Molecular Neurobiology (2019) 56:3937–3947
https://doi.org/10.1007/s12035-018-1347-7

Sodium Butyrate Exerts Neuroprotective Effects in Spinal Cord Injury

M. Lanza[1] · M. Campolo[1] · G. Casili[1] · A. Filippone[1] · I. Paterniti[1] · S. Cuzzocrea[1,2] · Emanuela Esposito[1]

Received: 3 August 2018 / Accepted: 7 September 2018 / Published online: 18 September 2018

Abstract

Sodium butyrate (SB) is a dietary microbial fermentation product and serves as an important neuromodulator in the central nervous system. Recent experimental evidence has suggested potential therapeutic applications for butyrate, including its utility in treating metabolic and inflammatory diseases. The aim of the present study was to evaluate the potential beneficial effects of SB in a mouse model of spinal cord injury (SCI) and its possible mechanism of action. SCI was induced by extradural compression for 1 min of the spinal cord at the T6–7 level using an aneurysm clip, and SB (10–30-100 mg/kg) was administered by oral gavage 1 and 6 h after SCI. For locomotor activity, study mice were treated with SB once daily for 10 days. Morphological examination was performed by light microscopy through hematoxylin-eosin (H&E) staining. In addition, NF-κB, IκB-α, COX-2, and iNOS expressions were assayed by western blot analysis and IL-1β and TNF-α levels by immunohistochemistry analysis. The results showed that SB treatment significantly ameliorated histopathology changes and improved recovery of motor function changes in spinal cord injury in a dose-dependent manner. Moreover, we demonstrated that SB modulated the NF-κB pathway showing a significant reduction in cytokine expression. Thus, this study showed that SB exerts neuroprotective effects anti-inflammatory properties following spinal cord injury suggesting that SB may serve as a potential candidate for future treatment of spinal cord injury.

Keywords Spinal cord injury · Sodium butyrate · Short-chain fatty acid · Inflammation · Neuroinflammation · Neurodegenerative disease

Introduction

Spinal cord injury (SCI) occurs with an annual incidence of 12.1–57.8 cases per million, and it is associated with permanent disability and decreased life expectancy. The incidence, prevalence, and causation of SCI differ between developing and developed countries and suggest that management and preventative strategies must be adapted to regional needs. Although several studies have attempted to quantify the worldwide prevalence and incidences, several issues have prevented an accurate estimate [1]. SCI is the damage caused to the spinal cord that compromise the major functions and actually remains the most important cause of mortality in the society.

SCI is known to result in neurological deficits through both the primary and secondary damage. The "primary" injury encompasses the immediate mechanical damage to the spinal cord tissue that occurs at the moment of impact, which is irreversible and not preventable. The "secondary" injury, by contrast, is incurred as a result of the pathological processes initiated at the time of the primary injury and continues for several days or months, characterized by infiltration of leukocytes and activation of glial cells that aggravate tissue damage by releasing proteases, reactive oxygen intermediates, lysosome enzymes, and pro-inflammatory cytokines/chemokines.

Recent studies have indicated sodium butyrate (SB) exerts anti-inflammatory and neuroprotective effects in neurodegenerative disorders [2]. SB is a natural short-chain fatty acid (SCFA) present in dairy products and produced in the colon by anaerobic fermentation of undigested carbohydrates. Fatty acids are building blocks of fats and our cells cannot be without them. This particular fatty acid is very small and is usually made by bacteria living in our gut mostly from fibers derived from grains, beans, onions, and bananas. This four-carbon acid plays an important role in maintaining normal function and integrity of the colonic mucosa [3]. Its anti-inflammatory

✉ Emanuela Esposito
eesposito@unime.it

[1] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale Ferdinando Stagno D'Alcontres n°31, 98166 Messina, Italy

[2] Department of Pharmacological and Physiological Science, Saint Louis University, Saint Louis, MO, USA

properties are also shown in an increasing number of animal and cellular models of inflammatory diseases. It has been shown that SCFA improved inflammation through activating NF-κB signaling pathways [4]; in fact, SB decreases the activation of NF-κB reducing inflammation and oxidative damage in the kidney of rats subjected to contrast-induced nephropathy. SCFAs have profound effects on gut function, mediated by the FFA2 and FFA3 receptors, by suppressing intestinal inflammation and maintaining intestinal homeostasis [5–7] On the other hand, SB belongs to the class of deacetylase inhibitors (HDAC) and acts as HDAC inhibitor in various types of cells [5]. SB suppresses the expression of pro-inflammatory cytokines in dendritic cells through inhibiting HDAC activity. Recent experimental evidence has suggested potential therapeutic applications for SB, including its utility in treating metabolic and inflammatory diseases [8, 9]. Moreover, the latest studies have highlighted a possible involving of SB on redox system, demonstrating the role of SB as an activator of NRF2 pathway [10]. Although, the mechanism by which SB activates NRF2 is not clarified.

Clinical trials suggest that SB exerts its anti-inflammatory properties in human inflammatory bowel disease, including ulcerative colitis, proctosigmoiditis, and chronic radiation proctitis [11–14]. Recently, Kukkar et al. showed that oral administration of butyrate attenuates neuropathic pain symptoms in a chronic constriction injury (CCI) model, which may be mainly attributed to its ability to decrease the release of pro-inflammatory mediators during neuropathy development [15]. Although the neuroprotective effect of SB has been well documented, its neuroprotective effects on SCI have never been studied. Therefore, since inflammation is a key element in pathophysiology on SCI, in the present study, we evaluated the effects of SB on the inflammatory and oxidative pathway in a mouse model of SCI.

Materials and Methods

In Vivo Experiments

Animals

Male adult CD1 mice (25 to 30 g, 6–8 weeks Envigo, Italy) were housed in a controlled environment and powered with standard rodent chow and water. Mice were housed in stainless steel cages in a room kept at 22 ± 1 °C with a 12-h light, 12-h dark cycle. Mice were acclimatized to their environment for 1 week and had ad libitum access to tap water and rodent standard diet. The study was accepted by the University of Messina Review Board for the care of animals. All animal experiments were in agreement with regulations in Italy (DM 116192) and EU regulations (OJ of EC L 358/1 12/18/1986).

SCI Animal Model

Mice were anesthetized with xylazine and ketamine (0.16 and 2.6 mg/kg body weight, respectively). A longitudinal incision was made in the midline of the back, exposing the paravertebral muscles. These muscles were dissected away exposing T5 to T8 vertebrae. SCI was produced by extradural compression of the spinal cord at T6 to T7 using an aneurysm clip with a closing force of 24 g as previously described. In all injured groups, the spinal cord was compressed for 1 min. Sham-injured animals were only subjected to laminectomy. Spinal cord tissues were collected 24 h after trauma.

Experimental Groups

Mice were divided randomly in the following groups:

1. Sham + vehicle group. Mice were subjected to the surgical procedures except for the aneurysm clip, which was not applied. These mice received orally saline.($N = 20$)
2. Sham + SB 100 mg. Identical to sham + vehicle group except for the administration of SB (100 mg/kg, *o.s.* 1 h and 6 h, after SCI) ($N = 20$)
3. SCI + vehicle: mice were subjected to SCI plus administration of saline ($N = 20$)
4. SCI + SB10mg/Kg: mice were subjected to SCI plus orally administration with SB at the dose of 10 mg/kg 1 h and 6 h after SCI ($N = 20$)
5. SCI + SB 30 mg/kg: mice were subjected to SCI plus orally administration of SB at the dose of 30 mg/kg, orally 1 h and 6 h after SCI ($N = 20$)
6. SCI + SB 100 mg/kg: mice were subjected to SCI plus orally administration of SB at the dose of 100 mg/kg, orally 1 h and 6 h after SCI ($N = 20$)

In a separate set of experiments, ten animals for each group were observed until 10 days after SCI, to evaluate the motor score. The animals received SB at the dose of 10, 30, and 100 mg/kg orally 1 and 6 h after SCI and daily until day 9; the motor score was assessed daily.

The dose and the route of administration of SB were based on a previous in vivo study [16].

Grading of Motor Disturbance

The motor function of mice subjected to compression damage was evaluated with the Basso Mouse Scale (BMS). That is a certified scale used to monitor the progress of hind-limb functional recovery following SCI. The scale ranges go from 0 that indicate complete paralysis to 9 points that indicate normal hind-limb function [17]. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. The evaluations of BMS score were

recorded daily until day 9 subsequent SCI and were made by two blind observers (without knowledge of the treatments) for all analyzed groups. To evaluate coordinated movement and stepping, hind-limb motion was used. The average of the two scores was used when differences were observed between the right and left hind limbs.

Histological Examination

For histopathological examination by standard hematoxylin and eosin (H&E) staining, 24 h after injury, mice were deeply anesthetized with pentobarbital sodium and then perfused transcardially with cold PBS (0.1 M). One centimeter of tissue on each side of the lesion was collected in 4% paraformaldehyde for 24 h, at room temperature, dehydrated by graded ethanol, and embedded in paraffin wax. Sections 7 μm thick were cut from paraffin-embedded tissue and deparaffinized with xylene, re-hydrated by graded ethanol, stained with hematoxylin/eosin, and examined by light microscopy (AxioVision, Zeiss, Milan, Italy). The segments of each spinal cord were estimated by an experienced histopathologist, and all the histological studies were performed by two independent examiners who were blind to the experimental conditions without knowledge of the treatments. Injured neurons were counted and the histopathologic modifications of the gray matter were scored on a six-point scale: 0, no lesion noticed; I, gray matter contained one-five eosinophilic neurons; II, gray matter contained five-ten eosinophilic neurons; III, gray matter contained more than ten eosinophilic neurons; IV, small infarction (less than 1/3 of the gray matter area); V, moderate infarction (1/3 to one 1/2 of the gray matter area); VI, large infarction (more than 1/2 of the gray matter area). The results from every section of all spinal cord were averaged to furnish a final score for distinct mice [18].

Localization of TNF-α and IL-1β by Immunohistochemistry Analysis

After deparaffinization and rehydration, endogenous peroxidase was removed with 0.3% H_2O_2 in 60% methanol for 30 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. The sections were then incubated overnight with primary IL-1β (Santa Cruz Biotechnology; 1:100 in PBS) and TNF-α(Santa Cruz Biotechnology; 1:100 in PBS). Sections were washed with PBS and incubated with peroxidase-conjugated bovine anti-mouse immunoglobulin G (IgG) secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2.000 Jackson Immuno Research, West Grove, PA, USA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG or biotin-conjugated goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA). To verify the binding specificity for IL-1β and TNF-α, control sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these controls, no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments. The immunohistochemical pictures were collected by Zeiss microscope using Axio Vision software. For graphic display of densitometric analyses, the % of positive staining (brown staining) was measured by computer-assisted color image analysis (Leica QWin V3, UK). The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as % of total tissue area (red staining) within five random fields at × 20 magnification. In particular, firstly, the colors of the images that have been stained to the molecule of interest were defined. Once these colors were defined, they were automatically detected in all samples. This is a semi-quantitative analysis that measures areas and not intensities [19–21]. In particular, the densitometry analysis was carried out a section in which the spinal cord tissues were orientated longitudinally in order to observe all the histological portions.

Western Blot Analysis for IκB-α, NF-κB, COX-2, iNOS, MnSOD, and Nrf-2

Levels of IκB-α, NF-κB, COX-2, iNOS and β-actin were studied, as previously described, in cytosolic and nuclear fraction from spinal cord tissue collected at the end of the experiment with minor modifications. Spinal cord tissue from each mouse was suspended in extraction buffer A containing 0.2 mM PMSF, 0.15 mM pepstatin A, 20 mM leupeptin, 1 mM sodium orthovanadate, homogenized at the maximum setting for 2 min, and centrifuged at 12,000× rpm for 4 min at 4 °C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 mM leupeptin, 0.2 mM sodium orthovanadate. After centrifugation for 10 min at 12,000 rpm at 4 °C, the supernatants containing the nuclear protein were stored at − 80 °C for further analysis. Proteins from cytoplasm and nuclear fraction were added to sample buffer (0.125 M Tris–HCl, (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue), and boiled in a water bath for 5 min. Protein samples were separated on denatured 12% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Non-specific binding to the membrane was blocked for 1 h at room temperature with 5% non-fat dry milk (PM) in PBS. Membranes were incubated at 4 °C overnight with primary antibody in milk–PBS–Tween 20, 0.1% (PMT) for IκB-α (1:500; Santa Cruz Biotechnology), NF-κB (1:500; Santa Cruz Biotechnology), iNOS (1:500;BD Trasduction) and COX-2 (1:500; Cayman Chemicals), MnSOD (1:500; Millipore), and Nrf-2 (1:500; Santa Cruz Biotechnology,

SC-722), washed three times with PBS–0.1% Tween, and then incubated for 1 h at room temperature with a secondary antibody (peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated anti-rabbit IgG, 1:2000; Jackson Immuno Research, West Grove, PA). Bands were detected by chemiluminescence (ECL) system (Thermo, USA), visualized with the ChemiDoc XRS (Bio-Rad, USA), and analyzed by using Image Lab 3.0 software (Bio-Rad, USA). The expression levels of β-actin and lamin A/C served as an internal control for protein loading.

Nitric Oxide Measurements

Tissue homogenate (100 μg/100 μl assay system) was incubated with a reaction mixture containing Griess reagent (1% sulfanilamide, 2% HCl, and 0.1% naphthyl ethelene diamine dihydrochloride) and vanadium (III) chloride-based reduction. Vanadium (III) in dilute acid solution causes reduction of nitrate to nitrite. The absorbance of the chromophore formed during diazotization of the nitrite with sulfanilamide, and subsequent coupling with naphthyl ethelene diamine, was read at 540 nm as previous described [22].

Materials

All other compounds were obtained from Sigma-Aldrich Company Ltd. (Milan, Italy). All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, 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 of histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different 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. BMS scale data were analyzed by the Mann–Whitney test and considered significant when p value was < 0.05.

Results

SB Reduces the Damage Caused by SCI

The severity of trauma in the peri-lesional area, as well as estimation of alterations of white matter, was evaluated by hematoxylin and eosin staining 24 h after trauma. Significant damage to the spinal cord was observed in tissue from mice subjected to SCI, characterized by loss of tissue architecture and presence of edema, when compared with sham-operated mice (Fig.1(A)); SB treatment significantly reduced spinal cord damage in a dose-dependent manner (Fig.1(D–F)). To evaluate whether histological damage to the spinal cord was associated with a loss of motor function, we used the BMS open-field score (1H). Motor function was not impaired in sham mice. SCI-injured mice showed significant deficits in hind-limb movement, when treatment with SB in a dose-dependent manner significantly enhanced the neurological score, compared with the SCI-vehicle mice (Fig. 1(H)).

Effects of SB on NF-kB Inflammatory Pathway

To investigate the molecular mechanisms by which SB treatment may attenuate the development of SCI, we evaluated IκB-α degradation and NF-κB translocation to the nucleus by western blot analysis. Basal expression of IκB-α was detected in spinal cord section homogenates from sham-operated mice, whereas IκB-α levels were substantially decreased in spinal cord tissue collected from SCI mice (Fig.2(A)). SB treatment prevented SCI-induced degradation of IκB-α (Fig.2(A)). Moreover, NF-κB translocation in the spinal cord nuclear fractions was also considerably increased 24 h after SCI compared with the sham-operated mice (Fig.2(B)), whereas SB treatment significantly reduced, in a dose-dependent manner, the nuclear translocation of NF-κB (Fig.2(B)).

Release of pro-inflammatory cytokines is an important mechanism responsible for spinal cord injury; also, NF-κB plays multiple roles in the induction of IL-1 beta and TNF-α transcription [23]. We investigated the role of TNF-α and IL-1β by immunohistochemical analysis. A substantial increase in TNF-α and IL-1β positive staining was found in spinal cord tissues collected from mice at 24 h after SCI (Figs. 3(C) and 4(C)). However, the treatment with SB reduced the positive staining of both TNF-α and IL-1β in a dose-dependent manner (respectively 3D, 3E, 3F and 4D, 4E, 4F, see densitometric analysis Figs. 3(G) and 4(G)).

Effect of SB on COX2 and iNOS Expression and NO Levels

To analyze how SB modulates two of major inflammatory mediators, we have assessed Cox-2 and iNOS expression by Western blot analysis and the NO levels (Fig. 5).

Total $NO^{\bullet}$ levels in spinal cord fractions were measured as an indicator of oxidative stress and/or inflammatory responses. Notably, at 24 h post-SCI, total NO· levels in tissue homogenate fractions were significantly higher compared to shams group (Fig. 5(A)). SB treatment, in a dose-dependent manner, significantly reduced total NO· levels in tissue homogenate fractions (Fig. 5(A)).

As showed, there was a significant increase in the expression of Cox-2 (Fig. 5(C), see densitometric analysis

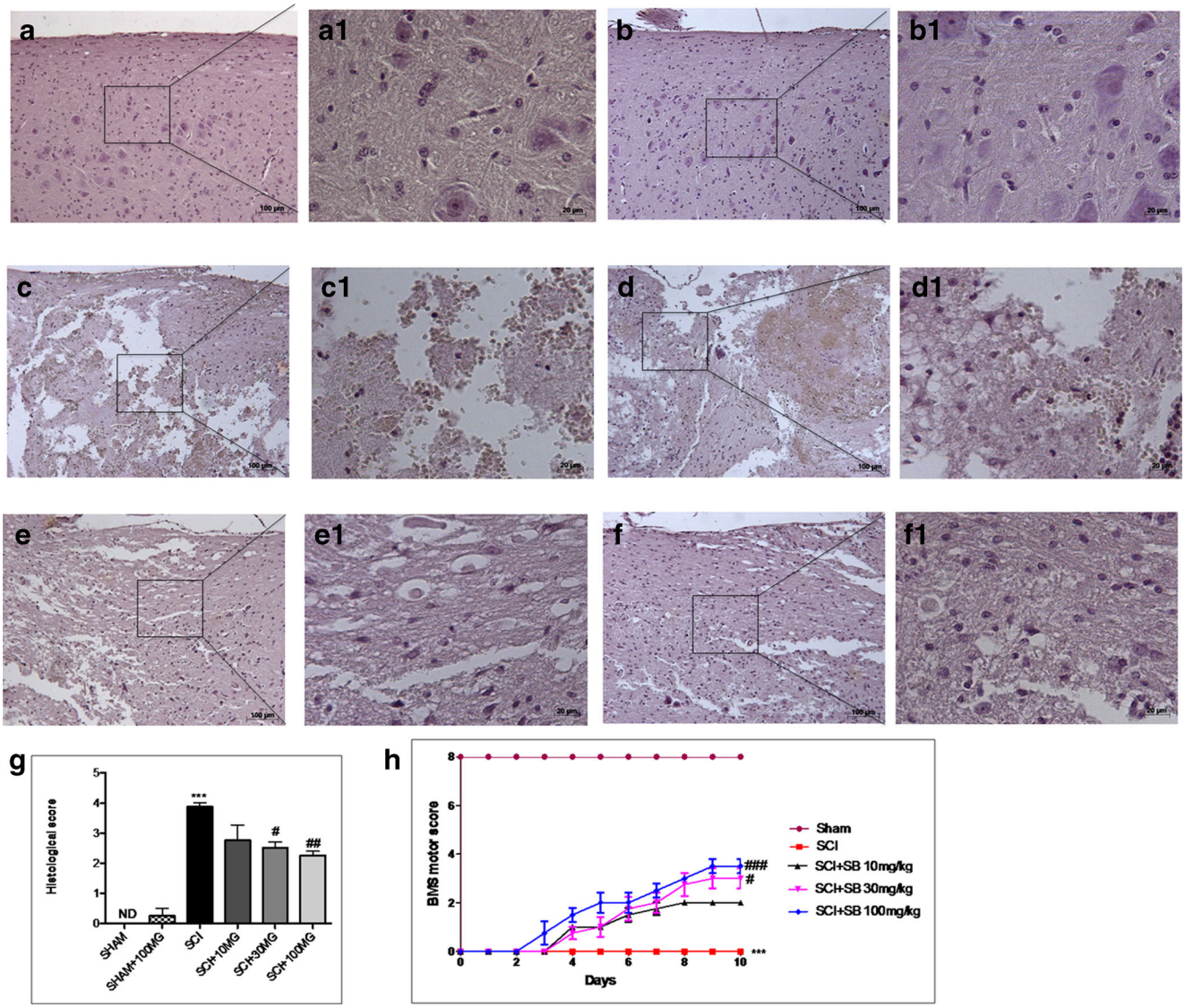

Fig. 1 SB reduces the severity of injury after SCI and accelerated recovery of motor function. No histological modifications have been found in the spinal cord tissue collected from sham-operated mice (A, see histological score G). A substantial injury to the spinal cord was assessed in SCI-operated mice stained with H & E (C, see histological score G). A significant protection against SCI was observed in SB-treated mice (E and F, see histological score G). Moreover, the degree of motor disturbance was assessed every day until 10 days after SCI by Basso mouse scale (BMS) open-field score. Motor function was intensely reduced in all mice subjected to SCI (H). Day-to-day treatment with SB resulted in a significant enhancement in motor function after SCI. However, daily treatment with SB at 30 and 100 mg/kg dose (E and F) resulted in a more rapid and substantive restoration of motor function than mice treated with SB 10 mg/kg dose (D) . The picture is demonstrative of at least three experiments executed on distinctive experimental days. The histological score was made by an independent observer. $^{***}p < 0.001$ vs. sham; $^{\#\#}p < 0.01$; $\#p < 0.05$; $^{\#\#\#}p < 0.001$ vs. SCI. ND not detectable

Fig. 5(C1)) and iNOS (Fig. 5(B), see densitometric analysis Fig. 5(B1)) 24 h after SCI. Instead, treatment with SB significantly attenuated Cox-2 and iNOS expression in a dose-dependent manner (respectively 5C and 5B).

Effects of SB Treatment on Antioxidant Response Activation

Oxidative stress plays a key role in determining neuronal cell damage. Nrf-2 is a transcription factor with strong antioxidant effects, which protects neurons from ROS-induced damage. We evaluated the effect of SB on Nrf-2 pathway and related protein MnSOD by Western blot analysis (Fig. 6).

A basal level of Nrf-2 was detected in the spinal cord sections from sham-operated animals (Fig. 6(A), see densitometric analysis A1); SCI increased Nrf-2 levels (Fig. 6(A), see densitometric analysis A1).Treatment with SB considerably increase Nrf-2 expression at the dose of 30–100 mg/kg (Fig. 6(A), see densitometric analysis A1).

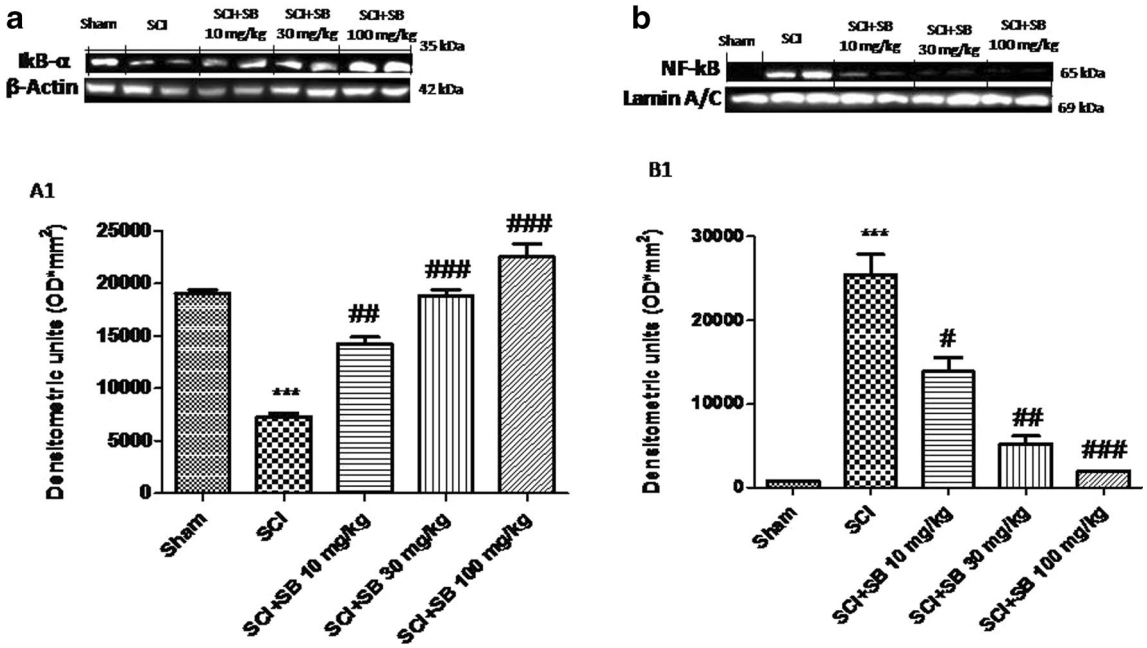

Fig. 2 Effects of SB on NF-κB inflammatory pathway. Representative Western blots showing the effects of SCI and SB treatment on IκB-α and NF-κB expression in spinal tissue. Basal expression of IκB-α was detected in spinal cord section homogenates from sham-operated mice, whereas IκB-α was substantially degraded in spinal cord tissue collected from SCI mice (B). SB treatment prevented SCI-induced degradation of IκB-α (B). Moreover, NF-κB translocation in the spinal cord nuclear fractions was also considerably increased 24 h after SCI compared with the sham-operated mice (A). SB treatment significantly reduced, in a dose-dependent manner, the nuclear translocation of NF-κB, and the degradation of IκB-α (A). $^{***}p < 0.001$ vs. sham; $^{\#\#}p < 0.01$; $\#p < 0.05$; $^{\#\#\#}p < 0.01$ vs. SCI

MnSOD expression showed a tendency to decrease following SCI as compared to control mice (Fig. 6(B), see densitometric analysis B1), while SB administration 24 h after SCI reported MnSOD expression to control levels (Fig. 6(B), see densitometric analysis B1).

Discussion

SCI results in the complete or partial loss of motor and sensory functions below the lesion site. This type of injury causes irreversible paralysis; chronic pain; and loss of the bladder, bowel, and sexual function. SCI consists of a two-step process involving a primary mechanical injury followed by an inflammatory process and apoptosis. Secondary insult leads to dramatic losses in neurons and synaptic connections and consequently functions [24].

Nowadays, methylprednisolone is the only drug available and is reported to have a limited effect. Several other compounds entering clinical trials including naloxone, nimodipine, tirilazad mesylate, and gacyclidine have been tested with largely negative consequences; therefore, an effective drug is urgently needed to treat SCI in clinical work.

Post-traumatic inflammatory reaction has been shown to contribute to progressive tissue damage after spinal cord injury. Following spinal cord injury, inflammatory cells such as polymorphonuclear neutrophils, macrophages, and lymphocytes quickly infiltrate into the traumatized cord, and inflammatory mediators, such as eicosanoids and cytokines, are accumulated [25–32]. For example, within the first hour, post-spinal cord injury, neutrophil infiltration [25, 26, 33], and cytokine expression such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) at and surrounding the site of injury occurred [29, 31, 32]. The interplay between inflammatory cells and mediators likely perpetuates a progressive course of secondary injury, resulting in neuronal and glial cell death, axonal destruction, and functional loss.

Traumatic SCI elicits a non-resolving neuroinflammatory response that is conserved across mammals; this response begins almost immediately upon mechanical injury and includes a configuration of events including intraparenchymal hemorrhage and glial activation with release of immune mediators [34, 35]. Other immune cells, including mast cell myeloid-derived suppressor cells, dendritic cells, and T cells also are present and can affect injury outcomes [36].

It has been seen that SCFA acts with neuroprotective effect; belonging to this class of compounds are sodium acetate, propionate, and butyrate, derived from fermentation of undigested and unabsorbed carbohydrates by microflora, and they are utilized by the colonic mucosa, contributing to host health and gut microbiota composition. Among all these, our attention has focused on that the butyrate does not only represent a local source of energy for colonocytes, but it also plays a role in the regulation of systemic functions, such as inflammation.

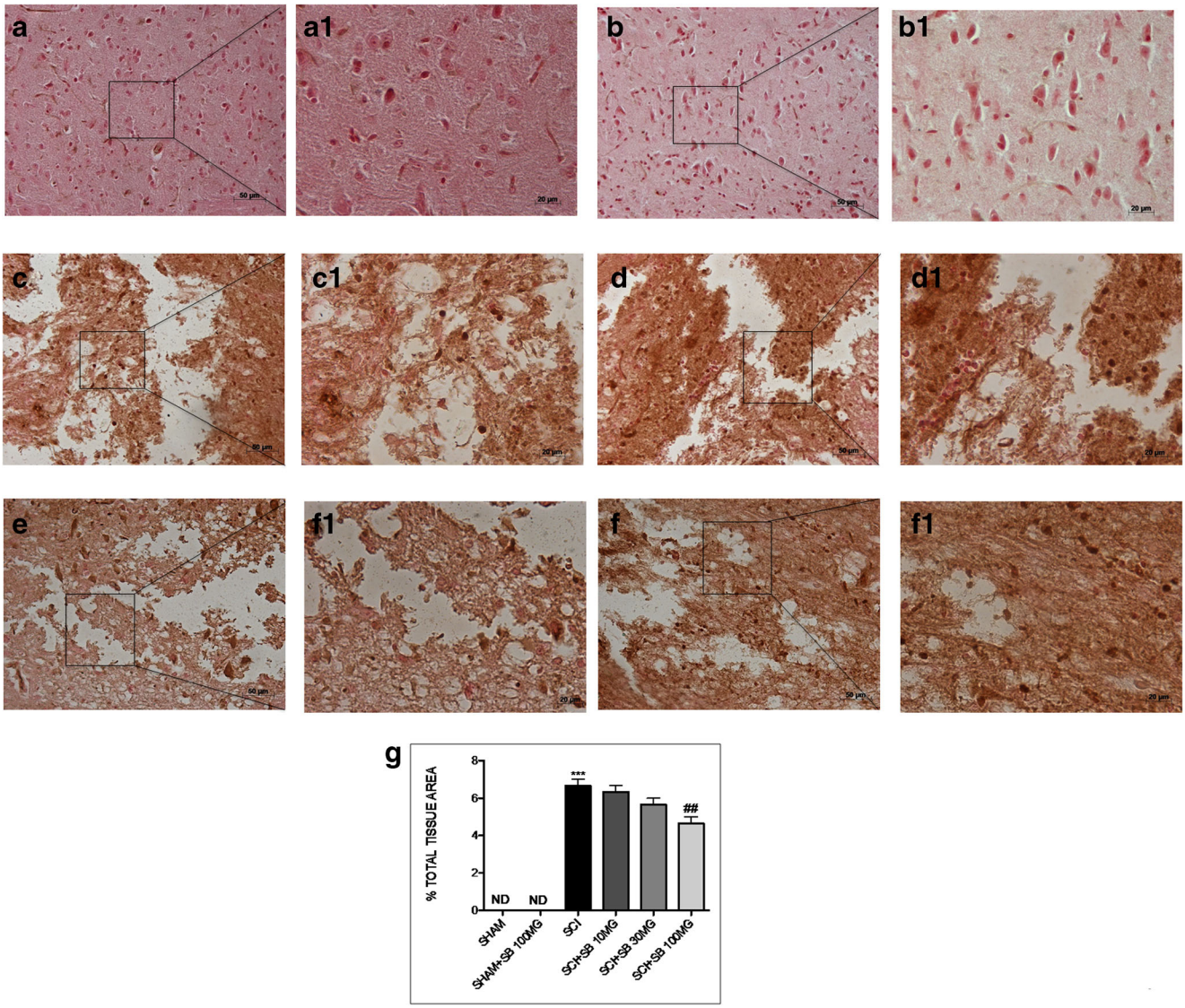

Fig. 3 Effects of SB on TNF-α A substantial increase in TNF-α positive staining was found in spinal cord tissues collected from mice at 24 h after SCI (C). However, the treatment with SB reduced the positive staining of TNF-α in a dose-dependent manner (E and F). $^{***}p < 0.001$ vs. sham; $^{\#\#}p < 0.01$ vs. SCI. ND not detectable

However, it competitively binds to the zinc sites of class I and II histone deacetylases (HDACs). This binding affects hyperacetylation of histones, resulting in a modified DNA conformation, which subsequently leads to the uncoiling or relaxing of chromatin. Enhanced accessibility of chromatin to transcription-regulatory complexes leads to increased transcriptional activation of various epigenetically suppressed genes. However, SB has been identified as having anticancer activity in a variety of human cancer cell lines, including neuroblastoma. SB has also been shown to restore the integrity of the blood-brain barrier and attenuate neurological deficits following traumatic brain injury in mice. Based on this, we wanted to investigate the protective effects of SB in spinal cord injury. It has been shown in several studies that the NF-kB pathway is the major pro-inflammatory transcription factor, activated in inflammation [37, 38] that plays a key role in pathophysiology of SCI. Different studies demonstrated a peak of NF-κB binding activity between 1 and 3 days post-injury [39]. The obtained results showed that SB could inhibit the nuclear activation of the NF-kB, inhibiting the cytoplasmic degradation of IkB-α, 24 h after damage. NF-κB pathway transactivates genes that code for pro-inflammatory proteins such as cytokines and adhesion molecules, pro-inflammatory enzymes including inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase II (COX-2), and pro-inflammatory proteases [37, 38, 40] demonstrated that after damage in spinal cord, neuroinflammatory cells expressed predominantly pro-inflammatory markers such as iNOS. Considering the extent of the inhibition of NF-κB by SB NF-κB pathway might be a major target of SB in iNOS inhibition [41]. Although NO production is indubitably

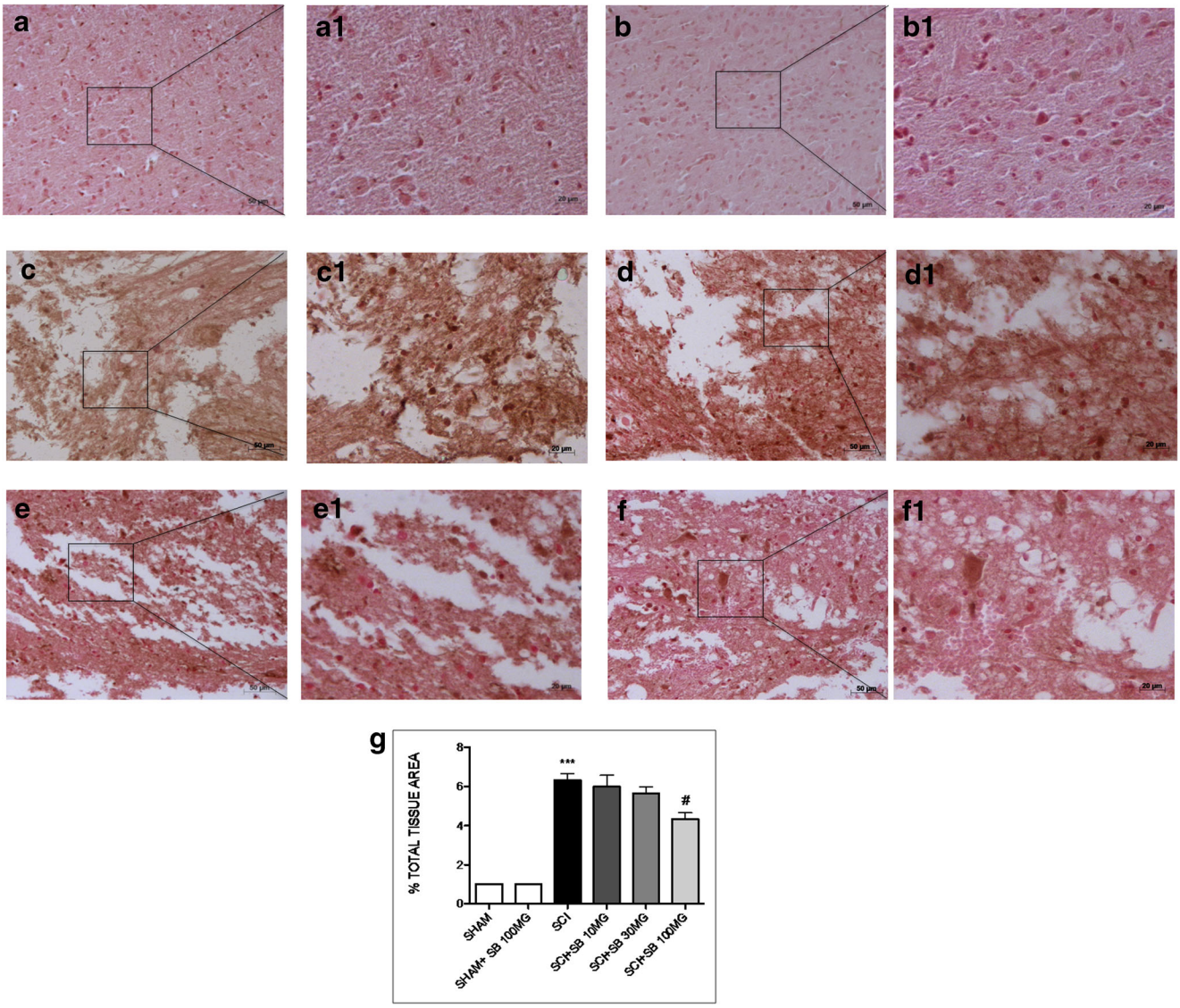

Fig. 4 Effects of SB on IL-1β levels. A substantial increase in IL-1β-positive staining was found in spinal cord tissues collected from mice at 24 h after SCI (C). However, the treatment with SB reduced the positive staining of IL-1β in a dose-dependent manner (E and F). ***$p < 0.001$ vs. sham; $^{\#}p < 0.05$ vs. SCI

involved in inflammation, it is known the mutual effect with Cox-2 activity to be fundamental in pro-inflammatory circumstances [42]. Moreover, it was demonstrated that Cox-2 mRNA and protein expression are induced by SCI and the selective inhibition of Cox-2 improved functional outcome following damage [43]. In this study, we demonstrated that SB modulated Cox-2 and iNOS expression, significantly reducing both mediators. Furthermore, Narita et al. [44], better demonstrated the endogenous pro-inflammatory cytokines IL-1β and TNF-α-dependent expression of Cox-2 mRNA within the spinal cord. In this work, we demonstrated that SB reduced the levels of both TNF-α and IL-1β in a dose-dependent manner. To evaluate the severity of traumatic SCI, we used hematoxylin and eosin staining which has clearly demonstrated in the perilesional area of post traumatic edema areas. The damage to the spinal cord causes alteration at the motor level. For this reason, we performed motor disturbance that was assessed every day until 10 days after SCI using the BMS score. Treatment with SB reduced the degree of motor disturbance more effectively than mice untreated. The principal finding in our present study is SB treatment showed brain-protective activity in SCI in a dose-dependent manner. This study demonstrated that SB may protect against SCI by improving neurological dysfunction and pathological changes. Recent studies demonstrated that SB not is only involved on Nf-KB inflammatory pathway but also on Nrf2 antioxidant system. In the nucleus, Nrf2 associates with small Maf and c-Jun, forming a heterodimer that binds to the ARE and stimulates antioxidant gene [45]. In our study, we showed that SB promoted the activation of Nrf2 pathway, reducing the oxidative damage caused by SCI.

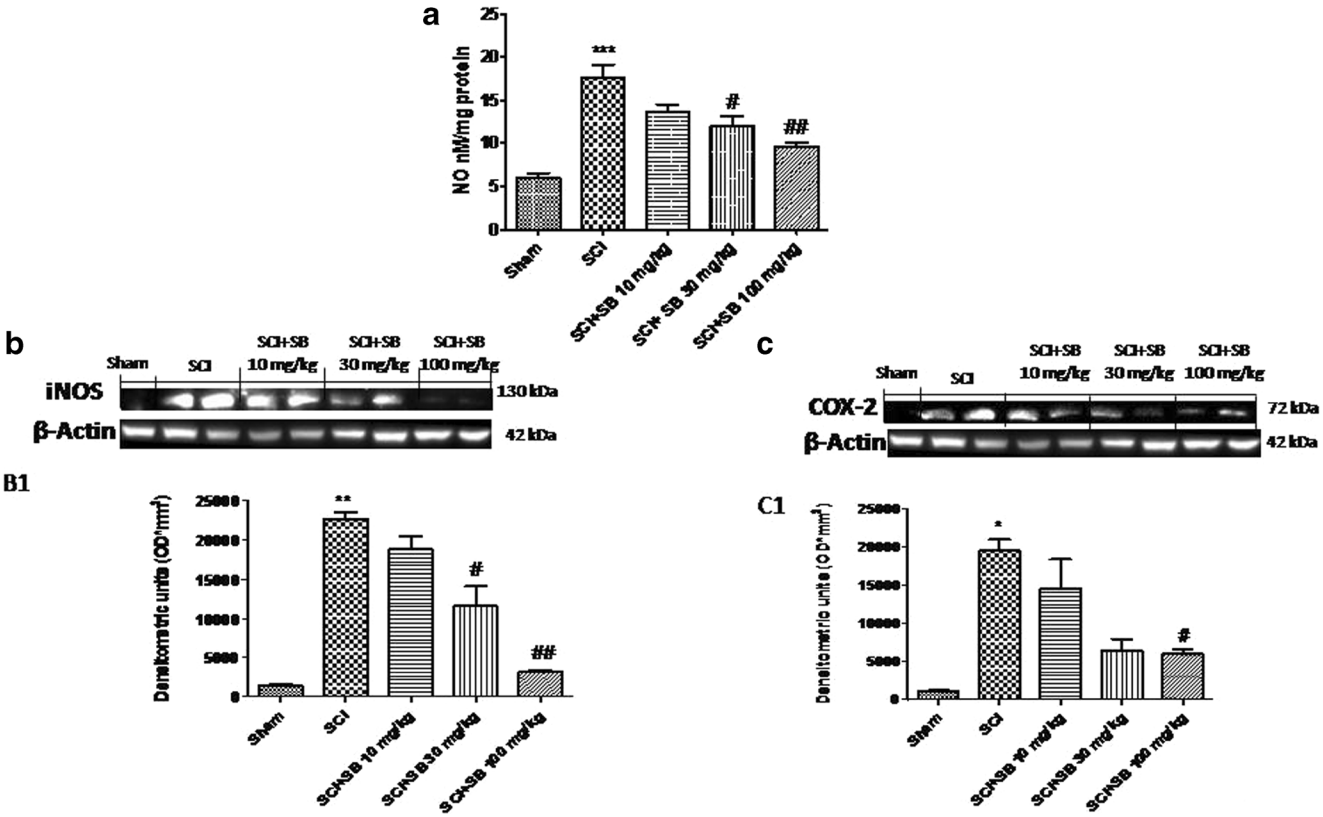

Fig. 5 Effects of SB on Cox2, iNOS expression and NO levels. Western blot analysis shows the expression of Cox-2 (C) and iNOS (B) 24 h after SCI. We observed a significant expression of these inflammatory mediators in SCI-injured mice, with respect to the control group. However, treatment with SB significantly attenuated Cox-2 and iNOS expression in a dose-dependent manner (C and B). They were assessed by standard Griess reagent and vanadium (III) chloride-based reduction assay. Spinal cord tissue homogenate (A) showed significantly increased NO levels 24 h following SCI compared to sham. $^{**}p < 0.01$; $^{*}p < 0.05$; $^{***}p < 0.001$ vs. sham; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs. SCI

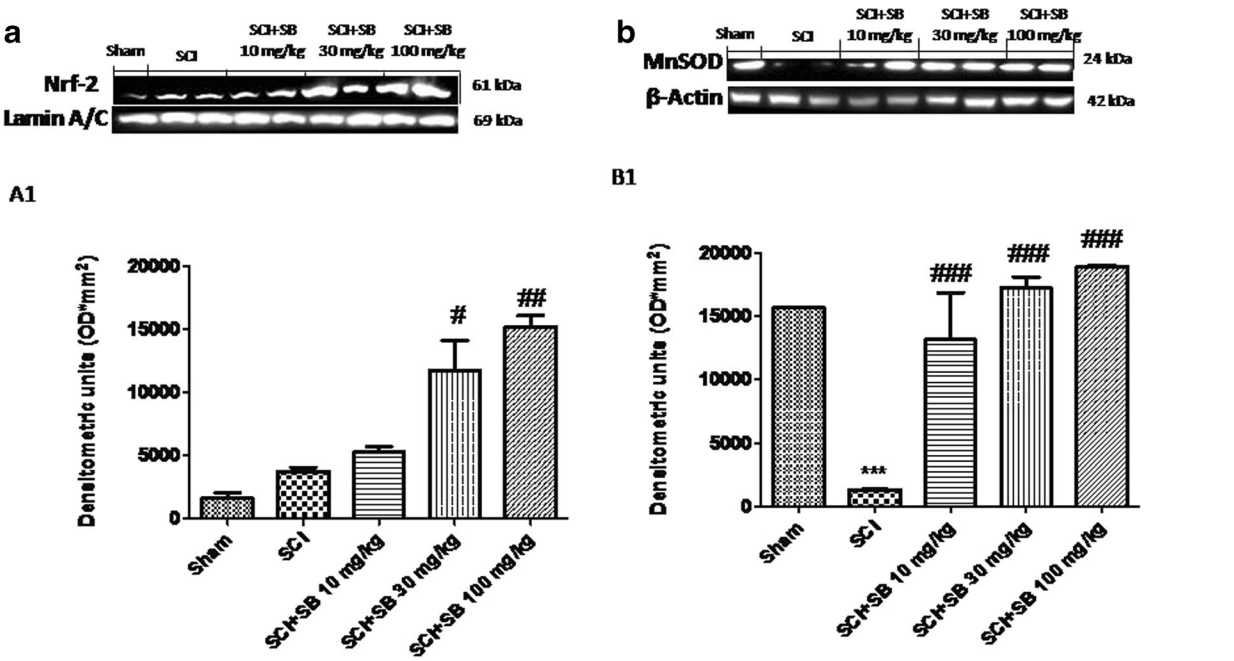

Fig. 6 Effects of SB treatment on antioxidant response activation. A basal level of Nrf-2 (A) was detected in the spinal cord sections from the sham-operated animals; SCI significantly increased Nrf-2 levels. Treatment with SB increased Nrf-2 expression. The expression of MnSOD levels showed a tendency to decrease following SCI damage as compared to control mice (B), to increase after SB treatment at the three doses (B). $^{**}p < 0.01$; $^{***}p < 0.001$ vs. sham; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs. SCI

Taken together, the data presented in this study demonstrated that treatment with SB exerts therapeutic activity of spinal cord injury, suggesting that SB could represent a target for therapeutic intervention in inflammatory disorders, such as oxidative stress and neurodegenerative disease.

Acknowledgments The authors would like to thank Antonietta Medici for the excellent technical assistance during this study and Miss Valentina Malvagni for the editorial assistance with the manuscript.

Compliance with Ethical Standards

Conflicts of Interest The authors declare that they do not have any conflicts of interest.

References

1. Zhang N, Fang M, Chen H, Gou F, Ding M (2014) Evaluation of spinal cord injury animal models. Neural Regen Res 9(22):2008–2012. https://doi.org/10.4103/1673-5374.143436
2. Chuang DM, Leng Y, Marinova Z, Kim HJ, Chiu CT (2009) Multiple roles of HDAC inhibition in neurodegenerative conditions. Trends Neurosci 32(11):591–601. https://doi.org/10.1016/j.tins.2009.06.002
3. Scheppach W (1994) Effects of short chain fatty acids on gut morphology and function. Gut 35(1 Suppl):S35–S38
4. Machado RA, Constantino Lde S, Tomasi CD, Rojas HA, Vuolo FS, Vitto MF, Cesconetto PA, de Souza CT et al Sodium butyrate decreases the activation of NF-kappaB reducing inflammation and oxidative damage in the kidney of rats subjected to contrast-induced nephropathy. Nephrol Dial Transplant 27(8):3136–3140. https://doi.org/10.1093/ndt/gfr807
5. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, de Roos P, Liu H, Cross JR et al Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 504(7480):451–455. https://doi.org/10.1038/nature12726
6. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, Glickman JN, Garrett WS The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science 341(6145):569–573. https://doi.org/10.1126/science.1241165
7. Sun J, Chang EB Exploring gut microbes in human health and disease: pushing the envelope. Genes Dis 1(2):132–139. https://doi.org/10.1016/j.gendis.2014.08.001
8. Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol 17(12):1519–1528. https://doi.org/10.3748/wjg.v17.i12
9. Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr Res Rev 23(2):366–384. https://doi.org/10.1017/S0954422410000247
10. Yaku K, Enami Y, Kurajyo C, Matsui-Yuasa I, Konishi Y, Kojima-Yuasa A (2012) The enhancement of phase 2 enzyme activities by sodium butyrate in normal intestinal epithelial cells is associated with Nrf2 and p53. Mol Cell Biochem 370(1–2):7–14. https://doi.org/10.1007/s11010-012-1392-x
11. Vernia P, Marcheggiano A, Caprilli R, Frieri G, Corrao G, Valpiani D, Di Paolo MC, Paoluzi P et al (1995) Short-chain fatty acid topical treatment in distal ulcerative colitis. Aliment Pharmacol Ther 9(3):309–313
12. Steinhart AH, Brzezinski A, Baker JP (1994) Treatment of refractory ulcerative proctosigmoiditis with butyrate enemas. Am J Gastroenterol 89(2):179–183
13. Patz J, Jacobsohn WZ, Gottschalk-Sabag S, Zeides S, Braverman DZ (1996) Treatment of refractory distal ulcerative colitis with short chain fatty acid enemas. Am J Gastroenterol 91(4):731–734
14. Pinto A, Fidalgo P, Cravo M, Midoes J, Chaves P, Rosa J, dos Anjos Brito M, Leitao CN (1999) Short chain fatty acids are effective in short-term treatment of chronic radiation proctitis: randomized, double-blind, controlled trial. Dis Colon Rectum 42(6):788–795 discussion 795-786
15. Kukkar A, Singh N, Jaggi AS Attenuation of neuropathic pain by sodium butyrate in an experimental model of chronic constriction injury in rats. J Formos Med Assoc 113(12):921–928. https://doi.org/10.1016/j.jfma.2013.05.013
16. Russo R, De Caro C, Avagliano C, Cristiano C, La Rana G, Mattace Raso G, Berni Canani R, Meli R et al Sodium butyrate and its synthetic amide derivative modulate nociceptive behaviors in mice. Pharmacol Res 103:279–291. https://doi.org/10.1016/j.phrs.2015.11.026
17. Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG (2006) Basso mouse scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 23(5):635–659. https://doi.org/10.1089/neu.2006.23.635
18. Lang-Lazdunski L, Blondeau N, Jarretou G, Lazdunski M, Heurteaux C (2003) Linolenic acid prevents neuronal cell death and paraplegia after transient spinal cord ischemia in rats. J Vasc Surg 38(3):564–575
19. Ferre N, Marsillach J, Camps J, Mackness B, Mackness M, Riu F, Coll B, Tous M et al (2006) Paraoxonase-1 is associated with oxidative stress, fibrosis and FAS expression in chronic liver diseases. J Hepatol 45(1):51–59. https://doi.org/10.1016/j.jhep.2005.12.018
20. Hernandez-Aguilera A, Sepulveda J, Rodriguez-Gallego E, Guirro M, Garcia-Heredia A, Cabre N, Luciano-Mateo F, Fort-Gallifa I et al (2015) Immunohistochemical analysis of paraoxonases and chemokines in arteries of patients with peripheral artery disease. Int J Mol Sci 16(5):11323–11338. https://doi.org/10.3390/ijms160511323
21. Rodriguez-Sanabria F, Rull A, Beltran-Debon R, Aragones G, Camps J, Mackness B, Mackness M, Joven J (2010) Tissue distribution and expression of paraoxonases and chemokines in mouse: the ubiquitous and joint localisation suggest a systemic and coordinated role. J Mol Histol 41(6):379–386. https://doi.org/10.1007/s10735-010-9299-x
22. Visavadiya NP, Patel SP, VanRooyen JL, Sullivan PG, Rabchevsky AG (2016) Cellular and subcellular oxidative stress parameters following severe spinal cord injury. Redox Biol 8:59–67. https://doi.org/10.1016/j.redox.2015.12.011
23. Cogswell JP, Godlevski MM, Wisely GB, Clay WC, Leesnitzer LM, Ways JP, Gray JG (1994) NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. J Immunol 153(2):712–723
24. Herman PE, Papatheodorou A, Bryant SA, Waterbury CKM, Herdy JR, Arcese AA, Buxbaum JD, Smith JJ et al Highly conserved molecular pathways, including Wnt signaling, promote functional recovery from spinal cord injury in lampreys. Sci Rep 8(1):742. https://doi.org/10.1038/s41598-017-18757-1
25. Carlson SL, Parrish ME, Springer JE, Doty K, Dossett L (1998) Acute inflammatory response in spinal cord following impact injury. Exp Neurol 151(1):77–88. https://doi.org/10.1006/exnr.1998.6785
26. Mautes AE, Weinzierl MR, Donovan F, Noble LJ (2000) Vascular events after spinal cord injury: contribution to secondary pathogenesis. Phys Ther 80(7):673–687

27. Popovich PG, Wei P, Stokes BT (1997) Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. J Comp Neurol 377(3):443–464. https://doi.org/10.1002/(SICI)1096-9861(19970120)377:3<443::AID-CNE10>3.0.CO;2-S
28. Resnick DK, Nguyen P, Cechvala CF (2001) Regional and temporal changes in prostaglandin E2 and thromboxane B2 concentrations after spinal cord injury. Spine J 1(6):432–436
29. Streit WJ, Semple-Rowland SL, Hurley SD, Miller RC, Popovich PG, Stokes BT (1998) Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. Exp Neurol 152(1):74–87. https://doi.org/10.1006/exnr.1998.6835
30. Tonai T, Taketani Y, Ueda N, Nishisho T, Ohmoto Y, Sakata Y, Muraguchi M, Wada K et al (1999) Possible involvement of interleukin-1 in cyclooxygenase-2 induction after spinal cord injury in rats. J Neurochem 72(1):302–309
31. Wang CX, Olschowka JA, Wrathall JR (1997) Increase of interleukin-1beta mRNA and protein in the spinal cord following experimental traumatic injury in the rat. Brain Res 759(2):190–196
32. Xu J, Fan G, Chen S, Wu Y, Xu XM, Hsu CY (1998) Methylprednisolone inhibition of TNF-alpha expression and NF-kB activation after spinal cord injury in rats. Brain Res Mol Brain Res 59(2):135–142
33. Dusart I, Schwab ME (1994) Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord. Eur J Neurosci 6(5):712–724
34. Pineau I, Sun L, Bastien D, Lacroix S Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion. Brain Behav Immun 24(4):540–553. https://doi.org/10.1016/j.bbi.2009.11.007
35. Beck KD, Nguyen HX, Galvan MD, Salazar DL, Woodruff TM, Anderson AJ Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment. Brain 133(Pt 2):433–447. https://doi.org/10.1093/brain/awp322
36. Brennan FH, Popovich PG Emerging targets for reprograming the immune response to promote repair and recovery of function after spinal cord injury. Curr Opin Neurol 31(3):334–344. https://doi.org/10.1097/WCO.0000000000000550
37. Barnes PJ, Karin M (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336(15):1066–1071. https://doi.org/10.1056/NEJM199704103361506
38. Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. Signal Transduct Target Ther 2. https://doi.org/10.1038/sigtrans.2017.23
39. Wang L, Yu WB, Tao LY, Xu Q Myeloid-derived suppressor cells mediate immune suppression in spinal cord injury. J Neuroimmunol 290:96–102. https://doi.org/10.1016/j.jneuroim.2015.11.023
40. Lago N, Pannunzio B, Amo-Aparicio J, Lopez-Vales R, Peluffo H (2018) CD200 modulates spinal cord injury neuroinflammation and outcome through CD200R1. Brain Behav Immun. https://doi.org/10.1016/j.bbi.2018.06.002
41. Park JS, Woo MS, Kim SY, Kim WK, Kim HS (2005) Repression of interferon-gamma-induced inducible nitric oxide synthase (iNOS) gene expression in microglia by sodium butyrate is mediated through specific inhibition of ERK signaling pathways. J Neuroimmunol 168(1–2):56–64. https://doi.org/10.1016/j.jneuroim.2005.07.003
42. Appleton I, Tomlinson A, Willoughby DA (1996) Induction of cyclo-oxygenase and nitric oxide synthase in inflammation. Adv Pharmacol 35:27–78
43. Resnick DK, Graham SH, Dixon CE, Marion DW (1998) Role of cyclooxygenase 2 in acute spinal cord injury. J Neurotrauma 15(12):1005–1013. https://doi.org/10.1089/neu.1998.15.1005
44. Narita M, Shimamura M, Imai S, Kubota C, Yajima Y, Takagi T, Shiokawa M, Inoue T et al (2008) Role of interleukin-1beta and tumor necrosis factor-alpha-dependent expression of cyclooxygenase-2 mRNA in thermal hyperalgesia induced by chronic inflammation in mice. Neuroscience 152(2):477–486. https://doi.org/10.1016/j.neuroscience.2007.10.039
45. Pool-Zobel B, Veeriah S, Bohmer FD (2005) Modulation of xenobiotic metabolising enzymes by anticarcinogens – focus on glutathione S-transferases and their role as targets of dietary chemoprevention in colorectal carcinogenesis. Mutat Res 591(1–2):74–92. https://doi.org/10.1016/j.mrfmmm.2005.04.020