acid-reactant substances (Figure 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., 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 (Figure 7a, see densitometry analysis d). PEA treatment reduced the degree of positive staining for PAR (Figure 7c, see densitometry analysis d) in the spinal cord. No positive staining for PAR was found in the spinal cord tissues from sham-operated mice (Figure 7a).

**Effect of PEA on activation of the NF-κB pathway.** We evaluated in the spinal cord tissues collected at 24 h after injury, IkB-α degradation, phosphorylation of Ser536 on the NF-κB subunit p65, nuclear NF-κB 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 IkB-α was detected in the spinal cord from sham-operated animals, whereas IkB-α levels were substantially reduced in SCI mice (Figure 8 panels A and A1). PEA administration prevented the SCI-induced IkB-α degradation (Figure 8 panels A and A1). In addition, SCI caused a significant increase in the phosphorylation of Ser536 at 24 h (Figure 8 panels B and B1). Treatment with PEA significantly reduced phosphorylation of p65 on Ser536 (Figure 8 panels B and B1). Moreover, NF-κB p65 levels in the nuclear fractions from spinal cord tissue were significantly increased 24 h after SCI,
compared to the sham-operated mice (Figure 8 panels C and C1), and were significantly reduced by PEA treatment (Figure 8 panels 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 (Figure 9a), whereas spinal cord sections obtained from SCI mice at 24 h after injury exhibited positive staining for FAS Ligand (Figure 9b, 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 (Figure 9c, 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 (Figure 10a). 24 h after the trauma, tissues from SCI mice demonstrated a marked appearance of apoptotic cells and intercellular apoptotic fragments (Figure 10b). In contrast, few or no apoptotic cells and fragments were noted in tissues obtained from mice treated with PEA (Figure 10c).

Western blot analysis and immunohistochemistry for Bax and Bcl-2. 24 h 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 (Figure 11, panels A and A1). PEA treatment prevented SCI-induced Bax expression (Figure 11, panels 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 (Figure 11, panels B and B1). Twenty-four hours after SCI, the Bcl-2 expression was significantly reduced in spinal cord from SCI mice (Figure 11, panels B and B1). Treatment of mice with PEA significantly blunted the SCI-induced inhibition of anti-apoptotic protein expression (Figure 11, panels 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 (Figure 12A) whereas spinal cord sections obtained from SCI mice exhibited a positive staining for Bax (Figure 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 (Figure 12c and see densitometry analysis D). In addition, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Figure 12E) while in SCI mice the staining significantly reduced (Figure 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 (Figure 12G and see densitometry analysis H).


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\textsuperscript{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; 2007).

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 T5-T8 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-κB pathway increasing IκB-α degradation, and enhancing NF-κB activation, as well as amplifying the expression of pro-inflammatory mediators, pro-inflammatory cytokines and nitrotyrosine and increased MPO activity. Our results show that PEA reduced (1) the degree of spinal cord damage, (2) neutrophils infiltration, (3) NF-κB activation, (4) IκB-α degradation, (5) nitrotyrosine formation , (6) pro-inflammatory cytokines production, (7) apoptosis as TUNEL staining, (8) Bax and Bcl-2 expression. All of these findings support the PEA exert potent anti-inflammatory effects.

Recent evidence suggests that the activation of NF-κB may also be under the control of oxidant/antioxidant balance (Haddad, 2002). Moreover, various experimental evidence have clearly suggested that NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in secondary inflammation associated with SCI (La Rosa et al.,...
NF-κB is normally sequestered in the cytoplasm, bound to regulatory proteins IkBs. In response to a wide range of stimuli including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, IkB is phosphorylated by the enzyme IkB kinase (Bowie and O'Neill, 2000). The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus. The mechanisms by which PEA suppress NF-κB 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 the IkB-α degradation as well as the NF-κB translocation. Moreover, we have also demonstrated in the present study that PEA treatment is able to prevent the PPAR-α degradation in the spinal cord at 24h after injury.

PPAR-α activation leads to a reduction in the formation of nuclear C/EBPβp50-NFκB complexes, and thereby reduces CRP promoter activation. Moreover, PPAR-α increases IkBa expression, thus preventing nuclear p50/p65 NF-kB translocation and arresting their nuclear transcriptional activity. Moreover, chronic treatment with fibrates decreases hepatic C/EBPβ and p50-NF-kB protein expression in mice in a PPAR-α dependent manner (Bregman et al., 1998). Thus, the effect of PEA treatment on NF-κB activation may be related to activation PPAR-α resulting in an upregulation of PPAR-α expression.

NF-κB 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-1β, iNOS and COX-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-1β) 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-1β in SCI. On the contrary, no significant expression of TNF-α and IL-1β was observed in the spinal cord sections obtained from SCI-operated mice which 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, while 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 at these authors that this isoform modulates central inflammation, possibly by regulation of cytokine production by astrocytes. Moreover, we have also reported that endogenous PPAR-α ligand reduces (among other effects) the biosynthesis and/or the effects of the pro-inflammatory cytokine TNF-α in 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 affects 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 hours post-injury at the lesion center and last 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 demonstrates that the treatment with PEA attenuates the degree of apoptosis, measured by TUNEL detection kit, in the spinal cord after the damage. Moreover various studies have postulated that preserving Bax, a pro-apoptotic gene, plays an important role in developmental cell death (Bar-Peled et al., 1999) and in CNS injury (Nesic-Taylor et al., 2005). Similarly, 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 (Casha et al., 2001). Based on these evidences, we have identified in SCI proapoptotic transcriptional changes, including upregulation 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, while on the contrary, Bcl-2 expressed much more in mice treated with PEA. A lot of number of studies has linked apoptosis to thoracic SCI. To such purpose, 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 co-localize 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 tissues injury. In the present study, we found that PEA treatment lead to a substantial reduction of FasL activation. However is not possible to exclude that anti-apoptotic effect observed after PEA treatment it 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 suppress the apoptosis of hepatocytes (Roberts et al., 1998). In addition, Inoue and colleagues have clearly demonstrated that apoptosis in human umbilical vein endothelial cells (HUVECs) was prevented by transfection with the gene for the human full-length peroxisome proliferator-activated receptor α (PPAR-α), or acyl-coenzyme A synthetase (AcylCS) into HUVECs (Inoue et al., 2003). Therefore, we have recently showed 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 tissues alteration as well as improve 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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Footnotes

These authors contributed equally to this work (T.G., E.E.).
Legends for Figures

Figure 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 (panels A and A1). A 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 (panels A and A1). The relative expression of the protein bands was standardized for densitometric analysis to β-actin levels, and reported in panel a1 are expressed as mean ± s.e.m. from n=5/6 spinal cord for each group. *P<0.01 versus sham, °P<0.01 versus SCI+vehicle.

Figure 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). Notably, 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 3 experiments performed on different experimental days. Values shown are mean ± s.e. mean of 10 mice for each group. *p<0.01 vs. SCI.

Figure 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 ± s.e. mean of 10 mice for each group. °p<0.01 vs. SCI.

Figure 4. Effects of PEA on MPO activity and spinal cord TNF-α and IL-1β levels.
Following the injury, MPO activity in spinal cord from SCI mice was significantly increased at 24 h after the damage in comparison to sham groups (A). In addition, a substantial increase in TNF-α (B) and IL-1β (C) production was found in spinal cord tissues from SCI mice 24 hours after SCI. Treatment with PEA significantly attenuated neutrophil infiltration as well as TNF-α and IL-1β levels into the spinal cord. Data are means ± s.e. means of 10 mice for each group. *p<0.01 vs. Sham. °p<0.01 vs SCI+vehicle.

Figure 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 24th hour 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 % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. *p<0.01 vs. Sham. °p<0.01 vs SCI+vehicle.

Figure 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, 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 % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. *p<0.01 vs. Sham. °p<0.01 vs SCI+vehicle.
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 % of total tissue area. In addition, a significant increase in thiobarbituric acid-reactant substances (e) were observed in the spinal cord collected at 24h from mice subjected to SCI when compared with sham-operated mice. Thiobarbituric acid-reactant substances were significantly attenuated by PEA treatment (e). Figures are representative of at least 3 experiments performed on different experimental days. Data are means ± s.e. means of 10 mice for each group. *p<0.01 vs. Sham. °p<0.01 vs SCI+vehicle.

Figure 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 hour 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 % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. *p<0.01 vs. Sham. °p<0.01 vs SCI+vehicle.

Figure 8. Effects of PEA treatment on IκB-α degradation, phosphorylation of Ser536 on the NF-κB subunit p65, 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 (panels A). In addition, SCI caused a significant increase in the phosphorylation of Ser536 at 24 h (panels B) and in nuclear NF-kB p65 compared to the sham-operated mice (panels C). PEA treatment significantly reduced the phosphorylation of p65 on Ser536 (panels B) and NF-kB p65 levels as shown in panels C. β-actin was used as internal control. A representative blot of lysates obtained from each group is shown, and densitometric analysis of all animals is reported (n=5 rats from each group). The relative expression of the protein bands from three separated experiments was standardized for densitometric analysis to α-tubulin levels, and reported in a1, b1, c1. *P<0.01 vs. Sham; °P<0.01 vs. SCI.

Figure 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 hour 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 % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. *p<0.01 vs. Sham. °p<0.01 vs SCI+vehicle.

Figure 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 24h 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). Positive KIT control section (D). Figure is representative of at least 3 experiments performed on different experimental days.

**Figure 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 (panels A and A1). On the contrary, PEA treatment prevented the SCI-induced Bax expression (panels A and A1). Moreover, a basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (panels B and B1). Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cord from SCI mice (panels B and B1). PEA significantly reduced the SCI-induced inhibition of Bcl-2 expression (panels B and B1).

The relative expression of the protein bands was standardized for densitometric analysis to α-tubulin levels, and reported in panel a1 and b1 are expressed as mean ± s.e.m. from n=5/6 spinal cord for each group. *P<0.01 versus sham, °P<0.01 versus SCI+vehicle.

**Figure 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) while 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 % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. *p<0.01 vs. Sham. °p<0.01 vs SCI+vehicle.
Figure 1

SHAM  SCI  SCI+PEA

PPAR-α  β-actin

a

a1

Arbitrary densitometric units

NAIVE  SCI  SCI+PEA

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Figure 2
**Figure 2**

![Bar graph showing histological score for Vehicle, PEA (pre-treatment), and PEA (post-treatment) groups.](image)

- **Vehicle**
- **PEA (pre-treatment)**
- **PEA (post-treatment)**

The graph illustrates the histological score for Sham and SCI conditions, with the post-treatment group showing a significant reduction in score compared to pre-treatment and controls.
Figure 3
Figure 4
Figure 5
Figure 6

Thiobarbituric acid-reactant substances
(μM/100mg tissue)

SHAM

SCI

Vehicle

*
Figure 8
Figure 8
Figure 8

|                | SHAM | SCI  | SCI+PEA |
|----------------|------|------|---------|
| NF-kBp65       |      |      |         |
| 65 kDa         |      |      |         |
| β-actin        |      |      |         |

**Figure 8**

- SHAM
- SCI
- SCI+PEA

**Graph:**
- X-axis: NAIVE, SCI, SCI+PEA
- Y-axis: Arbitrary densitometric units
- NAIVE: 0
- SCI: 9
- SCI+PEA: 2

Significant difference: *
Figure 11

(b) Western blot analysis of Bcl-2 and β-actin expression in SHAM, SCI, and SCI+PEA groups. The graph shows the arbitrary densitometric units for each group.

- **Bcl-2**
  - SHAM: Increased
  - SCI: Decreased
  - SCI+PEA: Increased

- **β-actin**
  - SHAM: Even distribution
  - SCI: Even distribution
  - SCI+PEA: Even distribution
Figure 12