Original Article
Pterostilbene protects against traumatic spinal cord injury via inhibiting inflammation and oxidative stress

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Abstract: To evaluate the protective role of pterostilbene (PTE) on spinal cord injury (SCI) in SD rats. SD rats were randomly divided into three groups: sham group (laminectomy only); SCI group; SCI + PTE (15 mg/kg) group. SCI was induced using the modified weight-drop method (10 g × 4 cm) at the 10th thoracic vertebral (T10) level. PTE (15 mg/kg) or vehicle was administered twice by subarachnoid injection at lumbar 4 level twenty minutes and two hours after SCI. After 4 weeks of SCI, the injury part of the spinal cord was removed for further experiments. To evaluate the oxidative stress, we detected the levels of MDA and 8-OHdG. In addition, cellular ROS levels were analyzed using the ROS fluorescent probe dihydroethidium (DHE). The expression levels of iNOS, COX-2, and nuclear factor-κB p65 were detected using Western blot. Moreover, expression of levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and HO-1 were detected using western blot. We observed a significant enhancement in oxidative stress and inflammation in rats with SCI injury. Pterostilbene treatment significantly attenuated the oxidative stress and inflammation in SCI rats. In conclusion, our findings suggest that pterostilbene significantly ameliorated spinal cord injury by mitigating oxidative stress and inflammation possibly via Nrf2/HO-1 signaling activation.

Keywords: Traumatic spinal cord injury, pterostilbene, inflammation, oxidative stress

Introduction

It has been reported that the estimated incidence of traumatic spinal cord injury (SCI) was 54 cases per 1 million population based on 3393 cases in 1993 in the United States [1]. Traumatic spinal cord injury is still a worldwide problem, resulting in disability and consuming a large amount of medical resources. Two processes are involved in the traumatic SCI. Mechanical trauma is the first damage to the spinal cord, which immediately leads to progressive morphologic changes, such as shear, stretch, laceration and more commonly contusion and compression on the spinal cord. These morphologic changes cause pathological changes, including the initial hemorrhage and necrosis in the gray matter [2, 3]. After mechanical injury to the spinal cord, the secondary injury follows, involving ischemia, edema, excitotoxicity, inflammatory response, free radical generation, lipid peroxidation, axon demyelination, neuronal loss, and cell apoptosis [4, 5]. The secondary injury is critical for the treat-

ment and prognosis of SCI; therefore, attenuation of secondary injury is a therapeutic target for the treatment of SCI.

Many researches have suggested that SCI in the acute period is characterized by the aggregation and activation of various inflammatory cells in the injured tissue. In rodent models, resident astrocytes and microglia can be seen as early as 2 h following injury and persist up to 6months [6]. Human studies have shown that neutrophils arrive as early as 4 h post injury; activated microglia were found at 1 day post injury, and macrophages were seen by day 5 [7]. In the meantime, pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, produced by inflammatory cells are also involved in the whole process of inflammation [8].

Additionally, oxidative stress, which could be defined as an imbalance between reactive oxygen species (ROS) and anti-oxidation, is also involved in the SCI. Studies have demonstrated that oxidative stress markers such as malondi-
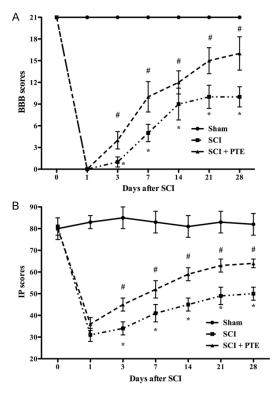
Figure 1. PTE treatment improved motor function after SCI. A. Open field assessment before and after SCI. B. The inclined-plane test before and after SCI. $^{*}P < 0.05$ vs. the Sham group, $^{#}P < 0.05$ vs. the SCI group, n = 6 for each group.

aldehyde (MDA) and advanced oxidation protein products (AOPP) increased and antioxidants such as glutathione (GSH) peroxidase, superoxide dismutase (SOD), catalase (CAT) and reduced GSH decreased [9, 10]. Oxidative stress contributes to tissue injury during a post-traumatic inflammatory response and cell death [11]. Therefore, targeted therapy against inflammation and oxidative stress has received more and more attention not only in clinical research of SCI but also in basic research of SCI.

Pterostilbene (PTE), a natural stilbene, primarily exists in blueberries, grapevines and heartwood of red sandalwood [12]. Several studies have indicated that PTE exhibits strong anti-inflammatory and antioxidant activities [13-17]. And PTE has been shown to exert beneficial effects against CNS diseases by altering several molecular targets [18]. However, there is still little research on whether PTE has neuro-protective effects against traumatic SCI.

Therefore, the aim of this study is to explore the protective roles of PTE in traumatic SCI and the potential mechanisms.

Materials and methods

Reagents

PTE, malondialdehyde (MDA) assay kit, dihydro-ethidium (DHE), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ELISA kits for measuring 8-hydroxy-2-deoxyguanosine (8-OHdG) was purchased from Cell Biolabs (San Diego, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime (Shanghai, China).

Animals

Seventy-two adult, male Sprague-Dawley rats (250-300 g) were used in this study. The rats were provided by the Experimental Animal Center of Guangxi Medical University. The animals were kept under standard housing conditions of 18-21°C room temperature, 65% humidity, and a 12-h light-dark cycle. They were allowed free access to food and water.

Spinal cord injury

SCI was induced as described previously [19, 20]. In brief, rats were anesthetized with pentobarbital (50 mg/kg i.p.) and received a laminectomy at the T10 (10th thoracic vertebra) level. After the spine was immobilized stereotaxically, a moderate SCI was triggered by dropping a weight of 10 g from a height of 4 cm onto an impounder (diameter, 0.2 cm) gently placed on the spinal cord.

Drug administration

PTE was first dissolved in DMSO and then diluted in 0.9% NaCl solution. A second laminectomy was performed at lumbar level 4 after SCI induction, as described previously [21]. PTE was administered into the subarachnoid space twice (20 min and 2 h after SCI induction) using an insulin syringe with a 29-gauge needle. Each injection was delivered over a 5-min period to ensure optimal delivery. The SCI group was administered the same volume of vehicle as the manner mentioned above.
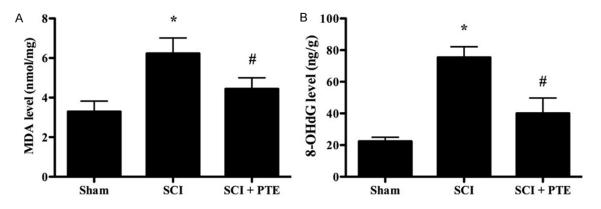
Figure 2. PTE treatment reduced MDA and 8-OHdG levels after SCI. A. MDA level. B. 8-OHdG level. *$P < 0.05$ vs. the Sham group, #$P < 0.05$ vs. the SCI group, n = 6 for each group.
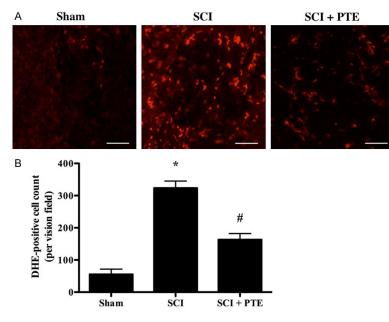

Figure 3. PTE treatment decreased ROS production after SCI. A. Representative confocal images of DHE staining. Scale bar = 50 μm. B. The histogram shows the percentages of DHE-positive cells. *$P < 0.05$ vs. the Sham group, #$P < 0.05$ vs. the SCI group, n = 6 for each group.

In addition, the rats' ability to maintain postural stability was evaluated using an inclined-plane test as previously described [23]. The rats were placed on an inclined plane, and the maximum inclination at which the rat could maintain its position for 5 s was recorded as the final angle, which was recorded IP scores.

Measurement of MDA and 8-OHdG levels

The animals were killed 7 d after SCI induction, and tissue homogenates were prepared for the detection of MDA and 8-OHdG levels. MDA levels were tested according to manufacturer instructions. The levels of 8-OHdG were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the instructions of the manufacturer.

ROS production assessment

Cellular ROS production was analyzed using the ROS fluorescent probe DHE 7 d after SCI induction. Rats were transcardially perfused with 50 mL ice-cold 0.1 M phosphate-buffered saline (PBS; pH 7.4) after being anesthetized. The spinal cords around the injury site were quickly removed and frozen at -80°C for 20 min, then

Behavioral test

The following tests were performed before and 1, 3, 7, 14, 21, 28 days after SCI induction by two examiners who are blind to the experimental groups. The open-field test assessed the movement, weight support, and coordination of the rats, and the results were scored using the BBB locomotor rating scale as previously reported [22]. The rats were allowed to walk around freely in a circular field for 4 min and the movements of the hind limbs were observed, and the results were recorded as BBB scores.
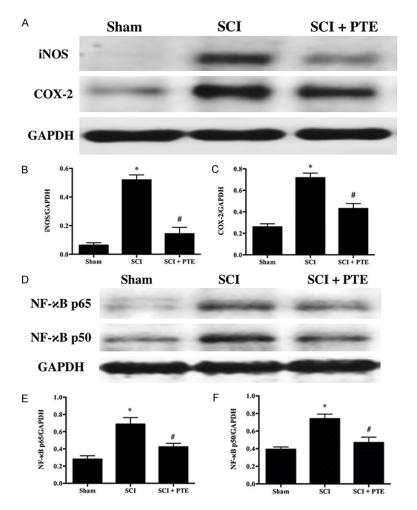
Figure 4. PTE treatment reduced inflammatory reaction after SCI. A. The expression levels of iNOS and COX-2 detected by Western blot. B. The relative level of iNOS. C. The relative level of COX-2. D. The expression levels of NF-κB p65 and NF-κB p50 detected by Western blot. E. The relative level of NF-κB p65. F. The relative level of NF-κB p50. *$P < 0.05$ vs. the Sham group, #$P < 0.05$ vs. the SCI group, n = 6 for each group.

blocked in 5% nonfat milk for 1 h at room temperature. Then, the membranes were incubated overnight at 4°C with rabbit anti-iNOS (Abcam, 1:800), rabbit anti-COX-2 (Abcam, 1:500), rabbit anti-NF-κB p65 (Abcam, 1:2000), rabbit anti-NF-κB p50 (Santa Cruz Biotechnology, 1:1000), rabbit anti-Nrf2 (Abcam, 1:1000), mouse anti-HO-1 (Abcam, 1:250), and rabbit anti-GAPDH (Abcam, 1:2,500) primary antibodies, and then incubated with horse- radish peroxidase-conjugated secondary antibody (1:5,000) for 1 h at room temperature. The protein bands were detected and quantified by the Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were presented as the mean ± SEM and analyzed using SPSS V.13.0 (SPSS, Chicago, IL, USA). The statistical significance of differences between the values was determined using an ANOVA followed by Bonferroni multiple comparisons test. $P < 0.05$ was considered statistically significant.

they were quickly sliced into 15-μm-thick coronal sections by suing a freezing microtome. The sections were then mounted onto polylysine-coated slides. After a 30-min incubation in DHE (10 μmol/L), the slices were observed and photographed under a laser confocal microscope.

Western blot

After 7 days of SCI, the rats were killed as mentioned above. The injured spinal cord was then removed for Western blot analysis. The tissues were lysed, and the protein concentrations were determined using a BCA protein assay kit. Equal amounts of proteins (50 μg) were separated using 8-15% SDS-PAGE and transferred onto PDVF membranes. The membranes were

Results

PTE improved locomotor function after SCI

All the rats had an BBB score of 21 before SCI induction, and SCI resulted in a score of 0. However, one week after SCI induction, rats in SCI + PTE group showed significant improvement compared to the SCI group ($P < 0.05$) (**Figure 1A**). In addition, the angle in the inclined-plane test was approximately 80°. After SCI, the angle decreased significantly. From one week after SCI, PTE treatment dramatically increased the angle compared with the SCI group ($P < 0.05$) (**Figure 1B**). These results indicated that PTE treatment signifi-
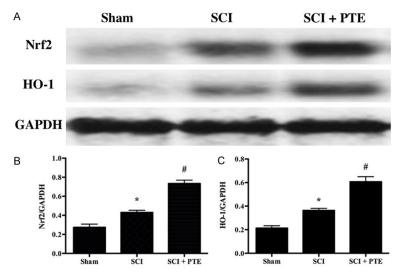
Figure 5. PTE treatment activated Nrf-2/HO-1 signaling after SCI. A. The expression levels of Nrf-2 and HO-1 detected by Western blot. B. The relative level of Nrf2. C. The relative level of HO-1. *$P < 0.05$ vs. the Sham group, #$P < 0.05$ vs. the SCI group, n = 6 for each group.

increased after SCI compared with the Sham group ($P < 0.05$). However, the increased expression was dramatically reduced by PTE treatment compared with the SCI group ($P < 0.05$). These results suggested that PTE exerted the protection against SCI via down-regulating inflammatory modulates in the spinal cord.

PTE activated Nrf2/HO-1 signaling pathway in the spinal cord of SCI rats

Finally, the effect of PTE on Nrf2/HO-1 signaling was evaluated. The results suggested that SCI up-regulated the expression of Nrf2 and HO-1. And the increased Nrf2 and HO-1 expression in the spinal cord after SCI was further increased by PTE treatment (**Figure 5**). These findings indicated that treatment with PTE activated the Nrf2 signaling pathway, leading to increased HO-1 expression in the spinal and contributing to its antioxidant activity.

Discussion

The present study indicates that PTE treatment administered by subarachnoid injection after SCI can dramatically improve neurological recovery in rats, as indicated by the increased scores of the BBB test and the inclined-plane test. In addition, PTE treatment significantly decreases oxidative stress inflammatory reaction, as evidenced by the decreased MDA and 8-OHdG levels in the spinal cord tissues, and the reduced expression levels of iNOS, COX-2, NF-κB p65, and NF-κB p50. More importantly, PTE treatment up-regulates expression of Nrf2 and HO-1, indicating that Nrf2/HO-1 signaling activation is involved in the protective effects of PTE. Our results suggest that PTE may be a promising drug for the treatment of SCI patients.

In the current study, we use a modified weight-drop method to induce the traumatic SCI. It has been suggested that locomotor loss after SCI involves interruption of descending spinal cord tracts, and recovery depends on those spared

cantly improved locomotor function recovery after SCI.

PTE reduced oxidative stress induced by SCI

Then, we investigated whether PTE treatment reduced oxidative stress post SCI. At 7 days post-SCI, the levels of MDA and 8-OHdG in the spinal cord tissue were determined. The results indicated that SCI induced a dramatic increase in MDA level, while PTE treatment markedly decreased MDA level compared with the SCI group ($P < 0.05$) (**Figure 2A**). Similarly, PTE treatment markedly decreased 8-OHdG level compared with the SCI group ($P < 0.05$) (**Figure 2B**). Additionally, we evaluated ROS production using DHE staining. The results revealed that the number of DHE-positive cells in the SCI + PTE group significantly decreased compared with the SCI group ($P < 0.05$) (**Figure 3**). These results indicated that PTE treatment significantly reduced oxidative stress induced SCI.

PTE down-regulated inflammatory modulates in the spinal cord of SCI rats

As inflammation is another important part in the pathogenesis of SCI, we investigated the effect of PTE on the expression levels of several inflammatory molecules (**Figure 4**). The results suggested that the expression levels of iNOS, COX-2, NF-κB p65, and NF-κB p50 significantly

descending fibers [24]. Our results suggest that BBB scores in the rats of the SCI group decrease dramatically 1 day after SCI induction, and increase gradually in the following days, suggesting that the spinal cord can repair itself to some extent. Notably, PTE treatment significantly help the recovery of locomotor function in the rats. The similar phenomenon is observed in the inclined-plane test. These results suggest that PTE treatment after SCI contributes to the recovery of locomotor function.

It has been reported that ischemia is induced after traumatic SCI, which is localized to the injury site [25]. Ischemia induces ATP loss, leading to the disruption of the mitochondrial respiratory chain. Consequently, large amounts of free radicals are produced and released into the injury tissue. Additionally, many inflammatory cells, including neutrophils and macrophages, are activated and can produce free radicals, contributing to oxidative stress after SCI. These free radicals trigger cell injury by inducing the peroxidation of polyunsaturated fatty acids on the biological membranes [26]. MDA level often reflects the degree of lipid peroxidation in tissue. Peroxidation takes place at the side chains of polyunsaturated fatty acids in biological membranes, which changes the fluidity and permeability of the cell membrane, ultimately resulting in loss of cell function. Moreover, 8-OHDG is one of the most sensitive DNA damage markers and is produced following hydroxyl radical induction during oxidative stress [27]. Our data suggests that PTE treatment after SCI dramatically decreases the levels of MDA, 8-OHDG, and ROS production.

Inflammation, another important process involved in the pathogenies of SCI, is a therapeutic target in the treatment of SCI. It has been indicated that inflammation can induce neurodegeneration after traumatic SCI [28]. In the current study, we evaluate the levels of iNOS, COX-2, NF-κB p65, and NF-κB p50. Our results suggest that PTE treatment decrease the expression of iNOS and COX-2. Moreover, PTE treatment reduced NF-κB activity (expression of NF-κB p65 and NF-κB p50). Taken together, PTE contributes to protection against SCI by diminishing iNOS, COX-2, and NF-κB expression, and the blocking of the NF-κB pathway might be associated with the anti-inflammatory effects of PTE.

Nrf2 (NF-E2-related factor 2) is a transcription factor that plays a key role in protecting cells from various stresses [29]. Nrf2 serves as a regulator of the antioxidant response, which is one of the most important molecules protecting against oxidative stress [30]. Moreover, Nrf2 plays a critical role as a negative regulator of inflammation [31]. The activation of Nrf2 can upregulate the expression of several anti-oxidative enzymes such as HO-1, superoxide dismutase 1 (SOD1), and glutathione S-transferase (GSTs) [32]. Previous studies have suggested that HO-1 is an inducible enzyme and has strong antioxidant and anti-inflammatory effects [33, 34]. Soy isoflavone can alleviate oxidative damage induced by beta-amyloid peptides 1-42 (Aβ1-42), which might result from the activation of HO-1 signaling [35]. In addition, aescin reduces oxidative stress in experimental traumatic spinal cord injury via HO-1 induction [36]. In this study, we investigate the effect of PTE on the Nrf2/HO-1 signaling. Our results indicate that the expression levels of Nrf2 and HO-1 increase after SCI, which are further up-regulated by PTE treatment. These results suggest that the neuroprotective effects of PTE are associated with the activation of Nrf2/HO-1 signaling pathway.

In summary, our findings suggest that PTE treatment exerts a profound neuroprotective effect against traumatic SCI. This protection appears to be largely due to the attenuation of oxidative stress, inflammation and the activation of Nrf2/HO-1 signaling. These data indicate that PTE may be a promising candidate for the treatment of traumatic SCI.

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Disclosure of conflict of interest

None.

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