

Inflammatory response and oxidative stress attenuated by sulfiredoxin-1 in neuron-like cells depends on nuclear factor erythroid-2-related factor 2

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Abstract. Sulfiredoxin-1 (SRX1) is a conserved endogenous antioxidative protein, which is involved in the response to cellular damage caused by oxidative stress. Oxidative stress and inflammation are the primary pathological changes in spinal cord injuries (SCI). The aim of present study was to explore the roles of SRX1 in SCI. Using reverse transcription-quantitative PCR and western blotting, the present study discovered that the expression levels of SRX1 were downregulated in the spinal cord tissues of SCI model rats. Massive irregular cavities and decreased Nissl bodies were observed in the model group compared with the sham group. Thus, to determine the underlying mechanisms, neuron-like PC12 cells were cultured *in vitro*. Western blotting analysis indicated that SRX1 expression levels were downregulated following the exposure of cells to lipopolysaccharide (LPS). Following the transfection with the SRX1 overexpression plasmid and stimulation with LPS, the results of the Cell Counting Kit-8 assay indicated that the cell viability was increased compared with LPS stimulation alone. Furthermore, the expression levels of proinflammatory cytokines secreted by LPS-treated PC12 cells were downregulated following SRX1 overexpression. Increased malondialdehyde content, decreased superoxide dismutase activity and reactive oxygen species production were also identified in PC12 cells treated with LPS using commercial detection kits, whereas the overexpression of SRX1 partially reversed the effects caused by LPS stimulation. The aforementioned results were further verified by determining the expression levels of antioxidative proteins using western blotting analysis. In addition, nuclear factor erythroid-2-related factor 2 (NRF2), a transcription factor known to regulate SRX1, was indicated to participate in the protective effect of SRX1 against oxidative stress.

Inhibition of NRF2 further downregulated the expression levels of SRX1, NAD(P)H dehydrogenase quinone 1 and heme oxygenase-1 in the presence of LPS, while activation of NRF2 reversed the effects of LPS on the expression levels of these proteins. In conclusion, the results of the present study indicated that the anti-inflammatory and antioxidative effects of SRX1 may depend on NRF2, providing evidence that SRX1 may serve as a novel molecular target to exert a neuroprotective effect in SCI.

Introduction

Spinal cord injury (SCI) affects millions of individuals worldwide (1), with between 16 and 19.4 new cases per million annually in Western European countries (2), and characteristically causes life-long neurological consequences (different degrees of paralysis and sensory impairment of limbs and trunk) with substantial socioeconomic implications (3). SCI is divided into primary injury and secondary injury; primary injury refers to the initial physical damage of the spinal cord caused by an indirect or direct external force, while the secondary injury is characterized by a series of physiological and pathological changes to the spinal cord, including inflammation, oxidative stress, necrosis and neuronal apoptosis on the basis of the primary damage, further deepening and expanding the degree and scope of the damage (4-6). The secondary injury is largely responsible for the neurological dysfunction associated with SCI. The current therapeutic strategies for SCI include surgical intervention (grafts and bridges), neural stem cell transplantation and the administration of high-dose methylprednisolone (7,8), the neurological recovery remains limited since there is no consensus about the beneficial effects. Both molecular therapies (modulation of inflammatory response and administration of growth-stimulating factors), rehabilitative training and combinatorial therapies (tissue engineering and searching for synergistic effects) have raised hopes in developing novel therapies for attenuating secondary damage (9). Previous studies have demonstrated that the pathophysiological processes of SCI involved neuronal inflammation, oxidative stress, neuronal degeneration and apoptosis, as well as reactive changes in the glia (10-13). Therefore, further investigations into the pathogenesis may improve the current understanding of the molecular mechanisms of SCI.

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The small-molecule protein sulfiredoxin-1 (SRX1), a conserved endogenous antioxidative protein, is found widely distributed in eukaryotes (14). It was previously reported that SRX1 served an important role in maintaining the pulmonary antioxidative defense against cigarette smoke via nuclear factor erythroid-2-related factor 2 (NRF2)-dependent transcriptional regulation (15). SRX1 was also identified to have a crucial role in cellular damage triggered by oxidative stress (16,17). Moreover, SRX1 protected cardiac progenitor cells from oxidative stress and promoted their survival via ERK/NRF2 signaling (18). Another study also illustrated the cardioprotective effect of SRX1 via the inhibition of mitochondrial apoptosis through the PI3K/AKT signaling pathway (19). However, to the best of our knowledge, the effects of SRX1 on nerve damage in SCI remain poorly understood. Therefore, the present study aimed to determine whether the functions of SRX1 in SCI were associated with oxidative stress and inflammation. In addition, the roles of NRF2 and the downstream target genes were investigated.

Materials and methods

Establishment of SCI model rats. All experiments were approved by the Institutional Animal Ethics Committees of University of South China (Hengyang, China) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 8023, revised 1978). In total, 32 male Sprague Dawley rats (6 weeks old, 200–220 g) were purchased from the Central South University Experiment Center. The animals were provided with free access to food and water, and maintained in standardized conditions with a 12-h light/dark cycle, at a temperature of $22\pm 2^{\circ}\text{C}$ and humidity of $55\pm 10\%$. Following one week of adaptive feeding, all rats received thoracic laminectomies at the 7, 8 and 9th thoracic vertebrae under aseptic conditions; 5% isoflurane was used to induce anesthesia and rats were maintained at a surgical plane by continuous inhalation of 2% isoflurane for the duration of surgery. Subsequently, the rats were randomly divided into two groups (16 rats per group): Sham and model. The model group was established by the clip compression technique as previously described (20,21) and the wound was sutured. The sham group contained rats who underwent a laminectomy without clip compression.

During the 8 weeks following surgery, the animal health and behavior were monitored and recorded once a day (behavioral assessment data not disclosed). In the sham group, two rats died within 3 days of the sham operation and one died within 8 to 14 days; no deaths were recorded after 2 weeks. In the model group, two rats died within 3 days following spinal trauma, none died from day 4 to day 7, three died on day 8 to day 14 and one died from day 15 to day 30; no deaths were reported after 31 days (Table SI). Therefore, there were 10 rats in the model group and 13 rats in the sham group. Autopsy was performed to identify the cause of death. Subsequently, 8 weeks after surgery, rats were euthanized through inhalation of 5% isoflurane until respiration ceased (within 5 min) and decapitation was used as a secondary method of euthanasia to ensure a humane death. Spinal cord tissues were then extracted after confirming cardiac arrest. The following humane

endpoints were adhered to through the study: Emaciation, lethargy, abdominal swelling and self-mutilation.

Histological analysis. Spinal cord tissues were fixed in 4% formaldehyde at 4°C for 24 h and embedded in paraffin. The paraffin-embedded tissues were cut into 5- μm thick sections and stained with hematoxylin for 10 min and eosin for 5 min at room temperature (H&E).

Spinal cord tissues were fixed in 4% formaldehyde at 4°C for 24 h and embedded in paraffin. A Nissl staining kit (Beijing Solarbio Science & Technology Co., Ltd.) was used to stain 8- μm thick spinal cord sections, according to the manufacturer's instructions. Tissues sections were observed under a light microscope (magnification, $\times 200$).

Cell culture and treatments. Rat pheochromocytoma PC12 cells (China Center for Type Culture Collection) were cultured in DMEM (Hyclone; Cytiva), supplemented with 10% FBS (Hyclone; Cytiva), and maintained at 37°C under humid conditions with 5% CO_2 and 95% air. For differentiation, PC12 cells were treated with 50 ng/ml nerve growth factor (Sigma-Aldrich; Merck KGaA) every other day for 6 days at 37°C , which is a widely used method for the *in vitro* study of nervous system diseases, including SCI (22–25).

To investigate the function of NRF2 in the differentiated PC12 cells, the cells were pretreated with the NRF2 activator tert-butylhydroquinone (25 μM , TBHQ; Sigma-Aldrich; Merck KGaA) or inhibitor ML385 (5 μM ; MedChemExpress) for 24 h prior to the experiments (26–28), then PC12 cells were exposed to 5 $\mu\text{g}/\text{ml}$ lipopolysaccharide (LPS; MedChemExpress) for 18 h.

Cell transfection. PC12 cells were seeded into a 6-well plate (5×10^5 cells per well). At a confluence of 60%, 8 μg SRX1 overexpression (Ov) pEX-1 plasmid (Ov-SRX1; Shanghai GenePharma Co., Ltd.) or an empty control pEX-1 plasmid (Ov-NC) was transfected into cells using 5 μl Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). PC12 cells were transfected with Ov-SRX1 or Ov-NC according to the manufacturer's protocol. After successful transfection, cells were used in subsequent experiments.

Cell viability assay. The viability of PC12 cells was analyzed using a Cell Counting Kit-8 (CCK-8) assay (MedChemExpress), according to the manufacturer's protocols. Briefly, cells at a density of 5×10^3 cells/ml were plated into a 96-well plate (100 μl per well) and treated with 0.625, 1.25, 2.5 and 5 $\mu\text{g}/\text{ml}$ LPS for 18 h. Subsequently, 10 μl CCK-8 solution was added/well and incubated for 4 h. Following the incubation, the absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Western blotting. Total protein was extracted from spinal cord tissues or PC12 cells using RIPA lysis buffer (Beyotime Institution of Biotechnology), while nuclear protein was extracted using a Nuclear and Cytoplasmic Extraction kit (Beyotime Institute of Biotechnology). Total protein was quantified using a bicinchoninic acid assay kit (Abcam) and 25 μg protein was separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto PVDF membranes

(EMD Millipore) and then membranes were blocked with 5% BSA Blocking Buffer (Beijing Solarbio Science & Technology Co., Ltd.). The membranes were then incubated with the following primary antibodies at 4°C overnight: Anti-SRX1 (cat. no. ab203613; 1:1,000; Abcam), anti-peroxiredoxin (PRDX)1 (cat. no. ab15571; 1:1,000; Abcam), anti-PRDX6 (cat. no. ab59543; 1:1,000; Abcam), anti-thioredoxin reductase (TXNRD) 1 (cat. no. ab16840; 1:1,000; Abcam), anti-superoxide dismutase (SOD)2 (cat. no. ab13534; 1:1,000; Abcam), anti-NRF2 (cat. no. ab89443; 1:1,000; Abcam), anti-NAD(P)H dehydrogenase quinone (NQO) 1 (cat. no. ab28947; 1:1,000; Abcam), anti-heme oxygenase 1 (cat. no. ab13248; 1:1,000; Abcam), anti-GAPDH (cat. no. ab181603; 1:1,000; Abcam) and anti-Lamin B1 (cat. no. ab16048; 1:1,000; Abcam). Following the primary antibody incubation, the membranes were washed with TBS with Tween-20 (0.05%) and incubated with a goat anti-mouse IgG HRP-conjugated secondary antibody (cat. no. 31430; 1:10,000; Thermo Fisher Scientific, Inc.) or a goat anti-rabbit IgG HRP-conjugated secondary antibody (cat. no. 31460; 1:10,000; Thermo Fisher Scientific, Inc.) at room temperature for 2 h. Protein bands were visualized using Immobilon Crescendo Western HRP substrate (cat. no. WBLUR0100; Merck KGaA) and the expression levels were semi-quantified using ImageJ v1.8.0 software (National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the spinal cord tissues or PC12 cells using TRI reagent® (Sigma-Aldrich; Merck KGaA). The reaction templates in PrimeScript™ RT reagent kit (Takara Bio, Inc.) were added into the tube according to the manufacturer's protocol, centrifuged for 5 sec (500 x g) at room temperature and total RNA was reversed transcribed into cDNA at 37°C for 15 min and 85°C for 5 sec. qPCR was subsequently performed using a SYBR® Premix Ex Taq II kit (Takara Bio, Inc.), and the following thermocycling conditions were used: One cycle of 95°C for 30 sec, 40 cycles of 95°C for 5 sec and 60°C for 34 sec. The following primers sequences were used for the qPCR: SRX1 forward, 5'-AATCCCCAACCCTGACTTT-3' and reverse, 5'-AATCCCCAACCCTGACTTT-3'; NQO1 forward, 5'-GCGTCTGGAGACTGTCTGGG-3' and reverse, 5'-CGGCTGGAATGGACTTGC-3'; HO-1 forward, 5'-GCGAACAAGCAGAACCCA-3' and reverse, 5'-GCTCAGGATGAGTACCTCCCA-3'; and GAPDH forward, 5'-TGGCCTCCAAGGAGTAAGAAAC-3' and reverse, 5'-GGCCTCTCTCTTGCTCTCAGTATC-3'. The expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method and normalized to the loading control GAPDH (29).

Determination of malondialdehyde (MDA) content, and the levels of SOD, reactive oxygen species (ROS) and inflammatory cytokines. The cell medium of PC12 cells was collected and centrifuged at 500 x g for 5 min at room temperature. The concentrations of tumor necrosis factor (TNF)-α (cat. no. PT516; Beyotime Institute of Biotechnology), interleukin (IL)-1β (cat. no. PI303; Beyotime Institute of Biotechnology), IL-18 (cat. no. SEKR-0054; Beijing Solarbio Science & Technology Co., Ltd.) and IL-10 (cat. no. PI525; Beyotime Institute of Biotechnology) in the medium supernatants were analyzed using ELISA kits. MDA and SOD in the cellular

supernatants were analyzed using commercial detection kits (Beyotime Institute of Biotechnology), according to the manufacturers' protocols. PC12 cells (2×10^5) were seeded into a 24-well plate, and loaded with $10 \mu\text{M}$ 2',7'-dichlorofluorescein diacetate (DCFH-DA; Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min in the dark at room temperature. Intracellular ROS production was observed under a fluorescence microscope (magnification, x100; Olympus Corporation) and analyzed using ImageJ v1.8.0 software (National Institutes of Health).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc.) and data was presented as the mean \pm SD. Each experiment was repeated three times. Statistical differences between groups were determined using a one-way ANOVA, followed by a Tukey's post hoc test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of SRX1 are downregulated in SCI model rats. SCI model rats were established as previously reported (20,21). The pathological changes in the spinal cord tissues and the expression levels of SRX1 were subsequently investigated. The expression levels of SRX1 in the model group were significantly downregulated at the mRNA and protein level compared with the sham group (Fig. 1A and B). H&E staining also revealed numerous irregular cavities in the injured spinal cords of the model group compared with the sham group (Fig. 1C). In addition, Nissl bodies in the sham group were larger in shape and more abundant compared with those in the SCI model group, indicating a weaker protein synthesis ability of Nissl bodies in the SCI model rats (Fig. 1D). Collectively, these results suggested that the rat model of SCI was successfully established, and that the expression levels of SRX1 may be downregulated in damaged spinal cord tissues.

SRX1 expression levels are downregulated in PC12 cells stimulated with LPS. Subsequently, *in vitro* experiments were performed using the PC12 cell line, a common cell model used for researching neurobiological events, including SCI (22-25). A range of concentrations of LPS (0.625 - $5 \mu\text{g/ml}$) were used to stimulate PC12 cells for 18 h as previously described (26) and the viability of PC12 cells was subsequently determined. The results revealed that 1.25 , 2.5 and $5 \mu\text{g/ml}$ LPS significantly impaired the viability of the PC12 cells compared with the control cells (Fig. 2A). Subsequently, SRX1 expression levels were analyzed following the stimulation with LPS; the expression levels of SRX1 were downregulated by LPS in a dose-dependent manner at both the protein and mRNA level (Fig. 2B and C). These results suggested that SRX1 expression levels may be downregulated in PC12 cells challenged with LPS.

Overexpression of SRX1 inhibits the inflammatory response in PC12 cells. To determine the association between LPS-induced inflammation and SRX1 in PC12 cells, Ov-SRX1 was constructed and transfected into PC12 cells; a significant upregulation in the mRNA expression levels of SRX1 were

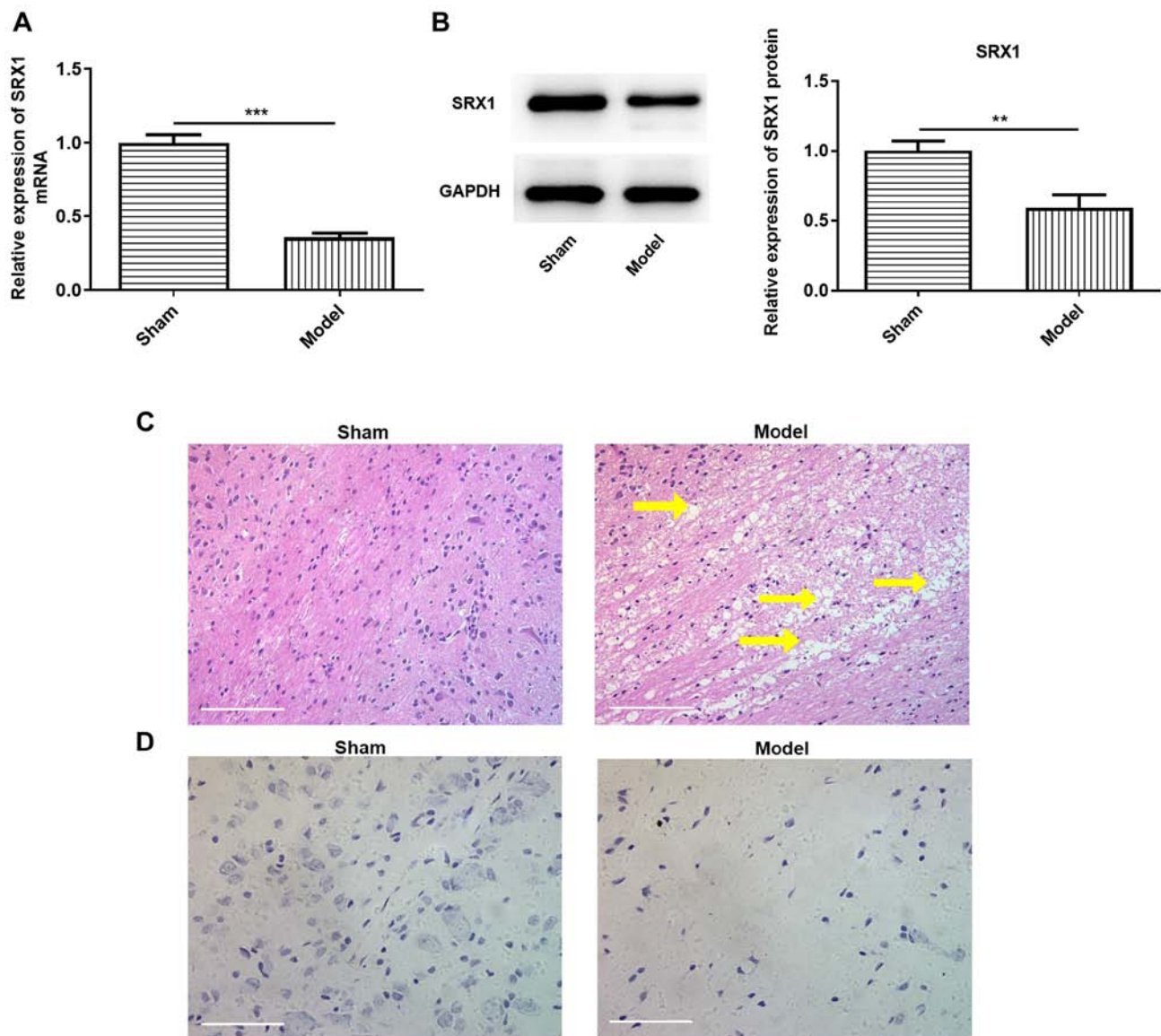


Figure 1. Expression levels of SRX1 are downregulated in spinal cord injury model rats. (A) mRNA and (B) protein expression levels of SRX1 in spinal cord tissues were determined using reverse transcription-quantitative PCR and western blotting, respectively. Spinal cord tissues were stained with (C) hematoxylin and eosin and (D) Nissl dye solution. Yellow arrow, irregular cavities in spinal cord tissue. Scale bar, 100 μ m. Data are expressed as the mean \pm SD from 3 independent experiments. ** P <0.01, *** P <0.001. SRX1, sulfiredoxin-1.

identified after the cells were transfected with Ov-SRX1 compared with the control and Ov-NC groups (Fig. 3A), indicating that the transfection with the overexpression plasmid was successful. Cell viability was significantly impaired after stimulation with 5 μ g/ml LPS, so 5 μ g/ml LPS was chosen for subsequent experiments. There was a significant increase in the expression of SRX1 mRNA and protein after cells were transfected with Ov-SRX1 (Fig. 3B and C). In addition, the results of the CCK-8 assay revealed that the Ov-SRX1 plasmid significantly increased the viability of LPS-induced cells compared with cells treated with LPS only (Fig. 3D).

Subsequently, the levels of inflammatory cytokines secreted by PC12 cells following the overexpression of SRX1 and LPS stimulation were analyzed. The levels of the proinflammatory factors, TNF- α , IL-1 β and IL-18, were all significantly increased following the stimulation of LPS compared with the control group, while the simultaneous transfection of cells

with Ov-SRX1 reversed this effect (Fig. 3E). Conversely, the secretion of IL-10 was significantly decreased following LPS treatment compared with the control group, while the simultaneous transfection with Ov-SRX1 significantly increased the secretion of IL-10 compared with the LPS treatment group (Fig. 3E). Taken together, these results indicated that the inflammatory response in PC12 cells may be attenuated by the overexpression of SRX1.

Overexpression of SRX1 reduces oxidative stress in PC12 cells.

The activation of the inflammatory response results in the production of ROS, which further exacerbates inflammation and leads to tissue damage (30). To investigate the antioxidative role of SRX1 in damaged neuron-like cells, the intracellular production of ROS was determined using the fluorescent probe DCFH-DA. Following the treatment of the cells with 5 μ g/ml LPS, the levels of ROS were significantly increased compared

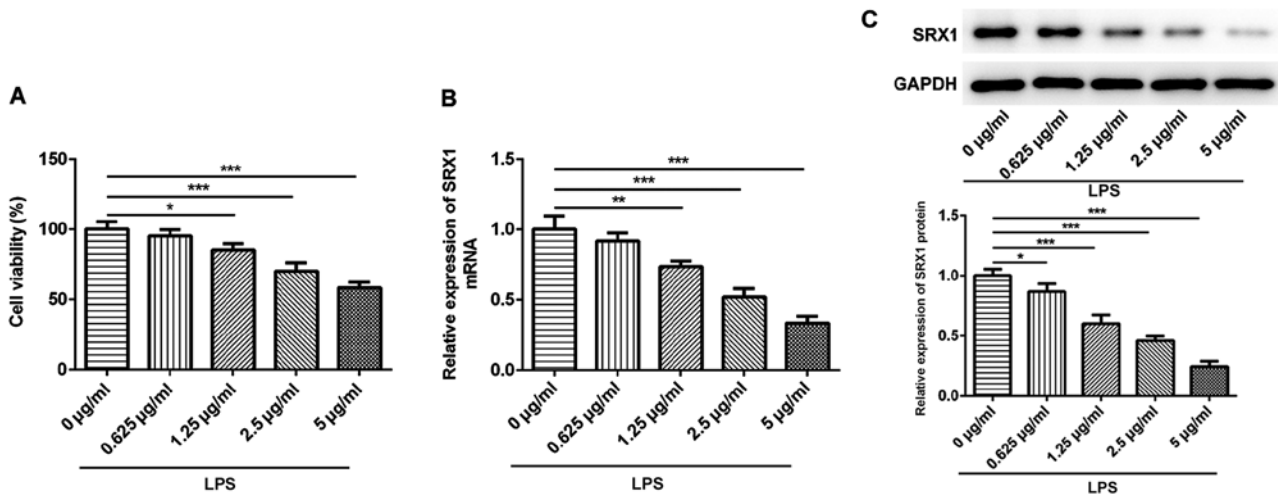


Figure 2. SRX1 expression levels are downregulated in PC12 cells stimulated with LPS. (A) Cell Counting Kit-8 assay was used to determine the cell viability of PC12 cells following the stimulation with a range of concentrations of LPS. (B) mRNA and (C) protein expression levels of SRX1 in PC12 cells stimulated with a range of concentrations of LPS were determined using reverse transcription-quantitative PCR and western blotting, respectively. Data are expressed as the mean \pm SD from 3 independent experiments. * P <0.05, ** P <0.01, *** P <0.001. SRX1, sulfiredoxin-1; LPS, lipopolysaccharide.

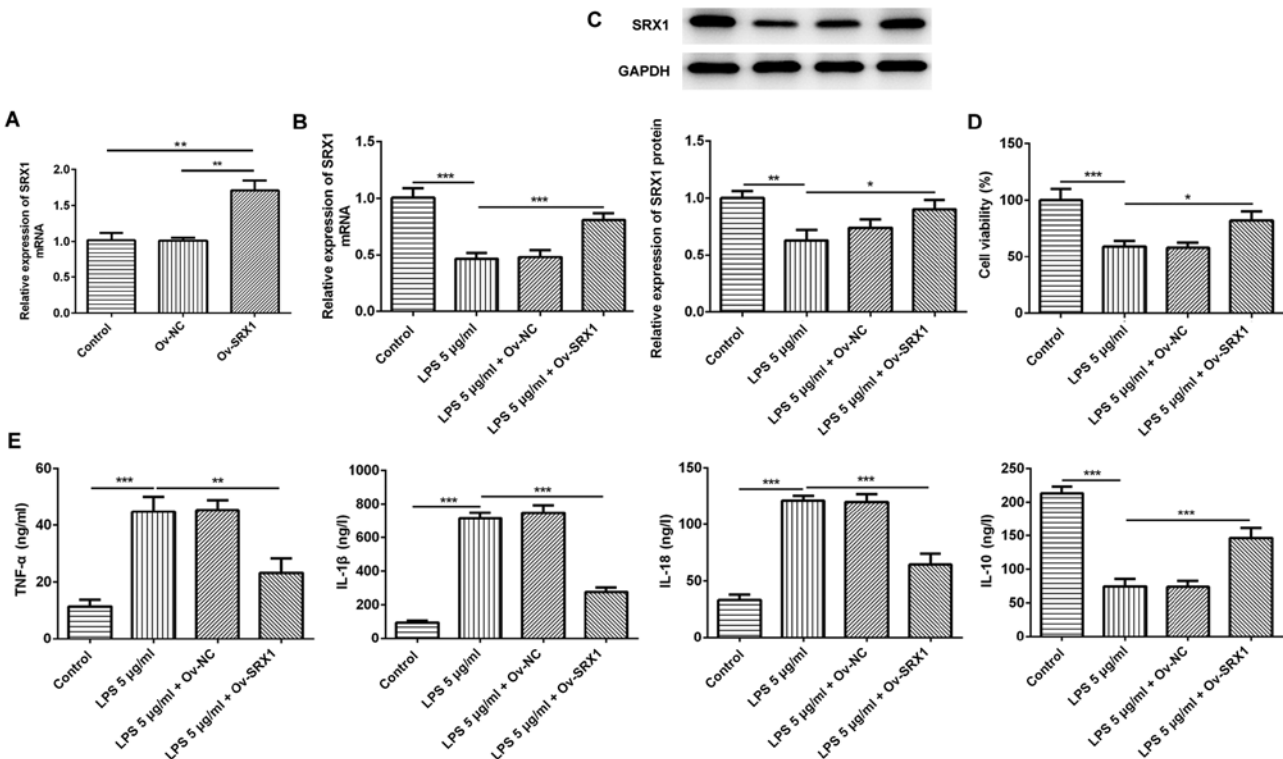


Figure 3. Overexpression of SRX1 reduces the inflammatory response in PC12 cells. (A) Transfection efficiency of Ov-SRX1 in PC12 cells was determined using RT-qPCR. (B) mRNA and (C) protein expression levels of SRX1 in PC12 cells transfected with Ov-SRX1 and stimulated with 5 μ g/ml LPS were determined using RT-qPCR and western blotting, respectively. (D) Cell Counting Kit-8 assay was used to determine the cell viability of PC12 cells transfected with Ov-SRX1 and stimulated with 5 μ g/ml LPS. (E) TNF- α , IL-1 β , IL-18 and IL-10 levels in PC12 cells transfected with Ov-SRX1 and stimulated with 5 μ g/ml LPS were detected using ELISA kits. Data are expressed as the mean \pm SD from 3 independent experiments. * P <0.05, ** P <0.01, *** P <0.001. SRX1, sulfiredoxin-1; LPS, lipopolysaccharide; Ov, overexpression; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; TNF- α , tumor necrosis factor α ; IL, interleukin.

with the control group, whereas this effect was significantly attenuated following the overexpression of SRX1 (Fig. 4A). Furthermore, the MDA content and SOD activity were also analyzed; LPS treatment significantly increased the generation of MDA compared with the control group; however, this effect was significantly impeded by the overexpression of

SRX1 (Fig. 4B). The overexpression of SRX1 also significantly reversed the inhibition over SOD activity induced by LPS (Fig. 4C). The expression levels of PRDX1, PRDX6, TXNRD1 and SOD2 were significantly downregulated in LPS-stimulated cells compared with the control group; however, a significant upregulation in the expression levels of these antioxidative

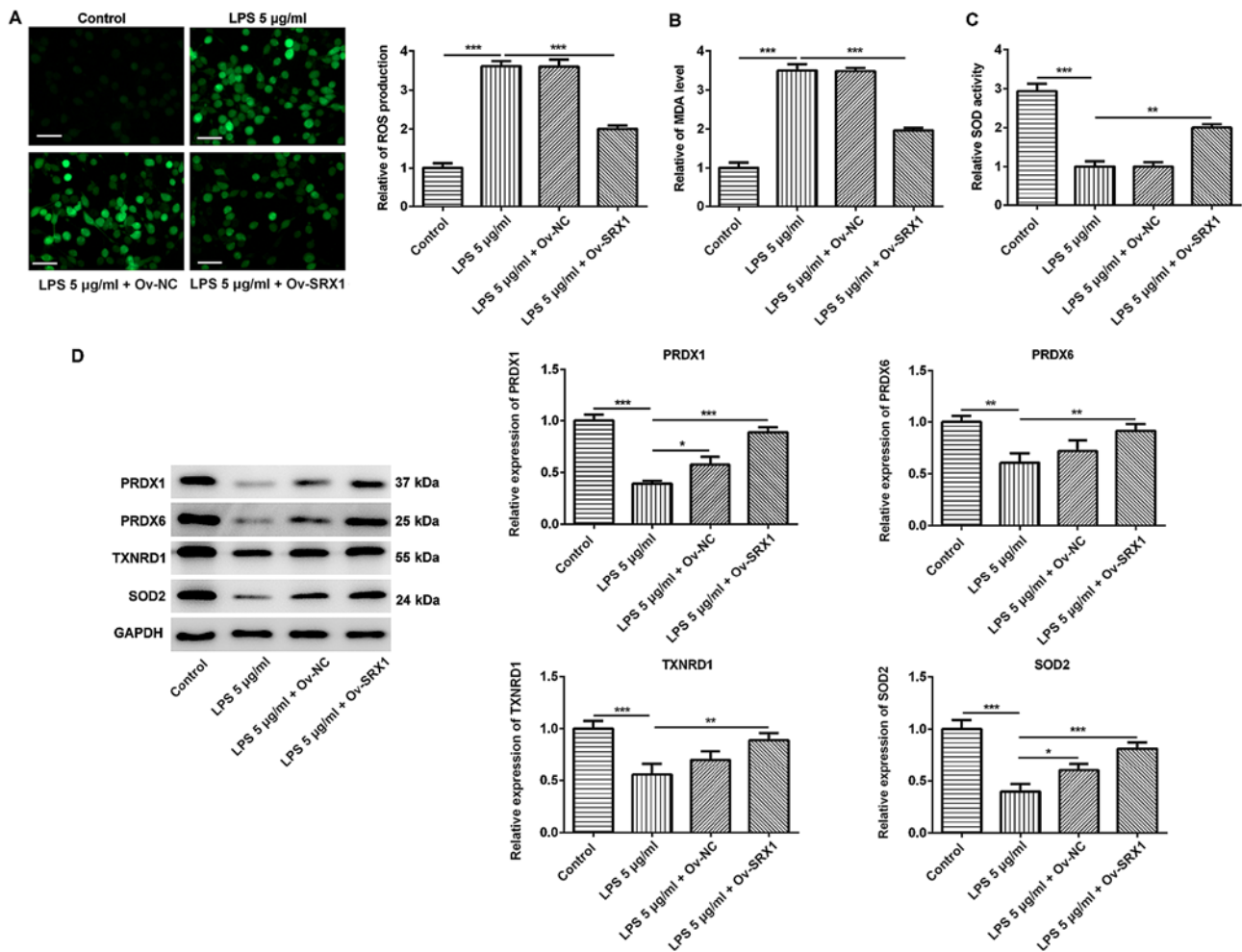


Figure 4. Overexpression of SRX1 relieves oxidative stress in PC12 cells. (A) Intracellular ROS in PC12 cells transfected with Ov-SRX1 and stimulated with 5 μ g/ml LPS was stained using 2',7'-dichlorofluorescein. Scale bar, 50 μ m. (B) MDA content and (C) SOD activity in PC12 cells transfected with Ov-SRX1 and stimulated with 5 μ g/ml LPS were analyzed using commercial detection kits. (D) Protein expression levels of antioxidative proteins PRDX1, PRDX6, TXNRD1 and SOD2 in PC12 cells transfected with Ov-SRX1 and stimulated with 5 μ g/ml LPS were analyzed using western blotting. Data are expressed as the mean \pm SD from 3 independent experiments. * P <0.05, ** P <0.01, *** P <0.001. SRX1, sulfiredoxin-1; LPS, lipopolysaccharide; Ov, overexpression; NC, negative control; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; PRDX, peroxiredoxin; TXNRD1, thioredoxin reductase 1.

proteins was observed in cells transfected with the Ov-SRX1 plasmid and stimulated with LPS compared with LPS treatment alone (Fig. 4D). Altogether, these findings suggested that SRX1 may exert an antioxidative role in injured PC12 cells.

NRF2 controls the expression levels of downstream target genes and SRX1. A previous study indicated that SRX1 exerted an antioxidative role, which relied on the activation of NRF2. SRX1 was discovered to protect neurons from ischemia/reperfusion-induced oxidative stress injury, which was regulated by NRF2 (31). The expression levels of NRF2 and its downstream target proteins NQO1 and HO-1 were all significantly downregulated in PC12 cells exposed to LPS compared with the control group (Fig. 5A), suggesting that NRF2 may participate in the neuronal damage triggered by LPS.

Subsequently, the NRF2 inducer, TBHQ, and inhibitor, ML385, were used to confirm the regulatory effects of NRF2 on SRX1. The mRNA expression levels of SRX1, NQO1 and HO-1 were then analyzed using RT-qPCR; TBHQ significantly reversed the suppressive effects of LPS on the expression levels of SRX1, NQO1 and HO-1, while ML385 enhanced the

inhibitory effects of LPS treatment (Fig. 5B). In addition, the protein expression levels of SRX1, NQO1 and HO-1, as well as nuclear NRF2, were significantly upregulated following the co-treatment of TBHQ and LPS compared to LPS treatment alone (Fig. 5C). However, ML385 treatment further downregulated the protein expression levels of SRX1, NQO1, HO-1 and NRF2 in PC12 cells exposed to LPS compared with LPS treatment alone. Overall, these findings suggested that the alleviation of the inflammatory response and oxidative stress by SRX1 may be modulated by NRF2.

Discussion

SCI is the most serious complication of spinal injury and often leads to severe dysfunction of the limb below the injury segment, which brings devastating physical and psychological harm to patients and imposes huge economic burdens to society (32). Currently, there are >1,000,000 patients with SCI in the United States and >12,000 new cases annually (33). Therefore, it remains an urgent requirement to determine effective therapeutic strategies for SCI. Inflammation and

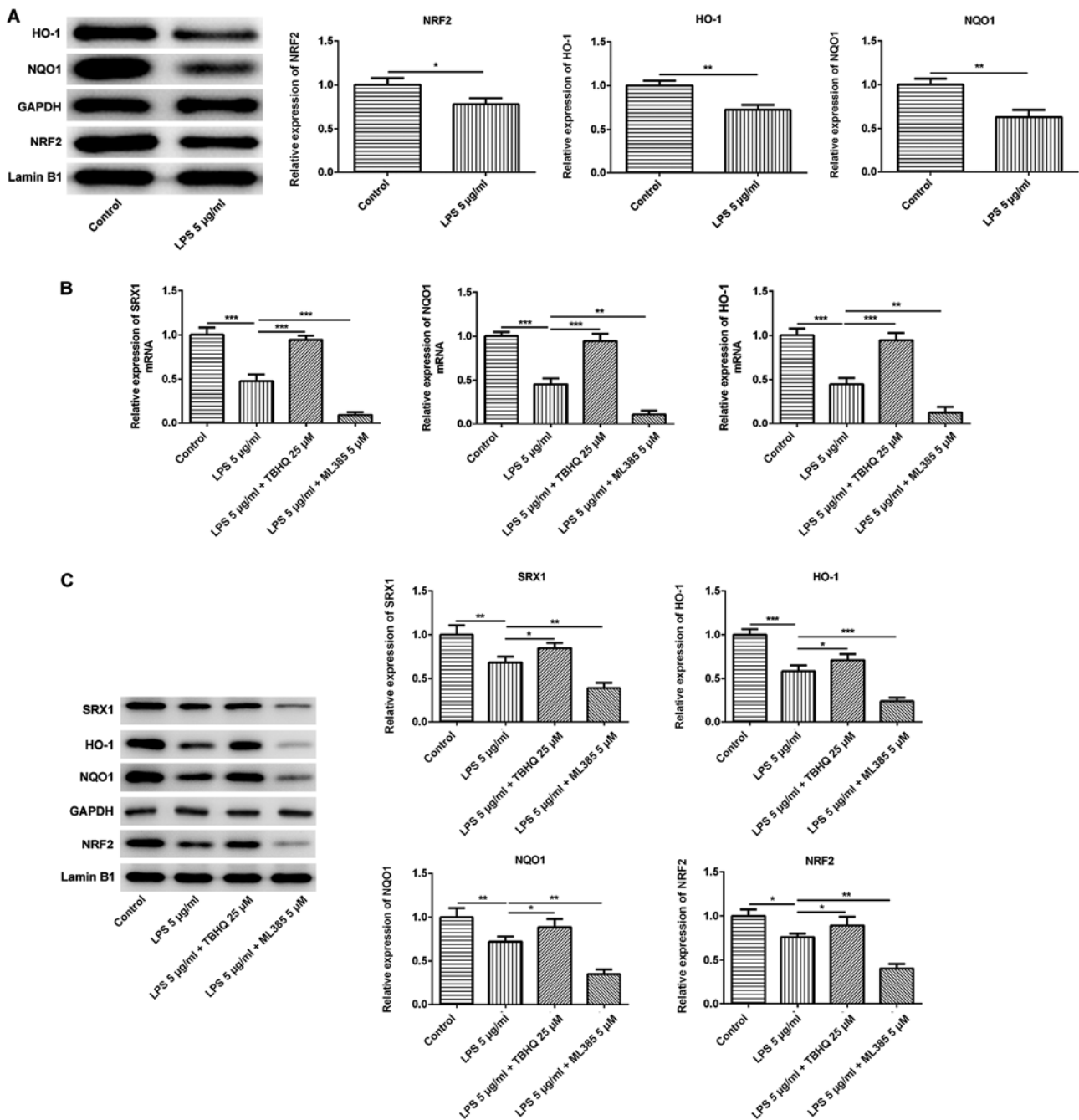


Figure 5. NRF2 controls the expression levels of downstream target genes and SRX1. (A) Expression levels of nuclear NRF2 and downstream target genes NQO1 and HO-1 in PC12 cells stimulated with 5 µg/ml LPS were analyzed using western blotting. (B) mRNA expression levels of SRX1, NQO1 and HO-1 in PC12 cells stimulated with 5 µg/ml LPS with or without 25 µM TBHQ or 5 µM ML385 treatment were determined using reverse transcription-quantitative PCR. (C) Western blotting analysis was performed to analyze the expression levels of nuclear NRF2, NQO1, HO-1 and SRX1 in PC12 cells stimulated with 5 µg/ml LPS with or without 25 µM TBHQ or 5 µM ML385 treatment. Data are expressed as the mean ± SD from 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001. SRX1, sulfiredoxin-1; LPS, lipopolysaccharide; Ov, overexpression; NC, negative control; NRF2, nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase 1; NQO1, NAD(P)H dehydrogenase quinone 1; TBHQ, tert-butylhydroquinone.

oxidative stress in neuron-like cells are commonly considered pathological alterations of SCI (10,11). However, the precise regulatory mechanisms of inflammation and oxidative stress in SCI remain poorly understood.

In the present study, SCI model rats were established by surgery in an aseptic environment. A large amount of cavities and decreased Nissl bodies were observed in the injured spinal cords of the model group compared with the sham group. In addition, the upregulated expression levels of SRX1 identified in

the spinal cord tissues were of great significance in the present study. A previous study illustrated that SRX1 protected against cardiomyocyte injury upon simulated ischemia/reperfusion by inhibiting mitochondrial apoptosis (19). SRX1 was also discovered to relieve apoptosis and oxidative stress in primary rat cortical astrocytes stimulated by oxygen-glucose deprivation or H₂O₂, which involved the activation of the mitochondrial apoptotic pathway (34). However, to the best of our knowledge, the definite role of SRX1 in SCI has remained elusive.

LPS, which is composed of lipids and polysaccharides, is a constituent of the outer cell wall of Gram-negative bacteria and typically induces oxidative stress and an inflammatory response in cells (35,36). In the present study, the mRNA and protein expression levels of SRX1 were downregulated in PC12 cells exposed to 0.625–5 µg/ml LPS in a dose-dependent manner. Therefore, to determine the role of SRX1 in SCI, SRX1 was overexpressed in PC12 cells. The secretion of the inflammatory cytokines TNF-α, IL-1β and IL-18 was reduced to differing degrees following the transfection of Ov-SRX1 and LPS stimulation, while the levels of IL-10 demonstrated the opposite trend.

In addition, the levels of oxidative stress were analyzed in PC12 cells transfected with Ov-SRX1 and stimulated with LPS; ROS production and MDA content were both inhibited following the overexpression of SRX1 and stimulation of LPS, whereas SOD activity was increased. ROS scavenging heavily relies on manganese-dependent SOD, a mitochondrial enzyme encoded by the SOD2 gene (37). PRDX1 and PRDX6 are members of the PRDX family that reduce the oxidative load and remove ROS (38,39), thus serving an important role in the antioxidant processes. In addition, TXNRD1 is an antioxidant enzyme, which is involved in antioxidant defense and redox regulation (40). SOD, PRDXs, glutathione and catalase are major ROS detoxification enzymes (14). In the current study, the production of glutathione and catalase was not investigated, thus future work should aim to investigate this. NRF2 is known to stimulate anti-inflammatory effects and redox homeostasis, conferring resistance to oxidative damage induced by exogenous chemicals and thereby promoting cell survival (41). SRX1 was identified as a pivotal NRF2-regulated gene responsible for the defense against oxidative injury in the lung induced by cigarette smoke (15). Another study demonstrated that SRX1 protected human cardiac stem/progenitor cells against oxidative stress-induced apoptosis through the activation of the ERK/NRF2 signaling pathway (18). NRF2 has also been reported to activate the antioxidant response element-dependent gene expression of HO-1, NQO1 and SOD through evading Kelch-like ECH-associated protein 1 (KEAP1)-mediated ubiquitination-proteasomal degradation and subsequent nuclear translocation (42). Thus, the precise regulatory mechanism between SRX1 and NRF2 in damaged PC12 cells was investigated in the present study. The results indicated that LPS reduced the expression levels of NRF2 in the nucleus, as well as the transcription and translation of the downstream target genes, NQO1 and HO-1. Conversely, the activation of NRF2 with TBHQ reduced the impact of LPS, while the inhibition of NRF2 with ML385 aggravated the effect caused by LPS, suggesting that the neuroprotective effect of SRX1 on PC12 cells challenged by LPS may depend on NRF2.

In conclusion, the findings of the present study discovered that the expression levels of SRX1 were downregulated in the injured spinal cord of rats. In addition, the data suggested that SRX1 may alleviate the inflammatory response and oxidative stress in neuron-like cells, which was dependent on the nuclear translocation of NRF2, providing a novel therapeutic target for nervous system damage. Neuron-like PC12 cells are commonly used to investigate neuronal damage resulting from spinal cord injury (22–24,43,44); however, primary spinal neurons derived from the rat spinal cord may be more appropriate for the study

of SCI and should be used in further investigations. Moreover, the influence of SRX1-related drugs on the SCI model rats and the influence of the ubiquitination of KEAP1 on NRF2 activation should be further studied to determine the functional recovery of the rats. Further studies addressing these limitations will help to validate the conclusions of the present study.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZW and ZL made substantial contributions to conception and design of the study, JO and XS analyzed and interpreted data, and JL conducted the experiments and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Ethics Committees of University of South China (Hengyang, China) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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