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Effect of Apocynin, an inhibitor of NADPH oxidase, in the inflammatory process induced by an experimental model of spinal cord injury

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Abstract
NADPH-oxidase is an enzyme responsible for reactive oxygen species production, and inhibition of this enzyme represents an attractive therapeutic target for the treatment of many diseases. The aim of this study was to investigate the effects of Apocynin, NADPH-oxidase inhibitor, in the modulation of secondary injury in the spinal cord. The injury was induced by application of vascular clips to the dura via a four-level T5–T8 laminectomy in mice. Treatment with Apocynin 1 and 6 h after the trauma significantly decreased (1) the degree of spinal cord inflammation and tissue injury, (2) neutrophil infiltration, (3) adhesion molecule expression, (4) nuclear transcription factor–κB expression, (5) nitrotyrosine and poly-ADP-ribose formation, (6) pro-inflammatory cytokines production, (7) apoptosis and (8) mitogen-activated protein kinase activation. Moreover, Apocynin significantly ameliorated the loss of limb function (evaluated by motor recovery score). Thus, it is proposed that Apocynin may be useful in the treatment of inflammation associated with spinal cord trauma.

Keywords: *Apocynin, inflammation, SCI, oxidative stress, apoptosis*

Introduction

Individuals paralysed by Spinal Cord Injury (SCI) are left with one of the most physically disabling and psychologically devastating conditions known to humans. Over 10 000 North Americans, most of them under the age of 30 years, experience such an injury each year [1]. Although enormous economic impact for the medical, surgical and rehabilitative care, the complex pathophysiology of SCI leads to the difficulty in finding a suitable therapy [2]. Typically, the centre of the spinal cord injury is predominantly characterized by necrotic death. The primary injury refers to the mechanical damage leading to direct cell death and bleeding. Further progressive destruction of the tissue surrounding the necrotic core is known as secondary injury [3] that is determined by a large number of vascular, biochemical and cellular cascades including the breakdown of blood–spinal cord barrier with oedema formation, ischaemia and hypoxia, the release of vasoactive substances leading to alteration of spinal cord perfusion, the excitotoxicity leading to Ca^{2+} dependent, glutamate-associated neuronal cell death, the formation of free radicals and nitric oxide (NO), a damage of mitochondrias with energy depletation, the invasion and activation of inflammatory cells such us (neutrophils, resident microglia, peripheral macrophages and astrocytes) which secrete lytic enzymes and cytokines contributing to further tissue damage, the apoptosis of oligodendrocytes and neurodegeneration [4].

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Neutrophils are the first inflammatory cells to arrive at the site of injury in non-neuronal and neuronal tissue. The greater degree of neutrophil recruitment in the spinal cord may be because of the stronger up-regulation of intercellular adhesion molecule (ICAM-1) and platelet–endothelial cell adhesion molecule (PECAM). Neutrophils are involved in the modulation of the secondary injury by release of neutrophil proteases and reactive oxygen species [4], which activate the transcription factors such as nuclear factor-κB (NF-κB) that plays a central and crucial role in inducing the expression of inflammatory cytokines and ICAM-1 [5].

Free radicals such as superoxide ($O_2^{\cdot -}$) and hydroxyl ($HO^{\cdot}$) are produced in small amounts by normal cellular processes as part of the mitochondrial electron transport chain and the microsomal cytochrome P-450 system. They are formed during traumatic or hypoxic injuries as a consequence of insufficient oxygenation [4]. Free radicals can react with and subsequently damage proteins, nucleic acids, lipids and extracellular matrix proteins. It was demonstrated that local and systemic inflammatory response, as well as neurodegenerative disease, are also associated with the production of free radicals such as superoxide anions, hydrogen peroxide and peroxynitrite ($ONOO^-$) [6], a cytotoxic molecule generated when nitric oxide and superoxide combine, in the secondary neuronal damage of SCI [7–9]. Increased reactive oxygen species (ROS) production is also implicated in the development of cellular hypertrophy and remodelling, at least in part through activation of redox-sensitive protein kinases such as the mitogen-activated protein kinase (MAPK) super-family [10].

To counteract oxidative stress, the body produces an armory of antioxidants to defend itself, which in fact are sometimes insufficient to effectively defend the organism from ROS [11]. There are a lot of substances that have been researched in order to find a way to inhibit production of ROS and thus protect the body from diseases.

Apocynin (4-hydroxy-3-methoxy-acetophenone) is a constituent of the Himalayan herb Picrorhiza kurrooa Royle (Scrophulariaceae) that is well known in traditional Indian medicine (Ayurveda). It is an acetophenone to which a range of biological activities is attributed [12]. It is a pro-drug that is converted by peroxidase-mediated oxidation to a dimer, which has been shown to be more efficient than Apocynin itself [13].

The structure of NADPH-oxidase is quite complex, consisting of two membrane-bounded elements (gp91phox or Nox 2 and p22phox), three cytosolic components (p67phox, p47phox and p40phox) and a low-molecular-weight G protein (either rac 2 or rac 1). The generation of superoxide anions by NADPH oxidase serves as a host defense mechanism against invading micro-organism infection and the enzyme is present in phagocytic cells, such as monocytes and neutrophils [14]. Recent studies have also suggested that NADPH oxidase is expressed in the central nervous system (CNS) [15] in neurons, astrocytes and microglia [15].

Apocynin is an inhibitor of the intracellular translocation of two critical cytosolic components of the NADPH-oxidase complex present in the cell membrane. The *in vitro* anti-inflammatory effects include the neutrophil-mediated oxidative damage [16], a reduction of polymorphonuclear granulocyte chemotaxis [17] and the inhibition of $ONOO^-$ [18].

In order to gain a better insight into the mechanism of action of Apocynin in the secondary injury associated with spinal cord trauma, in this study we have evaluated the following end-points of the inflammatory response: (1) histological damage, (2) motor recovery, (3) neutrophil infiltration, (4) ICAM-1 and platelet-adhesion molecule (P-selectin) expression, (5) nitrotyrosine and poly-ADP-ribose (PAR) formation, (6) pro-inflammatory cytokines production such as Tumour Necrosis Factor (TNF-α) and Interleuchin-1β (IL-1β), (7) NF-κB expression, (8) apoptosis (TUNEL staining, FAS ligand expression, Bax and Bcl-2 expression) and (9) MAPK activation.

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 anaesthetized using chloral hydrate (400 mg/kg body weight). We used the clip compression model described by Rivlin and Tator [19]. A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected away exposing T5–T8 vertebrae. The spinal cord was exposed via a four-level T5–T8 laminectomy and SCI was produced by extradural compression of the spinal cord using an aneurysm clip with a closing force of 24 g. In the injured groups, the cord was compressed for 1 min. Following surgery, 1.0 cc of saline was administered subcutaneously in order to replace the blood volume lost during the surgery. During recovery from anaesthesia, 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 of 10 mice/group (n = 40 total animals). Sham animals were subjected to the surgical procedure except that the aneurysm clip was not applied and treated intraperitoneally (i.p.) with vehicle (10% dimethylsulphoxide DMSO) or Apocynin (5 mg/kg 10% DMSO) 1 and 6 h after the surgical procedure. The remaining mice were subjected to SCI (as described above) and treated with an i.p. bolus of vehicle (saline) or Apocynin 1 and 6 h after SCI.

As described below mice (n = 10 from each group for each parameter) were sacrificed at 24 h after SCI in order to evaluate the various parameters. In a separate set of experiments another 10 animals for each group were observed until 10 days after SCI in order to evaluate the motor score.

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) or with silver impregnation for reticulum 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 histopathology changes of the gray matter were scored on a 7-point scale [20]: 0, no lesion observed, 1, gray matter contained 1–5 eosinophilic neurons; 2, gray matter contained 5–10 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 the histological studies were performed in a blinded fashion.

Determination of NADPH cytochrome c reductase activity

Portions of spinal cord tissues were homogenized in an isotonic buffer, pH 7.5. Centrifuge sequentially at 1000 g to obtain the post-nuclear supernatants. The NADPH cytochrome *c* reductase activity was measured utilizing a Cytochrome *c* Reductase (NADPH) Assay Kit (Sigma, Saint Louis, MO) following the manufacturer's instructions.

Measurement of spinal cord TNF-α and IL-1β levels

Portions of spinal cord tissues, collected at 24 h after SCI, were homogenized as previously described in phosphate buffered saline (PBS) containing 2 mmol/L of phenyl-methyl sulphonyl fluoride (PMSF, Sigma Chemical Co.) and tissue TNF-α and IL-1β levels were evaluated. The assay was carried out by using a colourimetric, commercial kit (Calbiochem-Novabiochem Corporation, Milan, Italy USA) according to the manufacturer instructions. All TNF-α and IL-1β determinations were performed in duplicate serial dilutions.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined in the spinal cord tissues as previously described [21] at 24 h after SCI. 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 H_2O_2. 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.

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 Basso Mouse Scale (BMS) [22].

Immunohistochemical localization of TNF-α, IL-1β, nitrotyrosine, Poli-ADP-Ribose-Polimerase (PARP), ICAM-1, P-selectin, FAS ligand, Bax and Bcl-2

At 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. 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 avidin-biotin peroxidase complex (DBA). Sections were incubated overnight with (1) goat polyclonal anti-TNF-α antibody (1:100 in PBS, wt/vol) (Santa Cruz Biotechnology Inc.), (2) rabbit polyclonal anti-IL-1β (1:100 in PBS, wt/vol) (Santa Cruz Biotechnology Inc.), (3) rabbit polyclonal anti-Bax (1:100 in PBS, wt/vol) (Santa Cruz Biotechnology Inc.), (4) rabbit polyclonal anti-Bcl-2 (1:100 in PBS, wt/vol) (Santa Cruz Biotechnology Inc.), (5) goat polyclonal anti-PARP-1 antibody (1:100 in PBS, wt/vol) (Santa Cruz Biotechnology Inc.), (6) rabbit polyclonal anti-ICAM-1 (1:100 in PBS, wt/vol) (Santa Cruz Biotechnology Inc.), (7) rabbit polyclonal anti-P-selectin (1:100 in PBS, wt/vol) (Santa Cruz Biotechnology Inc.), (8) mouse monoclonal anti Fas Ligand (1:100 in PBS, wt/vol) (Monosan), (9) rabbit polyclonal anti-nitrotyrosine (1:250 in PBS, wt/vol) (Millipore). Sections were washed with PBS and incubated with secondary antibody. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and DBA. The counter stain was developed with DAB (brown colour) and nuclear fast red (red background). A positive staining (brown colour) was found in the sections, indicating that the immunoreactions were positive. To verify the binding specificity for nitrotyrosine, TNF-α, IL-1β, nitrotyrosine, PARP, FAS-L, ICAM-1, P-selectin, 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) were assessed by densitometry using an Imaging Densitometer (AxioVision, Zeiss, Milan, Italy) and a computer program.

Terminal deoxynucleotidyltransferase-mediated UTP end labelling (TUNEL) assay

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Apotag, HRP kit DBA, Milan, Italy). 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% H_2O_2 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 5–10 fields for each coded slide.

Western blot analysis for IκB-α, NF-κB p65, Bax, Bcl-2, P-38 and Phospho-JNK. Cytosolic and nuclear extracts were prepared as previously described [23] with slight modifications. 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, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min and centrifuged at 1000 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°C for further analysis. The levels of IκB-α, iNOS, Bax, Bcl-2, P-38 and Phospho-JNK were quantified in cytosolic fraction from spinal cord tissue collected 24 h after SCI, while 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 (1:1000; Santa Cruz Biotechnology) or anti-Bax (1:500; Santa Cruz Biotechnology) or anti-Bcl-2 (1:500; Santa Cruz Biotechnology) or anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology) or anti-P38 (1:1000; Cell Signaling) or anti-phospho-JNK (Thr183/Tyr185) (1:1000; Cell Signaling) 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 (1:2000, Jackson ImmunoResearch, 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 (1:10000; Sigma-Aldrich Corp.).

The relative expression of the protein bands of IκB-α (~37 kDa), NF-κB p65 (~65 kDa), Bax (~23 kDa), Bcl-2 (~29 kDa), P-38 (~38 kDa) and phospho-JNK (~46 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).

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 [24] 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 colouration) performed on different experimental days on the tissues section collected from all the animals in each group. The results were analysed 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. BMS scale data were analysed by the Mann-Whitney test and considered significant when p was < 0.05.

Results

Apocynin reduces the severity of spinal cord trauma

The severity of the trauma at the level of the perilesional area, assessed by the presence of oedema as well as alteration of the white matter and infiltration of leukocytes, was evaluated 24 h after injury by haematoxylin/eosin (H&E) staining. Significant damage was observed in the spinal cord tissue collected from SCI (Figure 1B) when compared with sham-operated mice (Figure 1A). Significant protection against the SCI was observed in Apocynin-treated mice (Figure 1C). Moreover, to evaluate the severity of the trauma we also observed the alteration in the reticular and nervous fibre tissues structure by silver impregnation. In sham-treated mice was observed a normal presence of reticular and nervous fibres (Figure 1E). On the contrary, a significant alteration of reticular and nervous fibres was detected in the spinal cord tissues collected at 24 h after SCI (Figure 1F). Treatment with Apocynin significantly reduced the alteration of reticular and nervous fibres associated with SCI (Figure 1G). The histological score (Figure 1D) was evaluated by an independent observer.

In order to evaluate if histological damage to the spinal cord was associated with a loss of motor function, the modified BMS hind limb locomotor rating scale score was evaluated. While motor function was only slightly impaired in sham mice, mice subjected to SCI had significant deficits in movement (Figure 1H). Apocynin treatment significantly ameliorated the functional deficits induced by SCI (Figure 1H).

Effect of Apocynin on the activation of NADPH-oxidase

In this study we show that activation of spinal NADPH oxidase plays a critical role in the development of spinal cord injury. Indeed and as can be seen in Figure 2, the development of SCI was associated with activation of the NADPH oxidase in spinal cord tissues as measured by cytochrome *c* reductase (NADPH) activity. The administration of Apocynin blocked significantly spinal NADPH oxidase activation (Figure 2).

Apocynin modulate the expression of TNF-α and IL-1β

To test whether Apocynin modulates the inflammatory process through the regulation of secretion of pro-inflammatory cytokines, we analyzed spinal cord levels of TNF-α and IL-1β (Figures 3H and I). 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 (Figures 3H and I). Spinal cord levels of TNF-α and IL-1β were significantly attenuated by the intraperitoneal injection of Apocynin (Figures 3H and I). Spinal cord sections were also taken at 24 h after SCI to determine the immunohistological staining for TNF-α and IL-1β expression. Spinal cord tissues obtained from Sham-operated mice did not stain for TNF-α and IL-1β (Figures 3A, D and G). A substantial increase in TNF-α and IL-1β expression was found in inflammatory cells as well as in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues collected from SCI mice 24 h after SCI (Figures 3B, E and G). Apocynin treatment significantly reduced the degree of positive staining for these pro-inflammatory cytokines (Figures 3C, F and G).

Effect of Apocynin on the neutrophil infiltration and ICAM-1 and P-selectin expression

In this study, we investigated the effect of the treatment of Apocynin on the infiltration of neutrophils by measuring tissue MPO activity and ICAM-1 and P-selectin expression. MPO activity was significantly elevated in the spinal cord at 24 h after injury in mice subjected to SCI when compared with Sham-operated mice (Figure 4H). In Apocynin-treated mice, the MPO activity was significantly attenuated in comparison to that observed in SCI (Figure 4H).

Spinal cord sections were also taken at 24 h after SCI to determine the immunohistological staining for ICAM-1 and P-selectin expression. There was no staining for ICAM-1 and P-selectin in spinal cord obtained from the sham mice (Figures 4A, D and G). A substantial increase in ICAM-1 and P-selectin expression was found on the surface of endothelial cells of the spinal cord tissues collected from SCI mice 24 h after SCI (Figures 4B, E and G). Spinal cord expression of ICAM-1 and P-selectin were significantly attenuated in Apocynin-treated mice in comparison to SCI animals (Figures 4C, F and G).

Effects of Apocynin on nitrotyrosine and PAR formation

Spinal cord sections from sham-operated mice did not stain for nitrotyrosine and PAR (Figures 5A, D and

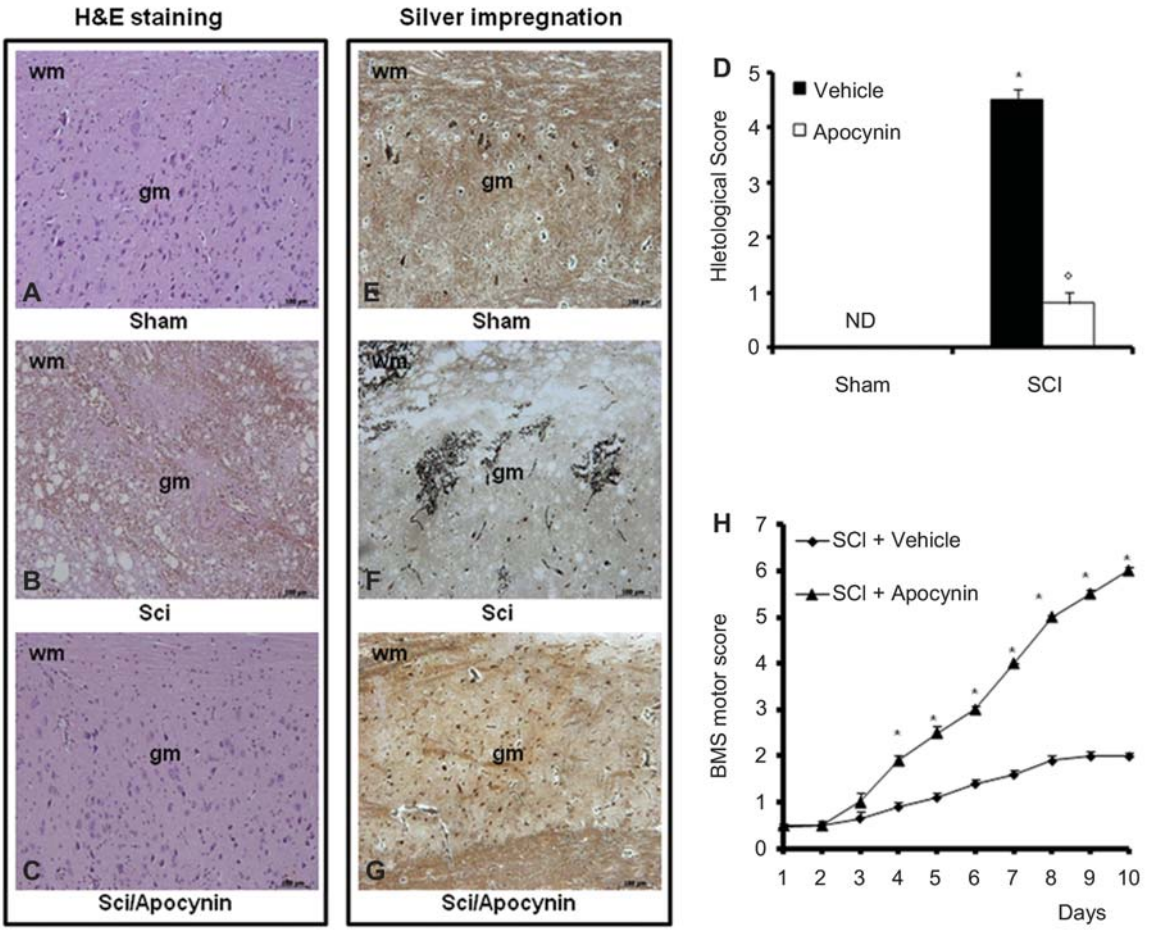

Figure 1. Effect of Apocynin treatment on histological alterations of the spinal cord tissue 24 h after injury. A significant damage to the spinal cord, from SCI operated mice at the perilesional area, was assessed by the presence of oedema as well as alteration of the white matter 24 h after injury (B). Notably, a significant protection from the SCI was observed in the tissue collected from Apocynin-treated mice (C) when compared with sham-operated mice (A). Moreover, to evaluate the severity of the trauma we also observed the alteration in the reticular and nervous fibre tissues structure by silver impregnation. In sham-treated mice was observed a normal presence of reticular and nervous fibres (E). On the contrary, a significant alteration of reticular and nervous fibres was detected in the spinal cord tissues collected at 24 h after SCI (F). Treatment with Apocynin significantly reduced the alteration of reticular and nervous fibres associated with SCI (G). The histological score 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 on the tissues section collected from all the animals in each group. Values shown are mean ± SE mean of 10 mice for each group. $^{*}p < 0.01$ vs *SCI* (D). 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 Basso Mouse Scale [22]. Treatment with Apocynin reduces the motor disturbance after SCI. Values shown are mean ± SE mean of 10 mice for each group. $^{\circ}$ $p < 0.01$ vs *SCI* (H).

G), whereas spinal cord sections obtained from SCI mice exhibited positive staining for nitrotyrosine and PAR (Figures 5B, E and G). The positive staining was mainly localized in inflammatory cells as well as in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues. Apocynin treatment reduced the degree of positive staining for nitrotyrosine and PAR (Figures 5C, F and G) in the spinal cord.

Effects of Apocynin on FAS ligand expression

Immunohistological staining for FAS ligand in the spinal cord was also determined 24 h after injury. Spinal cord sections from sham-operated mice did not stain for FAS ligand (Figures 6A and G), whereas spinal cord sections obtained from SCI mice exhibited positive staining for FAS ligand mainly localized in inflammatory cells as well as in nuclei of Schwann cells (Figures 6B and G). Apocynin treatment reduced the degree of positive staining for FAS ligand in the spinal cord (Figures 6C and G).

Effects of Apocynin in the apoptosis in spinal cord after injury

To test whether spinal cord damage was associated to cell death by apoptosis, we measured TUNEL-like staining in the perilesional spinal cord tissue. Almost no apoptotic cells were detected in the spinal cord from sham-operated mice (Figures 6D and H). At

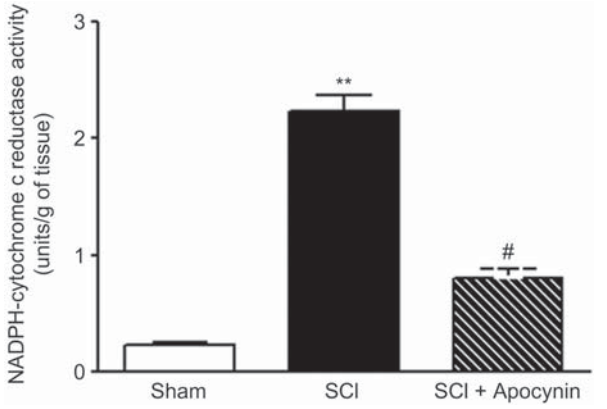

Figure 2. Effect of Apocynin on the activation of NADPH-oxidase. The activation of spinal NADPH oxidase plays a critical role in the development of spinal cord injury. The development of SCI was associated with activation of the NADPH oxidase ($p < 0.001$) in spinal cord tissues as measured by cytochrome *c* reductase (NADPH) activity. The administration of Apocynin blocked significantly spinal NADPH oxidase activation ($p < 0.01$). Morever, no significant activation of NADPH-oxidase was detected in the spinal cord from sham-operated mice.

24 h after the trauma, tissues from SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Figures 6E and H). In contrast, tissues obtained from mice treated with Apocynin demonstrated no apoptotic cells or fragments (Figures 6F and H).

Western blot analysis and immunohistochemistry for Bax and Bcl-2

At 24 h after SCI, the appearance of pro-apoptic protein, Bax, in spinal cord homogenates was investigated by Western blot. Bax levels were appreciably increased in the spinal cord from mice subjected to SCI (Figure 7C). On the contrary, Apocynin treatment prevented the SCI-induced Bax expression (Figure 7C).

By Western blot analysis we also analysed Bcl-2 expression in homogenates from spinal cord of each mice. A basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (Figure 7D). Twenty-four hours after SCI, the Bcl-2 expression was significantly reduced in spinal cord from SCI mice (Figure 7D). Treatment of mice with Apocynin significantly blunted the SCI-induced inhibition of anti-apoptotic protein expression (Figure 7D).

Moreover, samples of spinal cord tissue were taken at 24 h after SCI also to determine the immunohistological staining for Bax and Bcl-2. Spinal cord sections from sham-operated mice did not stain for Bax (Figures 8A and G), whereas spinal cord sections obtained from SCI mice exhibited a positive staining for Bax (Figures 8B and G). Apocynin treatment reduced the degree of positive staining for Bax in the spinal cord of mice subjected to SCI (Figures 8C and G). In addition, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Figures 8D and G), while in SCI mice the staining significantly reduced (Figures 8E and G). Apocynin treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI- subjected mice (Figures 8F and G).

Effect of Apocynin on IκB-α degradation and NF-κB p65 activation. We evaluated IκB-α degradation, nuclear NF-κB p65 activation by Western Blot analysis to investigate the cellular mechanisms by which treatment with Apocynin may attenuate the development of SCI.

A basal level of IκB-α was detected in the spinal cord from sham-operated animals (Figure 7A), whereas IκB-α levels were substantially reduced in SCI mice (Figure 7A). Apocynin administration prevented the SCI-induced IκB-α degradation (Figure 7A). In addition, NF-κB p65 levels in the nuclear fractions from spinal cord tissue were also significantly increased at 24 h after SCI compared to the sham-operated mice (Figure 7B). Apocynin treatment reduced the levels of NF-κB p65, as shown in Figure 7B.

Apocynin reduces the expression of P-38 and phospho-JNK kinase

To investigate the cellular mechanisms by which treatment with Apocynin may attenuate the development of spinal cord injury, we also evaluated the activation of MAP kinase such as P-38 and JNK by phosphorylation by Western blot. In spinal cord tissue homogenates after SCI a significant increase in P-38 and phospho-JNK levels were observed in SCI mice (Figures 9A and B). Treatment of mice with Apocynin significantly reduced the levels of P-38 and phospho-JNK (Figures 9A and B).

Discussion

Spinal cord injury is a highly debilitating pathology [25]. The pathological events following acute SCI are divided into two chronological phases [26]. The traumatic mechanical injury to the spinal cord, that is incurred following blunt impact and compression, is called 'primary injury'; it causes the death of a number of neurons that cannot be recovered and regenerated. 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. It is known that a progressive neuronal injury results from a combination of secondary injury factors including: ischemia, biochemical alterations, excitotoxicity, neurotransmitter accumulation, lipid peroxidation and generation of free radicals [27].

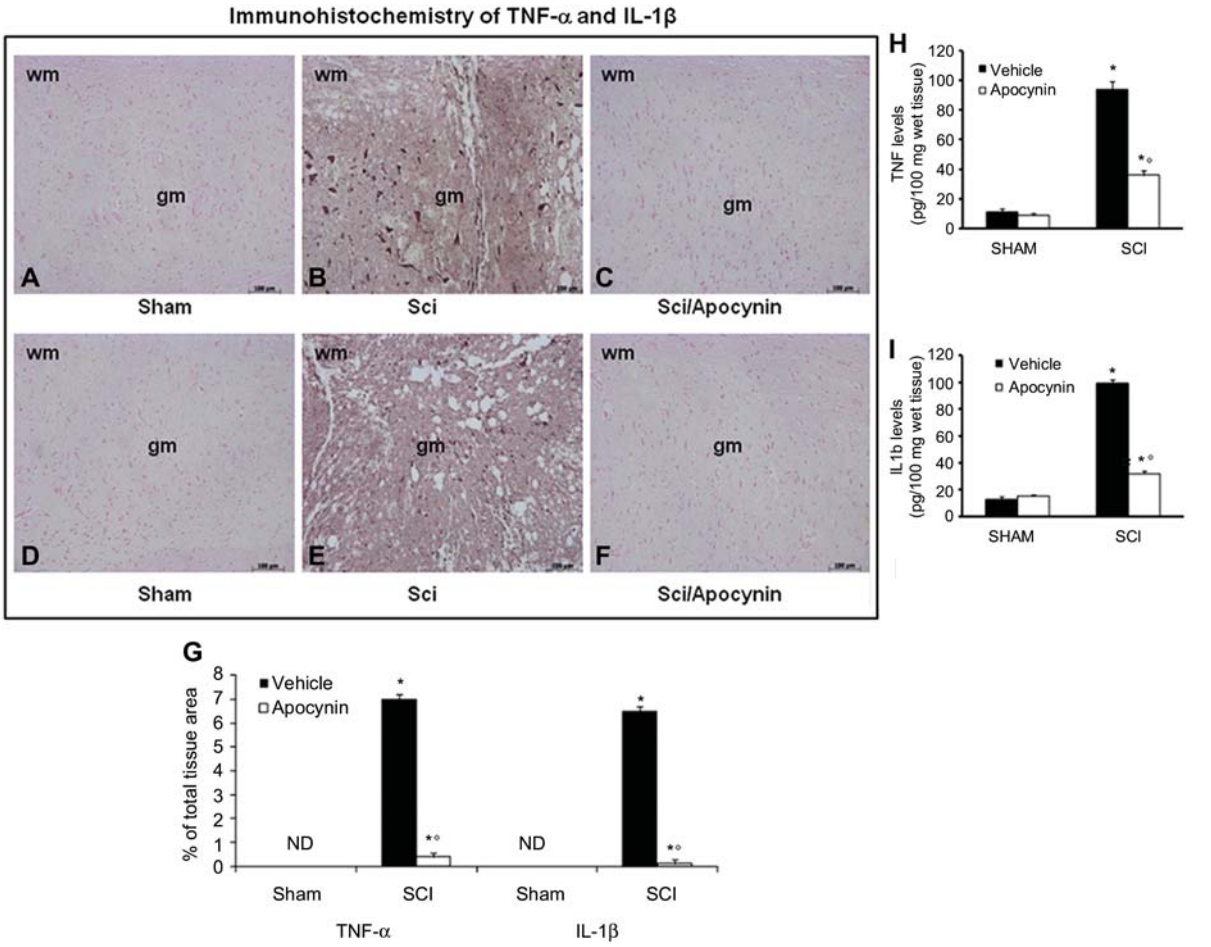

Figure 3. Effects of Apocynin on TNF-α and IL-1β expression. A substantial increase in TNF-α and IL-1β expression was found in inflammatory cells, in nuclei of Schwann cells in wm and gm of the spinal cord tissues from SCI mice at 24 h after SCI (B, E) in comparison to sham groups (A, D). Spinal cord levels of TNF-α and IL-1β were significantly attenuated in Apocynin-treated mice (C, F). In addition, a substantial increase in TNF-α (H) and IL-1β (I) production was found in spinal cord tissue collected from SCI mice at 24 h. Spinal cord levels of TNF-α and IL-1β were significantly attenuated by apocynin treatment (H, I). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for TNF-α and IL-1β (G) 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 a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}p < 0.01$ vs Sham; $^{\circ}p < 0.01$ vs *SCI*+vehicle.

The role of the NADPH oxidase was assessed by the use of Apocynin, a well-characterized inhibitor of the NADPH-oxidase [16,28]. Activation of NADPH-oxidase is associated with the migration of the cytosolic components to the cell membrane so that the complete oxidase can be assembled [29]. Apocynin prevents serine phosphorylation of p47phox and blocks its association with gp91phox, thus blunting NADPH oxidase activation [16,28].

In immune-stimulated phagocytes, the role of Apocynin is more complex. First, it stimulates ROS formation; however, after a certain period its behaviour changes and the inhibition of ROS yield occurs. When apocynin is pre-activated with hydrogen peroxide and a peroxidase prior to use, it immediately acts as an inhibitor both in phagocytes and non-phagocytes [30]. It is assumed that Apocynin is activated by H_2O_2 and MPO to form an Apocynin radical, which then oxidizes thiols in the NADPH-oxidase. Indeed, thiols are critical for the function of p47phox and thiol oxidizing agents have been shown to block NADPH-oxidase activation [13]. Therefore, Apocynin is activated in the locus of inflammation only where there is both free leukocyte myeloperoxidase and hydrogen peroxide. In this case Apocynin acts as an anti-inflammatory agent [30].

Generation of ROS appears to play a critical role in the induction of neurological dysfunctions in the course of SCI [31]. The CNS is particularly vulnerable to free radicals and this vulnerability is responsible for the deleterious post-traumatic ischaemia-induced lipid peroxidation [4]. Recent studies have also indicated that ROS produced by NADPH oxidase could promote neurodegeneration [32].

Apocynin exerts beneficial effects in animal models of nitroxidative stress including rheumatoid arthritis, diabetes, atherosclerosis, neurodegeneration, stroke and ischemia-reperfusion injuries [12,33–39].

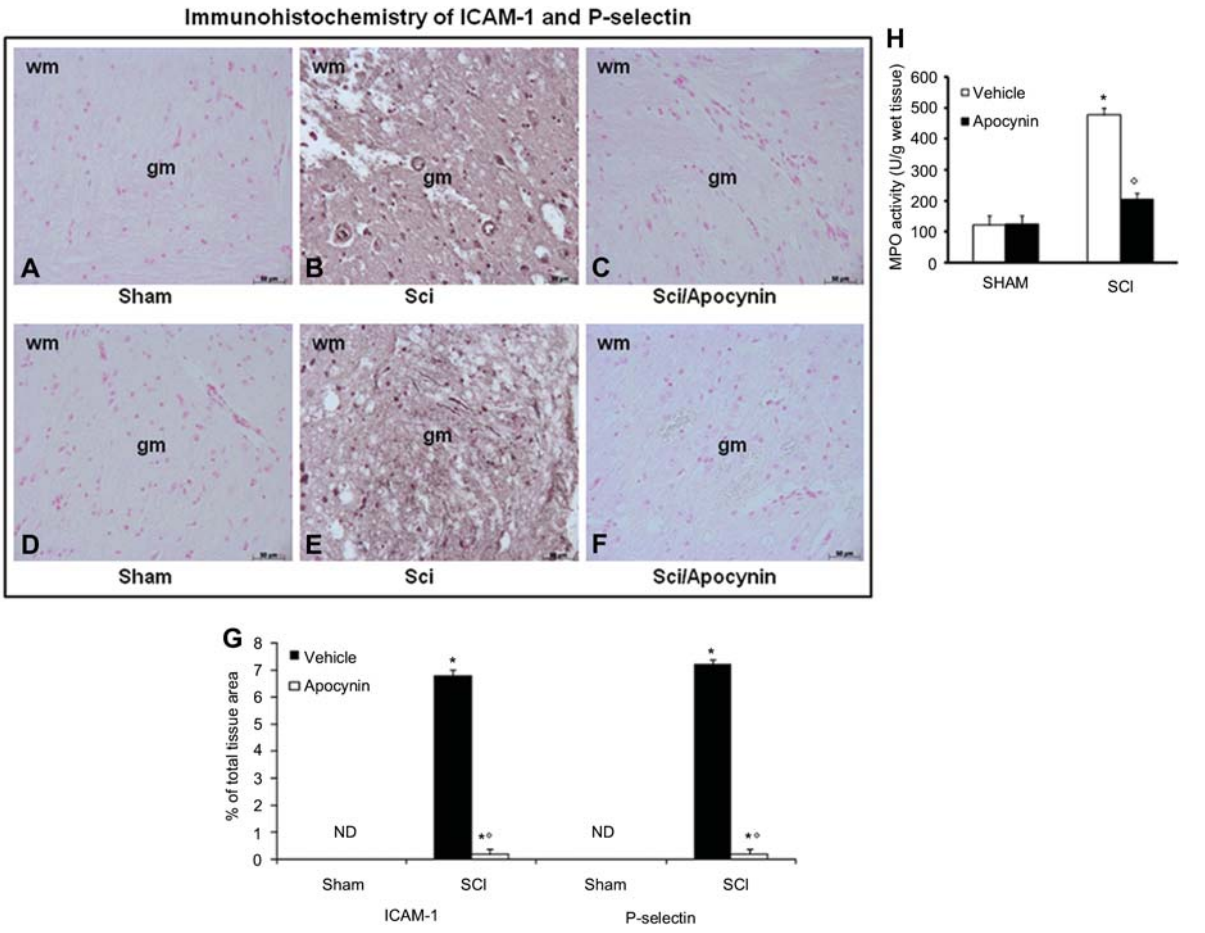

Figure 4. Effect of apocynin on adhesion molecules (ICAM-1 and P-selectin) expression and MPO activity. Spinal cord sections were also taken at 24 h after SCI to determine the immunohistological staining for ICAM-1 and P-selectin expression. There was no staining for ICAM-1 and P-selectin in spinal cord obtained from the apocynin treated sham-injured mice (A, D). A substantial increase in ICAM-1 and P-selectin expression was found on the surface of endothelial cells of the spinal cord tissues collected from SCI mice 24 h after SCI (B, E). Spinal cord expression of ICAM-1 and P-selectin were significantly attenuated in Apocynin-treated mice in comparison to SCI animals (C, F). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for ICAM-1 and P-selectin (G) 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 a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}p < 0.01$ vs Sham; $^{\circ}p < 0.01$ vs *SCI*+vehicle. 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 (H). Treatment i.p. with Apocynin significantly attenuated neutrophil infiltration. Data are means ± SE means of 10 mice for each group. $^{*}p < 0.01$ vs Sham; $^{\circ}p < 0.01$ vs *SCI*+vehicle.

In this study we demonstrate that Apocynin exerts beneficial effects in a mice model of spinal cord injury. We show here that SCI resulted in oedema 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 increased IκB-α degradation, enhanced NF-κB activation, substantial MAPK activation, amplified expression of pro-inflammatory mediators, adhesion molecules, pro-inflammatory cytokines, PAR and nitrotyrosine and increased MPO activity. Our results evidence that Apocynin reduced (1) the degree of spinal cord damage, (2) neutrophils infiltration, (3) ICAM-1 and P-selectin expression, (4) PAR and nitrotyrosine formation, (5) IkB-α degradation, (6) NF-κB activation, (7) pro-inflammatory cytokines production (TNF-α and IL-1β), (8) apoptosis (TUNEL staining, FAS ligand expression, Bax and Bcl-2 expression) and (9) MAPK activation (P-38 and phospho-JNK).

Recent study demonstrates that the development of morphine-induced hyperalgesia and anti-nociceptive tolerance was associated with activation of the NADPH oxidase in the lumbar portion of spinal cord and concomitant increased formation of superoxide [40]. Co-administration of morphine with apocynin, blocked NADPH oxidase activation, superoxide formation and the development of hyperalgesia and anti-nociceptive tolerance at doses devoid of behavioural side-effects [40].

In this work, we also show that activation of spinal NADPH oxidase plays a critical role in the development of spinal cord injury. The development of SCI was associated with activation of the NADPH oxidase

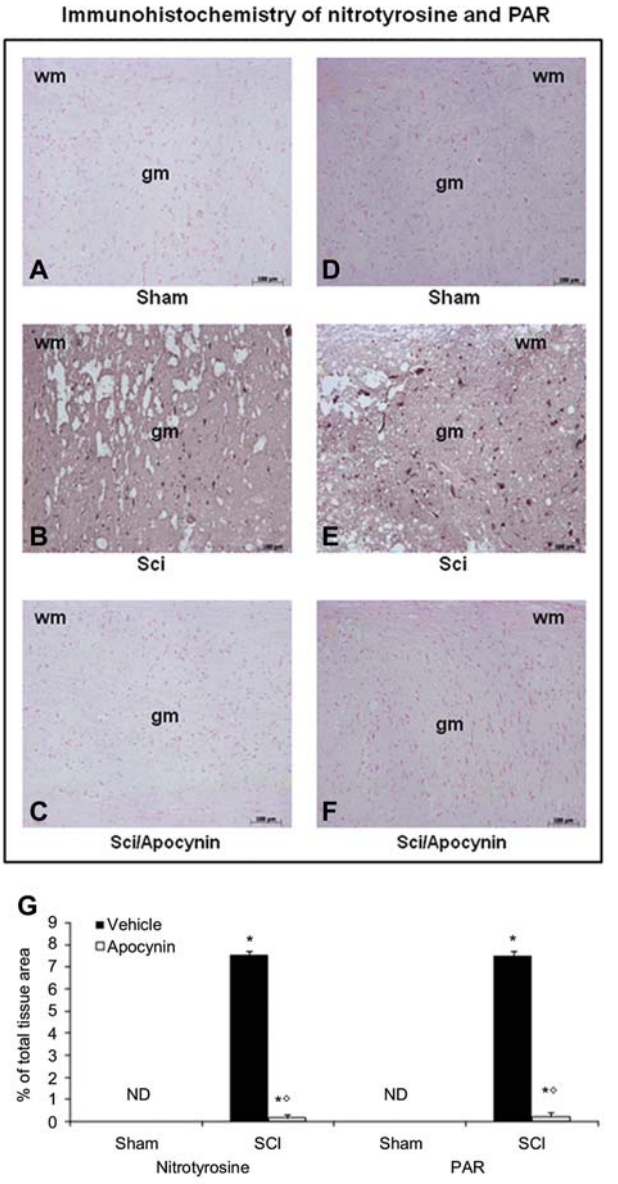

Figure 5. Effects of Apocynin on nitrotyrosine and PAR formation. Spinal cord sections from sham-operated mice did not stain for nitrotyrosine (A). Sections obtained from vehicle-treated animals after SCI demonstrate positive staining for nitrotyrosine mainly localized in inflammatory cells, in nuclei of Schwann cells in the white and gray matter (B). Apocynin treatment (5 mg/kg 10% DMSO, 1 and 6 h after SCI induction) reduced the degree of positive staining for nitrotyrosine (C) in the spinal cord. In addition, immunohistochemistry for PAR, an indicator of *in vivo* PARP activation, revealed the occurrence of positive staining for PAR localized in nuclei of Schwann cells in wm and gm of the spinal cord tissues from SCI mice (E). Spinal cord sections from Apocynin-treated sham-injured mice did not also stain for PAR (D). Apocynin treatment reduced the degree of positive staining for PAR (F) 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 and PAR (G) 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 a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}p < 0.01$ vs Sham; $^{\circ}p < 0.01$ vs *SCI*+vehicle.

in spinal cord tissues as measured by cytochrome *c* reductase (NADPH) activity. The administration of Apocynin blocked significantly spinal NADPH oxidase activation.

All of these findings support the view that Apocynin attenuates the degree of secondary inflammation and improves the motor recovery events.

One consequence of increased oxidative stress is the activation and inactivation of redox-sensitive proteins [41]. In that regard, MAPKs play a pivotal role. The inhibition of MAPK3/MAPK1 is believed to be beneficial in a number of experimental models of neurodegenerative diseases, diabetes type II, bipolar disorders, stroke, cancer, sepsis and chronic inflammatory disease [42]. Previous studies showed that the expression of activated ERK1/2 and p38 MAPK in microglia/macrophages may play a key role in production of CNS inflammatory cytokines and free radicals, such as NO [43]. Recent data have also demonstrated that NADPH oxidase activation mediates ROS production in cardiac hypertrophy and failure, in parallel with activation of MAPKs [10].

We confirm here that SCI leads to a substantial expression of P-38 MAP kinase and phospho-JNK in the spinal cord tissues at 24 h after SCI, on the contrary the apocynin treatment decreases P-38 and phospho-JNK expression in treated-mice.

Recent evidence suggests that the activation of NF-κB may also be under the control of oxidant/antioxidant balance [44]. NF-κB is normally sequestered in the cytoplasm, bound to regulatory proteins IκBs. In response to a wide range of stimuli including oxidative stress, infection, hypoxia, extracellular signals and inflammation, IκB is phosphorylated by the enzyme IκB kinase [41]. The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus and to active genic transcription of inflammatory proteins. In the study we report that SCI was associated with significant IκB-α degradation as well as increased nuclear expression of p65 in spinal cord tissue at 24 h after injury. Treatment with apocynin significantly reduced IκB-α degradation as well as the NF-κB translocation.

NF-κB has an important function in the regulation of many genes responsible for the generation of mediators or proteins in secondary inflammation associated with SCI [45] such as IL-1β, TNF-α and inducible nitric synthase iNOS) [46] and ICAM-1 [5]. In that regard, it has been well demonstrated that in SCI the expression of pro-inflammatory cytokines (TNF-α and IL-1β) at the site of injury regulates the precise cellular events after SCI [47]. We have clearly confirmed a significant increase in TNF-α and IL-1β 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 Apocynin.

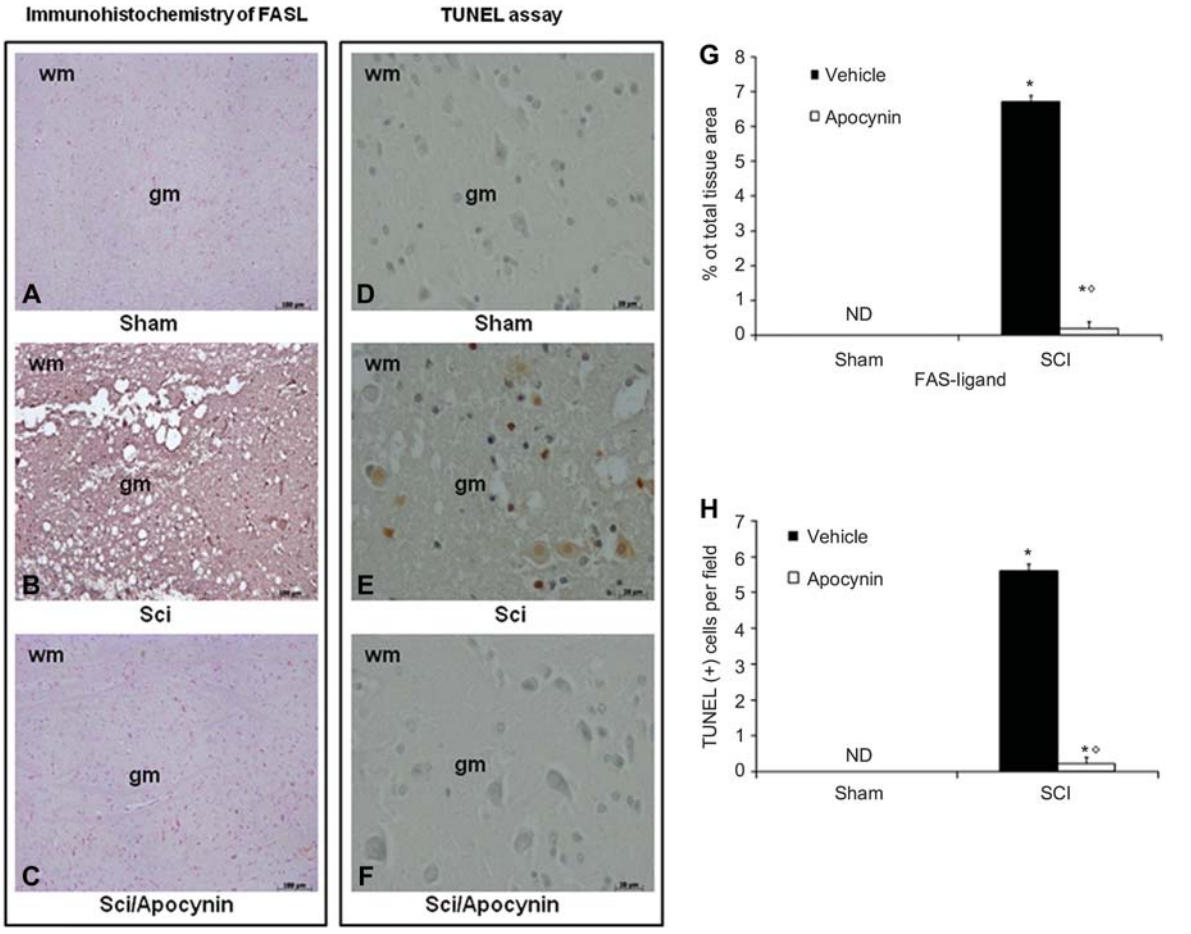

Figure 6. Effect of Apocynin on FAS-ligand expression and on TUNEL-like staining in the perilesional spinal cord tissue. Spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for Fas-ligand and TUNEL staining. Spinal cord sections from Apocynin-treated sham-injured mice did not stain for FAS ligand (A) whereas 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 24 h after SCI (B). Spinal cord levels of Fas-ligand were significantly attenuated in Apocynin-treated mice in comparison to SCI animals (C). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for Fas-ligand (G) 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 a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}p < 0.01$ vs Sham; $^{\circ}p < 0.01$ vs *SCI*+vehicle. Moreover, almost no apoptotic cells were detected in the spinal cord from sham-operated mice (D). At 24 h after the trauma, SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (E). In contrast, tissues obtained from mice treated with Apocynin (5 mg/kg 10% DMSO) demonstrated no apoptotic cells or fragments (F). The number of TUNEL positive cells/high-power field was counted in 5–10 fields for each coded slide (H). This figure is representative of at least three experiments performed on different experimental days on the tissues section collected from all the animals in each group.

During inflammation initiation, circulating leukocytes must first be able to adhere selectively and efficiently to vascular endothelium. This process is facilitated by induction of vascular cell adhesion molecules on the inflamed endothelium, such as vascular cell adhesion molecule VCAM-1, ICAM-1, E-selectin [48]. ICAM-1 is constitutively expressed in the endothelium of brain microvascular structure at low concentrations. Frijns and Kappelle [49] have demonstrated that, after a cerebral ischemia, it is up-regulated by cytokines and binds β_2 integrins on neutrophils, thus mediating neutrophil adhesion and transmigration into brain parenchyma.

We report here that SCI was associated with significant increase of ICAM-1 and P-selectin expression on the surface of endothelial cells and increase of MPO activity while, in apocynin-treated mice, the MPO activity, ICAM-1 and P-selectin expression were significantly attenuated in comparison to that observed in SCI. In this regard, Connolly et al. [50] have demonstrated that knockout mice of ICAM-1 caused a degree of brain protection similar to neutrophil depletion in a focal ischemia model and that ICAM-1 up-regulation was less significant in the gp91 KO and Apocynin-treated mice than in the WT mice at 24 h of reperfusion. Several studies also demonstrate that administration of apocynin (5–50 mg/kg; i.p.) in young rodents prior to middle cerebral artery occlusion (MCAO) attenuated superoxide production, infarct volume [51], neuronal death,

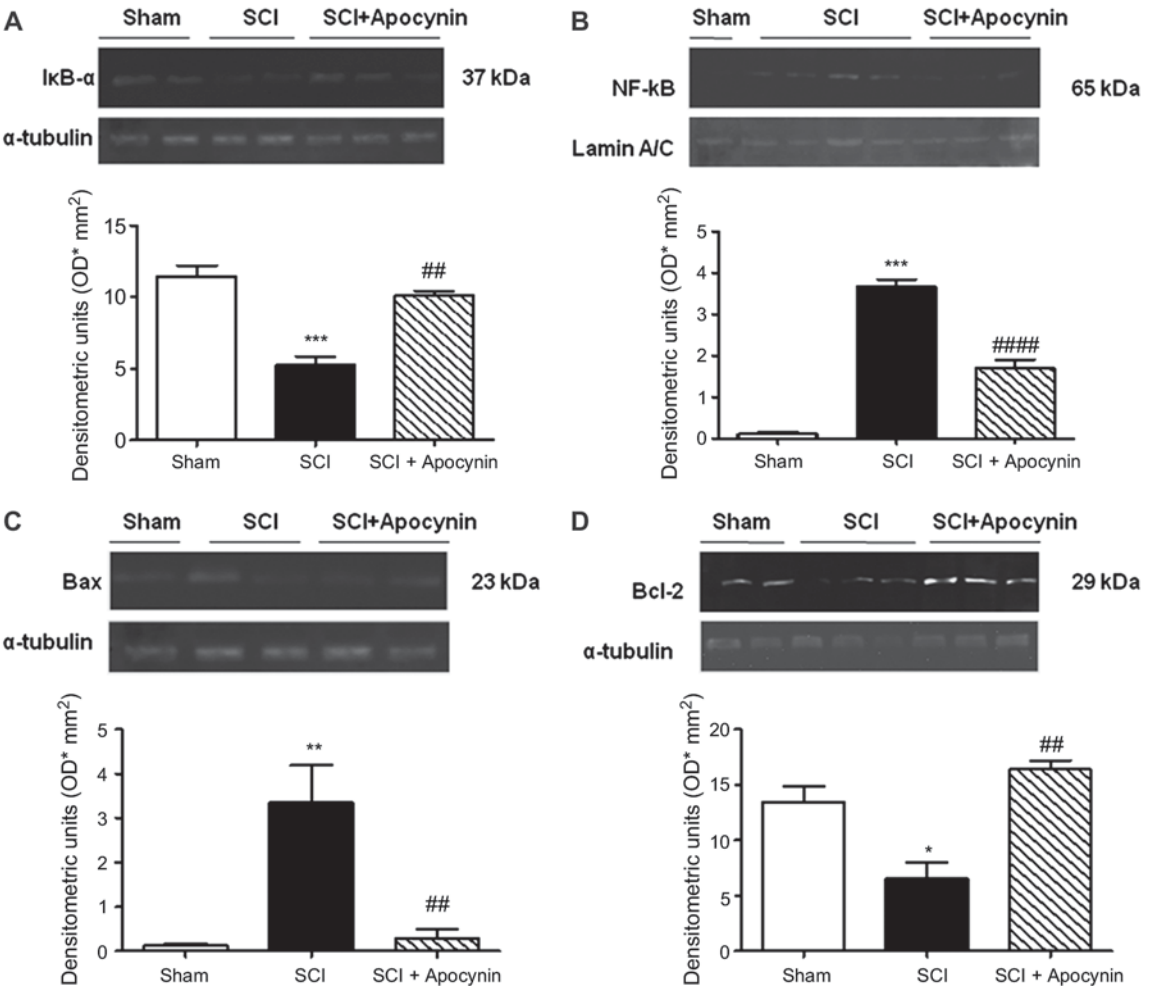

Figure 7. Western blot analysis for Bax, Bcl-2, IκB-α and NF-κB p65. Representative Western blots showing no significant Bax expression in spinal cord tissues obtained from sham-treated animals (C). Bax levels were appreciably increased in the spinal cord from SCI mice (C). On the contrary, Apocynin treatment (5 mg/kg 10% DMSO, 1 h and 6 h after SCI induction) prevented the SCI-induced Bax expression (C). Moreover, a basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (D). Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cord from SCI mice (D). Apocynin treatment (5 mg/kg 10% DMSO, 1 h and 6 h after SCI induction) significantly reduced the SCI-induced inhibition of Bcl-2 expression (D). A basal level of IκB-α was also detected in the spinal cord from sham-operated animals (A), whereas IκB-α levels were substantially reduced in SCI mice (A). Apocynin treatment (5 mg/kg 10% DMSO, 1 and 6 h after SCI induction) prevented the SCI-induced IκB-α degradation (A). In addition, SCI caused a significant increase in nuclear NF-κB p65 compared to the sham-operated mice (B). Apocynin treatment (5 mg/kg 10% DMSO) significantly reduced NF-κB p65 levels as shown in (B). β-actin was used as internal control. The relative expression of the protein bands was standardized for densitometric analysis to β-actin levels and reported in the figure are expressed as mean ± SEM from $n = 5/6$ spinal cord for each group. $^{*}p < 0.05$ vs Sham; $^{**}p < 0.01$ vs Sham; $^{***}p < 0.001$ vs Sham; $^{\#}p < 0.05$ vs *SCI*+vehicle; $^{\#\#}p < 0.01$ vs *SCI*+vehicle; $^{\#\#\#}p < 0.001$ vs *SCI*+vehicle.

activated microglia [52] and oedema formation [53]. On the contrary, a recent experiment reported that when Apocynin (5 mg/kg) was administered intravenously prior to MCAO, rodents exhibited larger cerebral haemorrhages in both hemispheres and increased mortality [54]. These opposing findings suggest that the activation, mechanism of action and toxicity of Apocynin are still poorly understood and further studies are necessary, especially before any therapeutic use of NADPH-oxidase inhibition is pursued in clinical trials.

Among the reactive oxygen species, $ONOO^-$ is known to play an important role in local and systemic inflammatory response as well as neurodegenerative disease [7,55]. It is one of a number of toxic factors produced in the spinal cord tissues after SCI [55] likely contributes to secondary neuronal damage through pathways resulting from the chemical modification of cellular proteins and lipids. To probe the pathological contributions of $ONOO^-$ to secondary damage after SCI, we have used the appearance of nitrotyrosine staining in the inflamed tissue. We have observed in this study that the immunoassaying of nitrotyrosine is reduced in SCI operated mice treated with apocynin when compared with SCI operated mice. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the

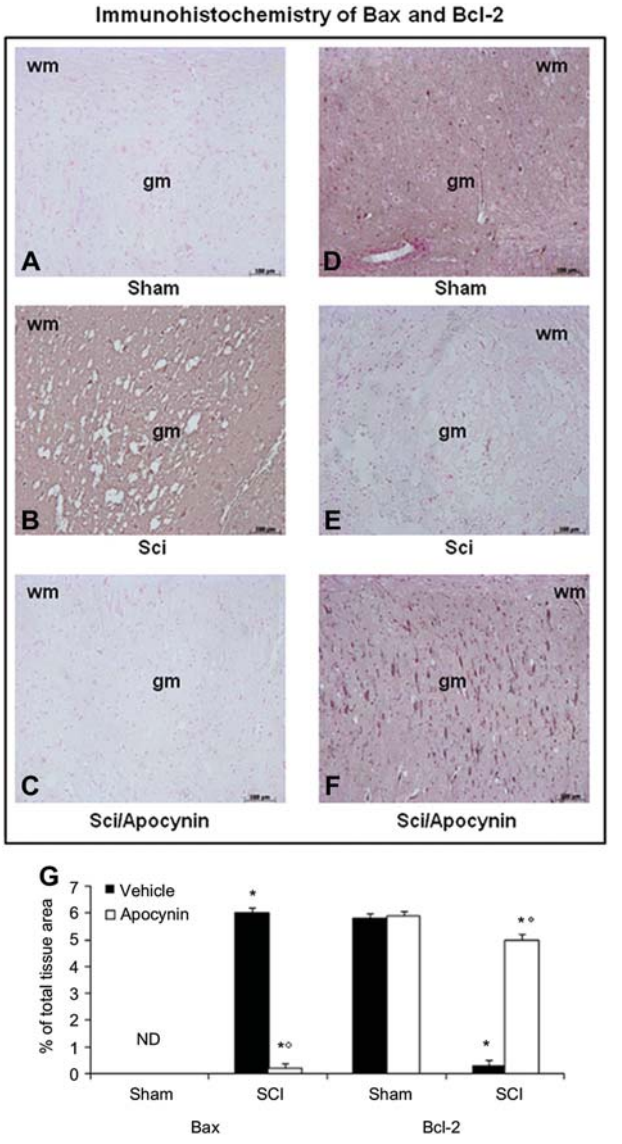

Figure 8. Immunohistochemical expression of Bax and Bcl-2. Spinal cord sections from Apocynin-treated sham-injured mice did not stain for Bax (A), whereas SCI caused, at 24 h, an increase in Bax expression (B). Apocynin (5 mg/kg 10% DMSO) treatment reduced the degree of positive staining for Bax in the spinal cord (C). On the contrary, positive staining for Bcl-2 was observed in the spinal cord tissues from Apocynin-treated sham-injured mice (D), while the staining was significantly reduced in SCI mice (E). Apocynin treatment (5 mg/kg 10% DMSO) attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (F). Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for Bax and for Bcl-2 (G) 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 a percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}p < 0.01$ vs Sham; $^{\circ}p < 0.01$ vs *SCI*+vehicle.

endogenous formation 'footprint' of peroxynitrite [56]. There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g. the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine [57]. Increased nitrotyrosine staining is considered, therefore, as an indication of 'increased nitrosative stress' rather than a specific marker of the peroxynitrite generation.

A novel pathway of inflammation associated to SCI, governed by the nuclear enzyme PARP, has been proposed in relation to hydroxyl radical and peroxynitrite-induced DNA single strand breakage [58]. Continuous or excessive activation of PARP produces extended chains of ADP-ribose on nuclear proteins and results in a substantial depletion of intracellular NAD+ and subsequently ATP leading to cellular dysfunction and, ultimately, cell death [59]. We demonstrate here that Apocynin reduced the increase of PARP activation in the spinal cord in animals subjected to SCI.

Apoptosis is an important mediator of secondary damage after SCI [60]. 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 [61]. Chronologically, apoptosis initially occurs 6 h post-injury at the lesion centre and lasts for several days associated with the steadily increased number of apoptotic cells in this region. An important intracellular signal transduction pathway that leads to apoptosis after SCI involves activation of the caspases, in particular caspase-3 [61]. In an effort to prevent or diminish levels of apoptosis, we demonstrate that the treatment with Apocynin attenuates the degree of apoptosis, measured by TUNEL detection kit, in the spinal cord after the damage. Recent studies have also shown that inhibition of NADPH oxidase by Apocynin reduces cardiomyocyte apoptosis in response to angiotensin [62]. Moreover, various studies have postulated that preserving Bax, a pro-apoptotic gene, plays an important role in developmental cell death [63] and in CNS injury [64]. Similarly, it has been shown that the administration of Bcl-xL fusion protein (Bcl-xL FP) (Bcl-2 is the most expressed anti-apoptotic 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 [65]. Based on these evidences, we have identified in SCI pro-apoptotic transcriptional changes, including up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2, by immunohistochemical staining. We report in this study that the pharmacological inhibition of NADPH-oxidase by Apocynin in SCI experimental model documents features of apoptotic cell death after SCI, suggesting that protection from apoptosis may be a pre-requisite for regenerative approaches to SCI. In particular, we demonstrated that the treatment with

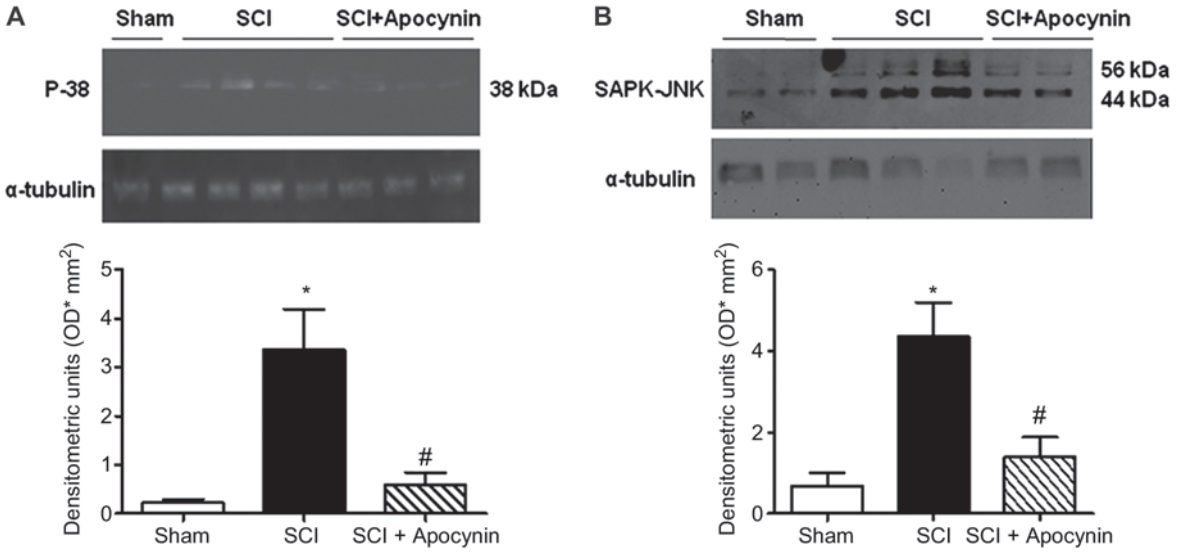

Figure 9. Effect of Apocynin on phospho-JNK and P38 expression. Representative Western blots showing no significant phospho-JNK and P38 expression in spinal cord tissues obtained from sham-treated animals (A, B). A significant increase in phospho-JNK (A) was observed in the spinal cord from mice subjected to SCI. On the contrary, Apocynin treatment prevented the SCI-induced (A) expression of this protein. Moreover, SCI also caused a significant increase in p38 expression at 24 h after trauma (B). The treatment with Apocynin significantly reduced p38 expression (B). The relative expression of the protein bands was standardized for densitometric analysis to β-actin levels, and reported in (A) and (B). $^{*}p < 0.05$ vs Sham; $^{**}p < 0.01$ vs Sham; $^{***}p < 0.001$ vs Sham; $^{\#}p < 0.05$ vs *SCI*+vehicle; $^{\#\#}p < 0.01$ vs *SCI*+vehicle; $^{\#\#\#}p < 0.001$ vs *SCI*+vehicle.

Apocynin reduced Bax expression, while on the contrary Bcl-2 is expressed much more in mice treated with Apocynin. A large 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 [66]. Recently, it has been pointed out that FasL signalling plays a central role in SCI [67]. 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 Apocynin treatment leads to a substantial reduction of FasL activation.

Finally, in this study we demonstrate that Apocynin treatment significantly reduced the SCI-induced spinal cord tissues alteration as well as improve the motor function. The results of the present study enhance our understanding of the role of NADPH oxidase dependent ROS generation in the pathophysiology of spinal cord cell and tissue injury following trauma, implying that inhibitors of the activity NADPH oxidase, such as Apocynin, may be useful in the therapy of spinal cord injury, trauma and inflammation.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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