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Celastrol inhibits microglial pyroptosis and attenuates inflammatory reaction in acute spinal cord injury rats

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ABSTRACT

Pyroptosis pathway is closely related to inflammation. However, Celastrol effect on pyroptosis pathway after spinal cord injury (SCI) are poorly understood. We studied the anti-inflammatory and neuroprotective effects of Celastrol on acute spinal cord injury in rats, and its anti-inflammatory effects on lipopolysaccharide (LPS)/ATP-induced microgliosis. Our results show that Celastrol can improve the recovery of hindlimb motor function after SCI in Sprague-Dawley (SD) rats, and reduce the cavity area of spinal cord injury along with the neuronal loss. Celastrol simultaneously reduced the activation of microglia (especially M1 microglia) in the spinal cord, inhibited the pyroptosis-related proteins (NLRP3 ASC Caspase-1 GSDMD), reduced the release of TNF-α IL-1β and IL-18 inflammatory factors, and increased the release of IL10 cytokines. In vitro studies showed that Celastrol reduced the toxicity resulting from the administration of LPS with ATP to BV-2 cells, inhibited the pyroptosis-related proteins (NLRP3 Caspase-1 GSDMD), and inhibited the release of corresponding inflammatory factors. Finally, Celastrol can inhibit the expression of NFκB/p-p65 in vitro and in vivo. Our results show that Celastrol can attenuate the inflammatory response of the spinal cord after SCI, which is associated with inhibition of microglial activation and pyroptosis pathway. Further study to explore the use of Celastrol to treat SCI is warranted.

1. Introduction

Traumatic spinal cord injury (SCI) is a severe and disabling trauma, which remains a challenging clinical problem [1,2]. SCI is largely a result of car accidents and falls at high altitudes [3]. Spinal cord injury can cause motor and neurological dysfunction, reducing quality of life and increasing the burden of social care. In developed countries, the incidence of SCI in the United States is 20.7–83 per million, and in Europe is 8.0–130.6 per million. Studies have shown that the incidence rate in China is similar to that in developed countries [4].

As a result of this drastic damage, the spinal cord undergoes a series of primary and secondary pathophysiological changes resulting from the trauma which includes shear stress, torsion, compression, and contusion. Primarily, the physical integrity of the spinal cord neurons and nerve fibers is destroyed and the continuity of the spinal cord interrupted. Cell edema, tissue ischemia, inflammation, intracellular ion homeostasis imbalance, apoptosis and other secondary changes may increase the degree of acute SCI, and contribute to the neurological dysfunction after injury [5]. Since the primary injury to the spinal nerve structure is difficult to reverse, current research focuses on the interventions to the “secondary injury” and aims to reduce the inflammatory response, reduce the necrosis and apoptosis of neurons, and to reduce further expansion of the lesion area, thereby creating a favorable microenvironment for axonal regeneration.

Neuroinflammation plays a key role in the secondary injury of acute SCI [6]. The cascade of secondary damage causes the inflammatory reaction at the lesion site to enlarge. While inflammation at the early stages is beneficial as well as harmful, extensive research has shown that inflammation can spread to surrounding tissues, leading to cell death, while inhibiting spontaneous regeneration and functional recovery of tissues [7].

Recent studies have shown that the activation of the inflammatory body pathway promotes cell pyroptosis, resulting in the release of a large number of inflammatory factors [8,9]. As an innate immune cell of the central nervous system, microglia can participate in inflammatory reactions through pyroptosis [10,11]. Pyroptosis, a specific form of cell death, is mainly mediated by caspase-1 and Gasdermin-d (GSDMD). It is a newly discovered programmed cell death and is accompanied by the production of various inflammatory mediators including interleukin-1β (IL-1β), IL-18 [12–14]. During pyroptosis, the cysteine protease-1 precursor (Pro-Caspase-1) may be indirectly linked to the pattern recognition receptor through the linker protein ASC

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(Apoptosis-associated speck-like protein contain a CARD) to form a macromolecular complex designated as an inflammasome. The inflammasome activates and cleaves Pro-Caspase-1 to form active caspase-1. Caspase-1 can cleave GSDMD protein to form active N-terminus and C-terminus moieties. The N-terminus product promotes cell membrane perforation and cell death, and expands the inflammatory response [15,16].

Microglial cells are the innate immune cells of the central nervous system (CNS) and serve an important role in mediating neuroinflammation [17,18]. They are heavily activated upon damage to the spinal cord [19]. SCI also produces many secretory factors (such as cytokines) that can bind cell surface receptors to further activate microglial cells and cause them to participate in cellular signaling pathways. These signaling pathways constitute a potent proinflammatory response that amplifies inflammation and aggravates secondary damage [20]. Therefore, controlling the activation of microglia, and the resultant production of inflammatory factors, may contribute to the treatment of SCI.

Celastrol is also known as phatosporine and is a triterpenoid methyl triterpene. It is widely distributed in the botanical family Cervidaceae, and is an important active ingredient present in Tripterygium wilfordii, where it was the first natural product isolated from the root bark. Studies in the past show that Celastrol has many beneficial biological effects such as anti-oxidation, anti-apoptosis, anti-inflammation, anti-cancer, and anti-obesity [21–24]. It has been used in the treatment of cerebral ischemia and Parkinson's disease, where its anti-inflammatory effects have been confirmed in the CNS [25,26]. However, the specific mechanism of the effects of Celastrol on SCI remains unclear.

We hypothesize that Celastrol reduces inflammation resulting from SCI by regulating glial activation and pyroptosis. Our results may provide new therapeutic targets and explain aspects of the scientific mechanisms involved in the treatment of SCI acute-phase inflammation.

2. Materials and methods

2.1. Animals

8-week-old female Sprague-Dawley (SD) rats weighing 220–250 g were purchased from the Animal Center of Chinese Academy of Sciences, Shanghai, China. All experimental operations and animal husbandry were approved by the institutional review board of the University of Wenzhou Medical University.

2.2. Spine cord injury model constructing and grouping

A total of 72 rats were randomly divided into 3 groups including sham group, SCI group, and SCI + Celastrol group. Rat SCI model was generated according to the method published by Rivlin [27]. Before surgery, we anesthetized rats with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The T8-T10 lamina was removed after spinal exposure and the spinal cord was fully exposed. The spinal cord was squeezed with a vascular clip (30 g forces, Oscar, Shanghai, China) for 1 min. The sham group underwent the same surgical procedure, except that the spinal cord was not damaged. Successful generation of an SCI model required manipulation of the rat's bladder to facilitate manual urination twice a day. Celastrol was dissolved in 0.9% sodium chloride (NaCI) containing 1% dimethyl sulfoxide. Postoperative rats were immediately treated with intraperitoneal injection of Celastrol, with subsequent Celastrol (1 mg/kg meilunbo, China) [28,29] being administered daily by intraperitoneal injection until sacrifice. In the control group and SCI group, an equal volume of vehicle was injected. And animal experiments were independently performed at least three times.

2.3. Locomotion recovery assessment

Rat hindlimb function was evaluated according to Basso Beattie Bresnahan (BBB) scores [30] and an inclined plane test [31]. The test time points included 0, 1, 7, 14 and 21 days in this experiment. The BBB scoring method assesses the hindlimb movement of rats on a scale of 0 to 21, with a total of 22 grades. A score of 0 indicates that the hind limbs have no exercise capacity, and 21 points indicate that the hindlimbs have a completely normal motor function. The inclined plane test was also used at the corresponding point in time. The capacity of the rat to stay on top of an angled plane for 5 s without dropping was recorded, and the maximum angle recorded. Three measurements were taken to obtain an average value for each rat. Three trained examiners who were blinded to the experimental conditions independently performed the tests to obtain the score.

2.4. Hematoxylin-Eosin (HE) and Nissl staining

On the 7th day after model establishment, rats were anesthetized with 2% sodium pentobarbital, the chest was opened. Cardiac perfusion was performed using physiological saline, followed by cardiac perfusion performed with 4% paraformaldehyde (Solarbio, China). And the length of the lesion centered at 1 cm was collected. The spinal cord was fixed in 4% paraformaldehyde fixative for 24 h, rinsed overnight with running water, subjected to stepwise dehydration with ethanol, rendered transparent in xylene, embedded in paraffin, and then serially sectioned (thickness 5 μm) for routine HE staining (Beyotime, China) [30] or Nissl staining (Solarbio, China) [30]. Stained sections were observed under an optical microscope (BX53, Olympus, Japan). The number of neurons containing Nissl bodies was counted.

2.5. Cell culture and drug treatment

BV-2 microglial cells were purchased from the Cell Storage Center of otwo (Guangzhou, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) with 10% fetal bovine serum (Gibco, Grand Island, NY) and 1% streptomycin/penicillin (Gibco, Grand Island, NY) and maintained at 37 °C under 5% CO_2. Prior to the experiment, cells were plated into 96-well plates at a density of 0.5×10^5 or plated into 6-well plates at 2×10^5 densities. Celastrol was dissolved in cell culture medium containing 0.1% dimethyl sulfoxide. After culturing for 24 h, Celastrol (100 nM) was first added for 1 h, then LPS (100 ng/ml Sigma, USA) was added for 24 h, and finally, ATP (1 mM Sigma, USA) was added for 3 h [32–34]. Cell experiments were independently performed at least three times.

2.6. Cell viability measurements

Prior to the experiment, cells were plated in 96-well plates at a density of 0.5×10^5, and 100 μl of cell suspension was added to each well and incubated in the incubator for 24 h. Cells were treated with different concentrations of Celastrol (50 mM, 100 mM, 200 mM) for 1 h, then LPS (100 ng/ml) was added for 24 h, and finally, ATP (1 mM) was added for 3 h. Cell viability was then measured with Cell Counting Kit-8 (CCK-8 Dojindo, Japan). After the cells were treated as described above, 10 μl of CCK-8 solution was added to each well and the plates were incubated in the incubator for 2 h. Finally, the absorbance was measured at 450 nm with a microplate reader (SpectraMax® Plus384, Molecular Devices, USA).

2.7. Immunoblotting

Spinal cord tissue was dissected 0.5 cm above and below the injury site, and RIPA lysis buffer (containing PMSF; Beyotime Biotechnology, China) was added and the tissue homogenized with a homogenizer. The supernatant was collected by centrifugation (Sorvall Legend Micro 21,

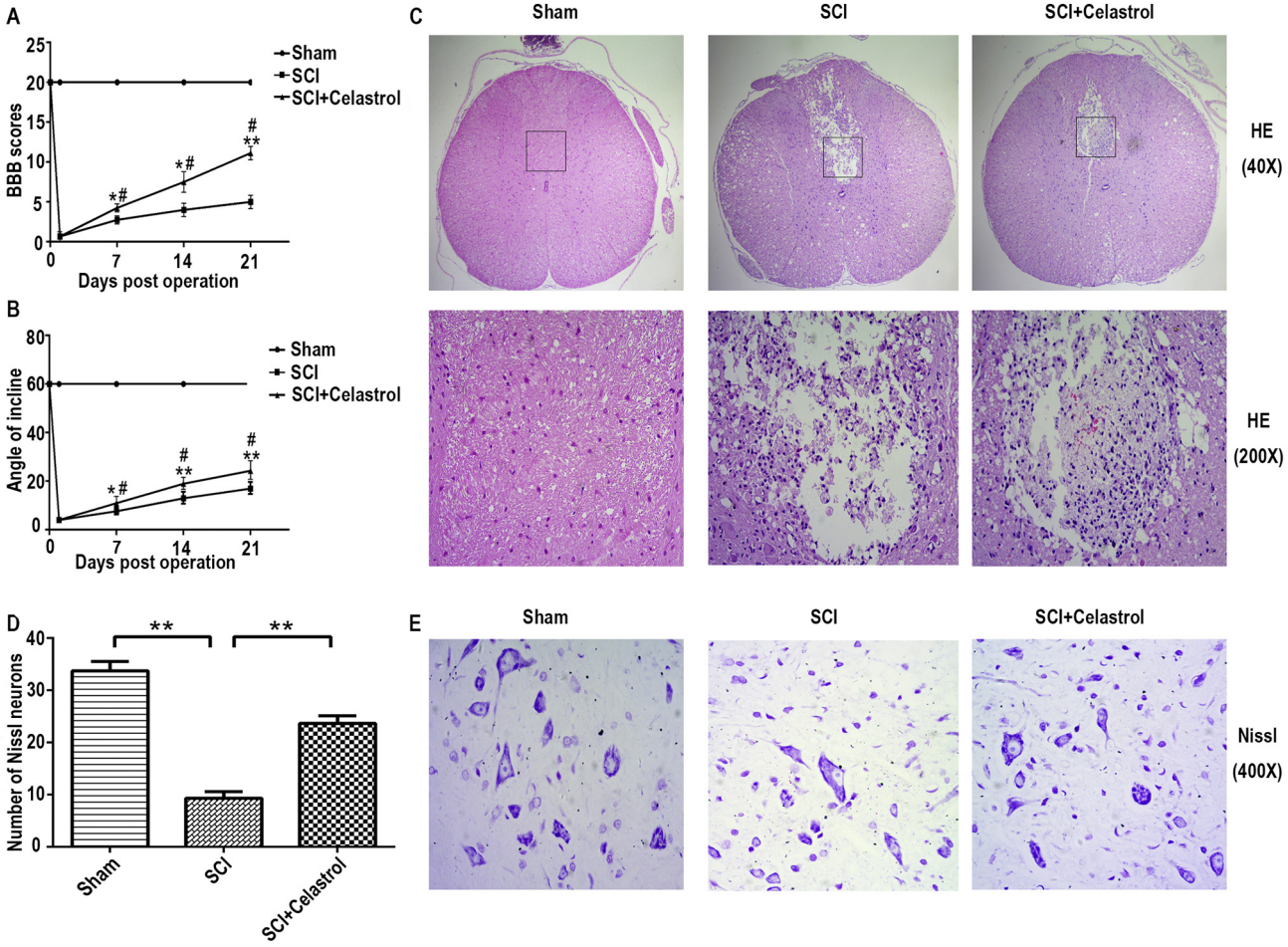

Fig. 1. Celastrol reduces motor neuron loss and promotes recovery of hindlimb motor function in SCI rats.
(A and B) The BBB scores and angles of inclined plane at 0, 1, 7, 14, and 21 days after SCI were shown for all groups of rats. (C) In the 3 groups, the representative sections were stained with HE on the 7th day after spinal cord injury. (D) 7 days after spinal cord injury in rats, the number of surviving neurons was counted after Nissl staining. (E) Representative Nissl staining of spinal motor neurons on day 7 after SCI. *P < 0.05, **P < 0.01, compared with SCI group, n = 6 per group, #P < 0.01, Compared with the previous time. And the data comes from three independent experiments.
SCI: spinal cord injury; BBB scores: The Basso, Beattie and Bresnahan scores; HE: Hematoxylin-Eosin.

Thermo Fisher Scientific, USA) at 12000 rpm for 30 min at 4 °C. For cultured cells, following a similar protocol, cells were lysed with RIPA lysis buffer on ice for 30 min, and the whole cell extracts were obtained after the supernatant was collected by centrifugation at 12000 rpm for 20 min at 4 °C. The protein concentration in the supernatant was measured with a BCA Protein Assay Kit (Thermo Fisher Scientific, USA). SDS-PAGE loading buffer was added to the supernatant, and heated at 100 °C for 5 min. A normalized amount of protein (30 μg) was loaded on 10% SDS-PAGE gels for electrophoresis. The proteins were then transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). The membrane was blocked with skimmed milk powder at room temperature for 2 h. then incubated overnight with the following primary antibody: rabbit anti-Pro-Caspase-1 (1:1000 Abcam, UK), rabbit anti-Caspase-1 (1:500 Affinity Biosciences, USA), rabbit anti-NLRP3 (1:1000 Abcam, UK), rabbit anti-GSDMD (1:500 Bioworld, USA), rabbit anti- ASC (1:500 Affinity Biosciences, USA), rabbit anti-NF-κB (1:1000 Cell Signaling, USA), rabbit anti-P-NF-κB (1:1000 Cell Signaling, USA), rabbit anti-β-Actin (1:1000 Cell Signaling, USA) at 4 °C. After washing with Tris Buffered saline Tween (TBST) 3 times (10 min each), the membrane was incubated with a secondary antibody room temperature for 1 h, and again washed with TBST 3 times (10 min each). After enhanced chemiluminescence (ECL; Thermo Fisher Scientific, USA) was added, proteins on the membrane were detected by ECL Western blotting detection system (Thermo Fisher Scientific, USA) and gray value analysis performed.

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA (3 μg) was extracted from cultured cells or spinal cord tissue using TRIzol reagent (ThermoFisher Scientific, Waltham, MA USA), according to the manufacturer's instructions. RNA was quantified by determining the absorbance at 260/280 nm using a spectrophotometer. Reverse transcription was performed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA USA). A sample of cDNA was used to quantify gene expression by qPCR using an SYBR Green (BioRad, California, USA)–based PCR reaction mixture on a CFX96™ Real-Time system. The amount of mRNA for each gene was normalized by β-Actin, and the relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The sequences of all primers used for the real-time quantitative PCR experiments is as follows: Iba-1 (Ionized calcium-binding adapter molecule 1) forward: ATGTCCTTGA AGCGAATGCTGGAG, reverse: AACGTCTCCTCGGAGCCACTG; iNOS (Inducible nitric oxide synthase) forward: GAGACGCACAGGCAGAGG TTG, reverse: CAGGAAGGCAGCAGGCACAC; Arg-1 (Arginase-1) forward: ACATCAACACTCCGCTGACAACC, reverse: GCCGATGTACACGA TGTCCTTGG; IL-10 (Interleukin 10) forward: AGCAAAGGCCATTCCA TCCG, reverse: CACTTGACTGAAGGCAGCCC; TNF-α (tumor necrosis factor α) forward: GCATGATCCGAGATGTGGAACTGG, reverse: CGCC

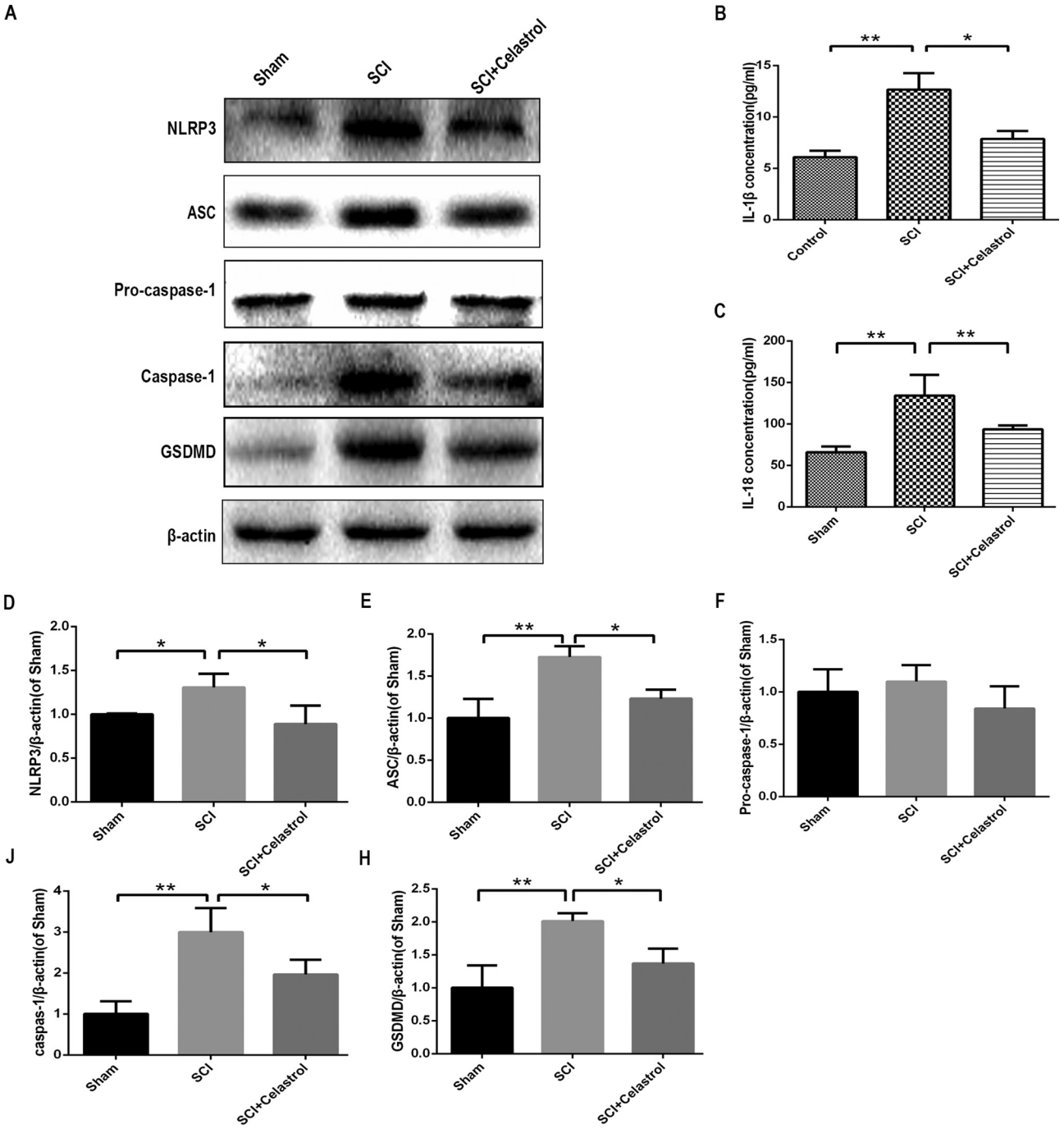

Fig. 2. Celastrol inhibits key proteins in the pyroptosis pathway, and reduces inflammation in the spinal cord at 7 days after spinal cord injury.
(A) Representative immunoblot bands for the NLRP3, ASC, Pro-Caspase-1, Caspase-1, and GSDMD proteins. β-actin was used as a loading control; (B–C) The expression levels of IL-1β and IL-18 in spinal cord tissue were detected by ELISA kit at 7 days after spinal cord injury. (D–H) Histogram of Relative Expression of NLRP3, ASC, Pro-Caspase-1, Caspase-1, and GSDMD; *P < 0.05, **P < 0.01 compared with SCI group, n = 6 per group. And the data comes from three independent experiments.
qRT-PCR: Quantitative Real-Time PCR.

ACGAGCAGGAATGAGAAG.

2.9. ELISA

Spinal cord tissue was dissected 0.5 cm above and below the site of injury, placed in pre-cooled PBS buffer, and homogenized. The supernatant was collected by centrifugation at 3000 revolutions/min (r/min) for 20 min at 4 °C. For cultured cells, the supernatant was collected after treatment under the same conditions as described above, and the supernatant was carefully collected by centrifugation at 2000 r/min for 20 min. The expression of IL-1β and IL-18 in the spinal cord homogenate and cell supernatant was quantitated according to the instructions of the ELISA kit (eBioscience USA), using a standard curve.

2.10. Statistical analysis

Experimental data were analyzed using SPSS 20.0 software and expressed as the mean ± SEM. Student *t*-test determined statistical

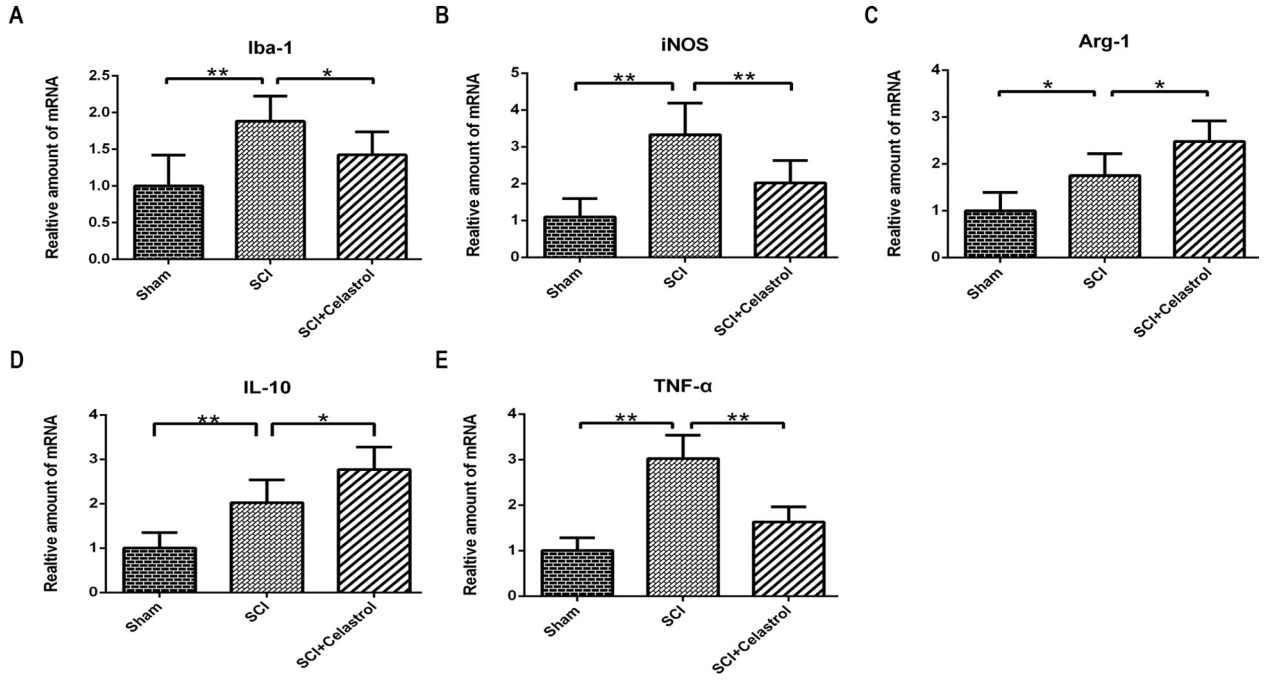

Fig. 3. Celastrol inhibits the activation of microglial.
qRT-PCR was used to detect microglia content and state of activation in the spinal cord on the 7th day after spinal cord injury. (A) iba-1 expression level in each group (microglia marker); (B) iNOS expression level in each group (M1 microglia marker); (C) Arg-1 expression level in each group (M2 microglia marker); (D) IL-10 expression level in each group; (E) TNF-α expression level in each group. *P < 0.05, **P < 0.01 compared with SCI group, n = 6 per group. And the data comes from three independent experiments.

significance for comparison between data from two experimental groups. For more than two group, statistical significance was determined with one-way analysis of variance (ANOVA) test, followed by Dunnett's LSD post hoc testing to calculate the longitudinal differences between groups. A value of P < 0.05 was considered statistically significant.

3. Results

3.1. Celastrol promotes recovery of hindlimb motor function in rats with spinal cord injury

In order to explore the therapeutic efficacy of Celastrol, we first established a rat SCI model. The functional recovery of the hind limbs of rats was measured with BBB scoring and an inclined plane test. As shown in Fig. 1, the treat group scored better than the SCI group in the BBB test (P < 0.05 or P < 0.01). Similarly, in the inclined plane test, the slope angle of the treat group was greater than the SCI group (P < 0.05 or P < 0.01). In addition, the scores of the BBB and inclined plane tests in the treat group, gradually increased as post-treatment time elapsed (1. 7. 14. 21 days) (P < 0.01). These data show that Celastrol can effectively promote the recovery of hindlimb motor function in SCI rats (Fig. 1).

3.2. Celastrol alleviates the severity of spinal cord injury in rats

To more comprehensively evaluate the protective effect of Celastrol on spinal cord injury, we performed HE staining and Nissl staining on adjacent paraffin sections of spinal cord. Normal spinal cord tissue structure was observed in the HE staining of the sham group, while the SCI group had a disordered spinal cord structure and cavity formation. In the treat group, the architecture of the spinal cord was better preserved. As seen in a Nissl stain, fewer neurons were lost in the treated group than in the SCI group, and this was quantitated by the counting of intact neurons located in the ventral horn (P < 0.01). The above data shows that Celastrol has a protective effect on spinal cord neurons (Fig. 1).

3.3. Celastrol inhibited the activation of microglia, inhibited the pyroptosis pathway and decreased the release of proinflammatory factors in SCI rats

To understand the specific mechanism of the protective effect of Celastrol on spinal cord in SCI rats, we measured the expression of Iba-1, iNOS, Arg-1, IL-10 and TNF-α by qRT-PCR. The data showed that compared with the sham group, the microglial cells in the SCI group increased significantly (P < 0.01), and the activation was significantly reduced in the presence of Celastrol (P < 0.05). The expression of iNOS, which is a marker of M1 microglia cells, was significantly increased in the SCI group (P < 0.01), and the expression of iNOS in the treat group was significantly decreased (P < 0.01). The expression of the marker Arg-1, which represents M2 microglia, was elevated in the SCI group (P < 0.05). And it also an increase in the treat group compared with the SCI group (P < 0.05). The level of inflammatory factor IL-10 in the SCI group was increased (P < 0.01). And IL-10 was further improved after drug treatment (P < 0.05). However, TNF-α was elevated in the SCI group and decreased in the treat group. Immunoblotting was used to determine the relationship between Celastrol and the pyroptosis pathway. The data shows that NLRP3, ASC, Caspase-1, GSDMD in the spinal cord of the treated group is significantly lower than that of the SCI group (P < 0.05 or P < 0.01). Pro-caspase-1 did not change significantly in the three groups (P > 0.05). By ELISA, IL-1β and IL-18 in the treat group were significantly lower than those in the SCI group (P < 0.05 or P < 0.01). Combining the above experimental results, it can be shown that the protective effect of Celastrol on tissue and the reduction of inflammation is related to the inhibition of microglia activation and pyroptosis pathways (Figs. 2 and 3).

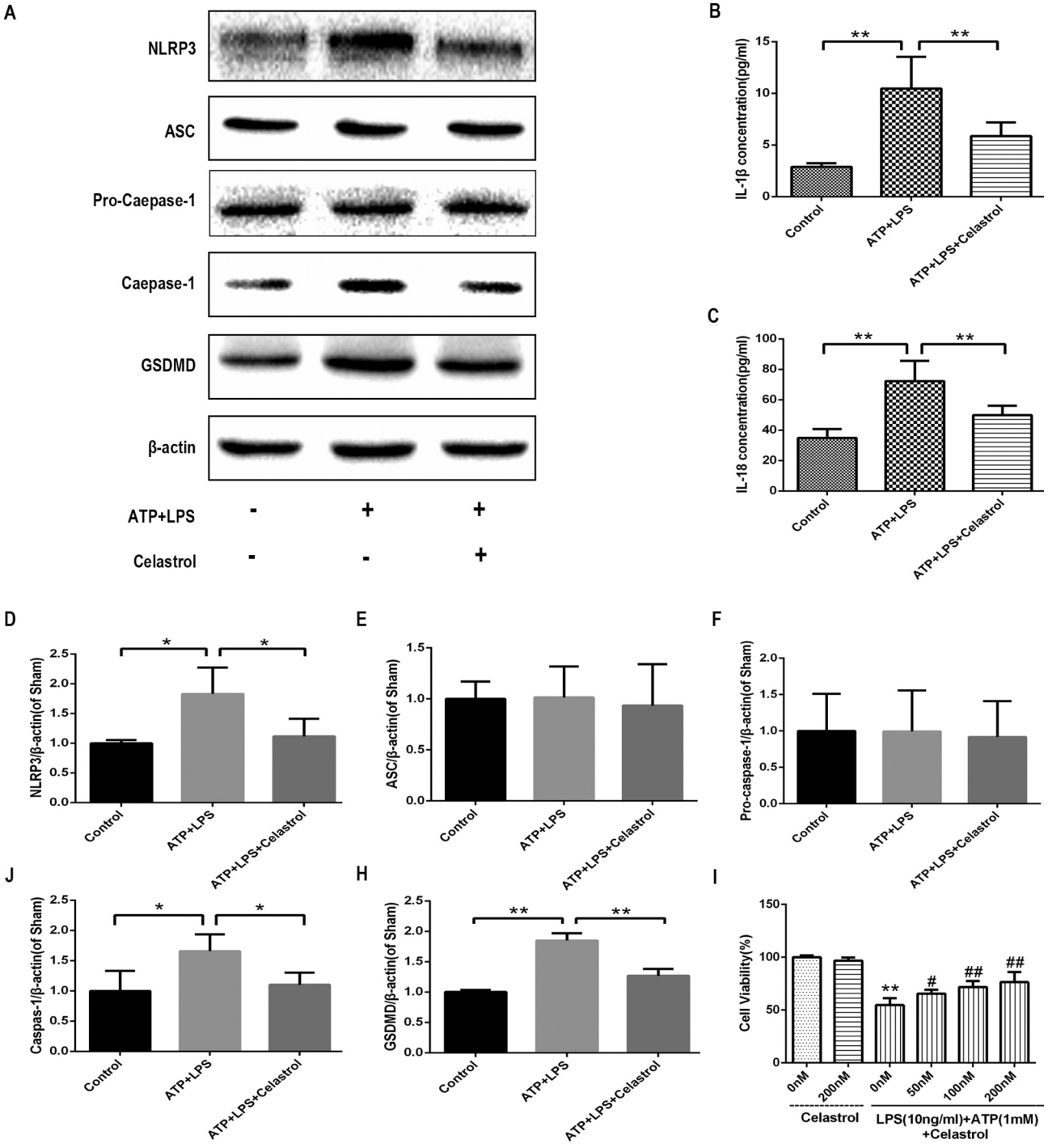

Fig. 4. Celastrol inhibits pyroptosis in LPS + ATP-induced BV-2 cells and reduces IL-1β and IL-18 inflammatory factor release.
After culturing for 24 h, Celastrol (100 nM) was first added for 1 h, then LPS (100 ng/ml) was added for 24 h, and finally, ATP (1 mM) was added for 3 h. (A) representative immunoblot bands for NLRP3, ASC, Pro-Caspase-1, Caspase-1, and GSDMD proteins. β-actin was used as a loading control; (B–C) The content of IL-1β and IL-18 in the cell supernatant was detected by ELISA kit. *P < 0.05, **P < 0.01 compared with SCI group, n = 6 per group; (D–H) Histogram of Relative Expression of NLRP3, ASC, Pro-Caspase-1, Caspase-1, and GSDMD. *P < 0.05, **P < 0.01 compared with SCI group, n = 6 per group.; (I) To evaluate the inhibitory effect of celastrol on LPS + ATP-induced BV-2 cell death. BV-2 cells were pretreated with celastrol (0, 50, 100 and 200 nM) for 1 h. Cell viability was measured by CCK-8 and presented as a percentage of sham, and each value represents the mean ± SEM of three independent experiments. (n = 3 experiments, *P < 0.05 versus control and #P < 0.05, ##P < 0.01 versus LPS + ATP group).
CCK-8: Cell Counting Kit-8.

3.4. Celastrol inhibited microglial pyroptosis and reduced the release of pro-inflammatory factors

In order to have a more comprehensive understanding of the effects of celastrol on pyroptosis in microglial cells, in vitro experimental tests were performed using BV-2 cells. Using immunoblotting, the expression of NLRP3, Caspase-1, and GSDMD proteins were significantly increased in LPS + ATP-stimulated microglia, while it was significantly decreased after intervention with Celastrol (P < 0.05 or P < 0.01). Pro-caspase-1 and ASC did not change significantly (P > 0.05). Similarly, ELISA

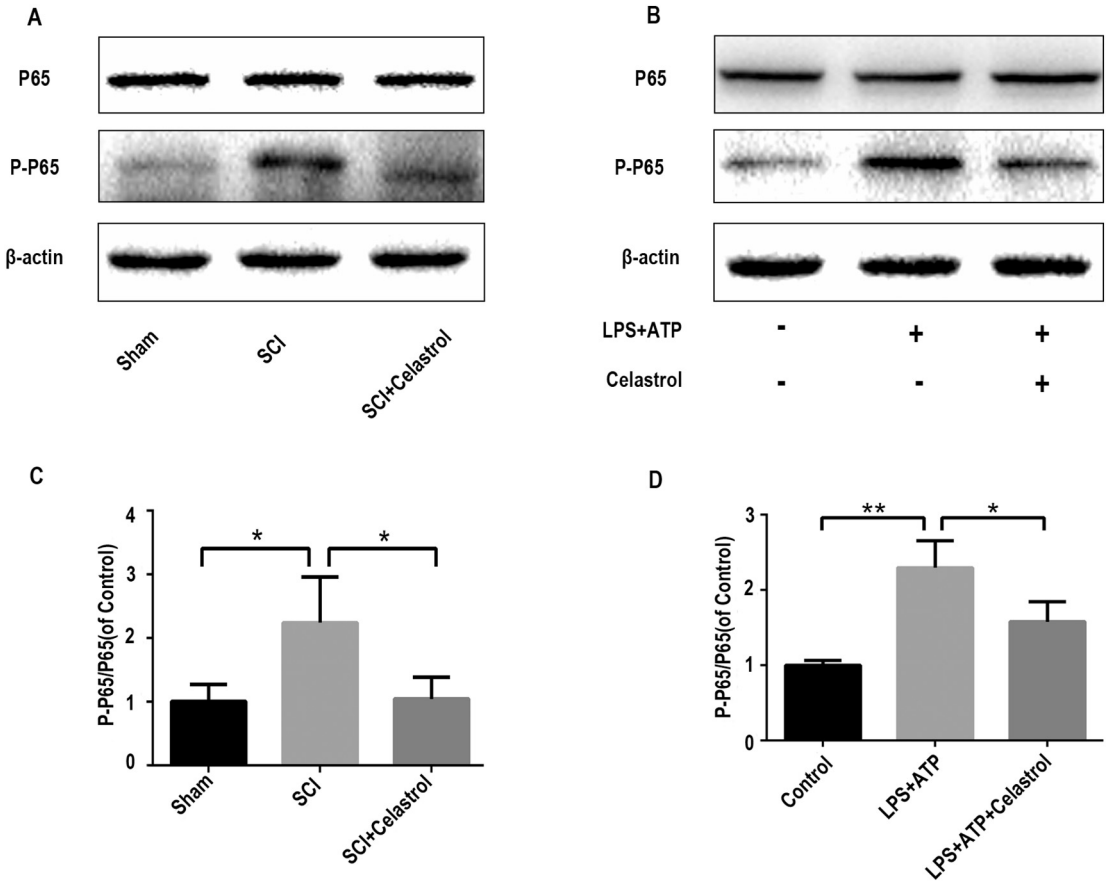

Fig. 5. Celastrol inhibits the activation of NF-κB in vitro and in vivo.
(A) Expression of NF-κB/P65 and NF-κB/P-P65 in spinal cord tissue after 7 days of spinal cord injury in rats; (B) Representative WB analysis of NF-κB/P65 and NF-κB/P-P65 expression in BV-2 cells in different groups; (C) Histogram of relative expression of NF-κB/P65 and NF-κB/P-P65 in rat spinal cord; (D) Histogram of Relative Expression of NF-κB/P65 and NF-κB/P-P65 in BV-2 cells. *P < 0.05, **P < 0.01 compared with SCI group. And the data comes from three independent experiments.

assays of inflammatory cytokines in cell supernatants were also performed and it was found that IL-1β and IL-18 were significantly lower in the treat group than those in the LPAS + ATP group (P < 0.01). The above trends of the key proteins and inflammatory factors in the pyroptosis pathway are consistent with the in vivo experiments. In addition, the results of CCK-8 showed that Celastrol can inhibit the death of microglia (P < 0.05 or P < 0.01). Thus Celastrol may reduce inflammatory factors by inhibiting pyroptosis in microglial cells (Fig. 4).

3.5. Effects of celastrol on NF-κB activation

The NF-κB pathway is a key pathway required for NLRP3 expression. Our results showed that the expression of NFKB/P-P65 decreased after treatment with Celastrol in vivo (P < 0.05), and the expression of NFKB/P-P65 also decreased in the presence of Celastrol in vitro (P < 0.05 or P < 0.01). This indicates that Celastrol can inhibit the NF-κB pathway and bring about a reduction of the expression of NLRP3 (Fig. 5).

4. Discussion

In traumatic SCI, as against irreversible primary injury, treatment is directed at the secondary injury with an aim to provide a microenvironment which may promote neuron survival. Inflammatory response is an important factor of secondary injury in SCI [35,36]. The production of specific factors has become an important indicator of the degree of damage. The adoption of various measures to prevent or suppress inflammation has become a means of SCI treatment. However, the inflammatory response is a “double-edged sword” in the secondary injury of SCI, and, the course of treatment needs to be optimized while it is in progress, such that adverse aspects of the inflammatory process can be reduced to the minimum and beneficial aspects enhanced, rather than attempting to completely prevent the occurrence of the inflammatory reaction. The purpose of this study was to investigate the effect of Celastrol in reducing the inflammatory response in SCI. Our data indicate that it may relieve the acute phase of the inflammatory response by inhibiting the pyroptosis in the cells of microglia, while promoting the recovery of neurological function in rats with SCI.

Some current studies have demonstrated that Celastrol has therapeutic effects on neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [37,38]. In vitro studies have shown that Celastrol can inhibit NF-κB and inactivate caspase-1, thereby reducing IL-1β and TNF secretion in THP-1, a macrophage-like cell line. Celastrol can attenuate inflammation in an adjuvant-induced early arthritis rat model in vivo. It inhibits the infiltration and proliferation of inflammation cells, and reduces the level of IL-6 in serum [39]. Celastrol can inhibit the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF) in LPS-activated PBMCs (Peripheral Blood Mononuclear Cells) and biopsies of patients with Crohn's disease by inhibiting NF-κB and p38 MAPK [40]. However, the purpose of this study was to study the effects of Celastrol on SCI in rats. Our in vitro and in vivo studies have shown that both IL-1β and IL-18 pro-inflammatory cytokines in the tissues and cell supernatants after drug intervention are reduced. These results are in line with previous studies. Compared with the BBB score and inclined plane test in the rat spinal cord injury group, the scores and slope angles of the rats in the treat group increased,

indicating that the motor function of the lower limbs had improved. In the HE staining, the recovery of the treat group was observed. Nissl staining indicated that the corresponding nerve cells were protected and the number of neurons in the treated group was greater than that of the injury group.

In the central nervous system injury, there are many kinds of cells involved in the development of the disease. And microglia play an important role in the inflammatory response [10,11]. It is well-known that microglia are the main innate immune defense cells of the CNS. After undergoing SCI, microglia maybe activated and undergo changes in morphology and function [41]. In the acute phase of SCI, microglial cells in the injured area are induced to activate and produce pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, proteases, and other cytotoxic substances, thus enhancing damage and impairing nerve regeneration [42]. A recent survey found that reducing inflammation and immune cell infiltration have a certain role in promoting the regeneration of neurons in the damaged central nervous system [43]. Therefore, the inhibition of the activation of microglia and the resultant release of inflammatory factors, and the stabilization of the internal environment of nerve cells in SCI, may play a role in promoting the recovery of damaged nerves. In our in vivo studies we detected Iba-1 (microglia marker) by PCR and found that Celastrol can inhibit the activation of microglia. In addition, in the past studies showed that the M1 phenotype of microglia released a large number of harmful substances such as inflammatory factors and oxygen free radicals in the early stage of inflammation, destroying the structure of neurons and affecting the normal function of neurons; The M2 phenotype microglia secrete some neurotrophic substances in the late stage of inflammation, remove necrotic or apoptotic neuron fragments, and promote the formation of tissue glial scars. In this study, the expression of iNOS (marker of M1 microglia) was elevated in the SCI group and decreased in the SCI + Celastrol group. However, Arg-1 (marker of M2 microglia) were slightly elevated in the SCI group. And it is more expressed in the SCI + Celastrol group than in the SCI group. The results also show that the expression of TNF-α and IL-1β harmful cytokines was decreased and the IL-10 cytokine was increased after drug treatment. This indicates that Celastrol inhibits microglial hyperactivation (especially M1 microglia) and may reduce the release of inflammatory factors.

Pyroptosis is a type of programmed cell death characterized by pro-inflammatory activity. When the body is subjected to noxious stimuli, intracellular and extracellular signaling pathways induce the formation of intracytoplasmic inflammasome through a caspase-1-dependent classical apoptotic pathway and/or caspase-4/5/11-dependent non-classical pyroptosis pathway. This results in the activation of caspase-1 or caspase-4/5/11 to promote the secretion of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18), leading to cell pyroptosis [44–46]. Numerous studies have shown that pyroptosis occurs extensively in diseases of the central nervous system [47,48]. Therefore, targeting the regulation of cell pyroptosis, may lead to the regulation of disease inflammation. In this study, we demonstrate the validity of this hypothesis. Both SCI rats and LPS + ATP-induced BV-2 cells had decreased expression of NLRP3, Caspase-1, and GSDMD after Celastrol treatment.

Previous studies have shown that the NLRP3 inflammasome exists in microglia [49]. Its activation requires two signals: the first signal is the pathogen-associated molecular pattern (PAMP), for example, lipopolysaccharide (LPS) is recognized and bound by the cell membrane pattern recognition receptor (PRR). The PAMP activates the NF-κB signaling pathway, and induces the expression of the inflammatory response related proteins such as NLRP3 and pro-interleukin-1β (pro-IL-1β);The second signal is the damage-associated molecular pattern (DAMP) such as adenosine triphosphate (ATP) that activates NLRP3, recruits pro-caspase-1 through the linker protein ASC, which leads to self-catalyzed processing to form activated caspase-1 [50]. Therefore, it is becoming increasingly clear that the NF-κ B signaling pathway plays an important role in reducing NLRP 3 inflammation and inflammatory factors. Our experiments demonstrate that intervention with Celastrol, leads not only to a decrease in the expression of proteins related to the pyroptosis pathway, but also to a reduction in the expression of NFkB/P-P65.

While studies relating to pyroptosis in the CNS were few, most of them focused on the brain [51,52]. The specific role of cell pyroptosis in SCI has not yet been fully elucidated. Therefore, in this study, we explored the correlation between inflammation in SCI and pyroptosis in microglia. The study was performed in rat SCI models and BV-2 cells stimulated with LPS + ATP. In in vivo experiments, by detecting the key proteins and inflammatory factors of the pyroptosis pathway, we found that the NLRP3, ASC, Caspase-1, GSDMD increased significantly in SCI rats, and the IL-1β and IL-18 in these animals also increased. Our in vitro experimental results were consistent with the in vivo data. Intervention with Celastrol, caused a decline in the levels of these proteins. There is no tendency of ASC in BV-2 glial cells, which is similar to that reported by Alexander Slowik [53].

In summary, our research found that the Celastrol plays a role in the inhibition of inflammation that is induced by microglial pyroptosis and microglial hyperactivation in SCI.

Our study has a few limitations which requires future attention. 1. Our experiments focus on the classical pathway of pyroptosis, and the non-classical pathway remains to be explored. 2. We selected time points and concentrations based on previous literature. The therapeutic effects of Celastrol on other time points remains to be studied, and it remains to be determined whether the treatment has dose dependence. 3. The interaction between microglia and nerve cells has not been thoroughly investigated. 4. There are many mechanisms that cause SCI inflammation, but this study focused on the pyroptosis of microglia in SCI inflammation.

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Conflict of interest

The authors have no conflict of interest to declare.

References

[1] P. Cheng, F. Kuang, H. Zhang, et al., Beneficial effects of thymosin beta4 on spinal cord injury in the rat, Neuropharmacology 85 (2014) 408–416, https://doi.org/10.1016/j.neuropharm.2014.06.004.
[2] G. Li, M.T. Che, K. Zhang, et al., Graft of the NT-3 persistent delivery gelatin sponge scaffold promotes axon regeneration, attenuates inflammation, and induces cell migration in rat and canine with spinal cord injury, Biomaterials 83 (2016) 233–248, https://doi.org/10.1016/j.biomaterials.2015.11.059.
[3] J. Li, G. Liu, Y. Zheng, et al., The epidemiological survey of acute traumatic spinal cord injury (ATSCI) of 2002 in Beijing municipality, Spinal Cord 49 (2011) 777–782, https://doi.org/10.1038/sc.2011.8.
[4] S. Yuan, Z. Shi, F. Cao, et al., Epidemiological features of spinal cord injury in China: a systematic review, Front. Neurol. 9 (2018) 1–12, https://doi.org/10.3389/fneur.2018.00683.
[5] L.A. Poniatowski, P. Wojdasiewicz, M. Krawczyk, et al., Analysis of the role of CX3CL1 (fractalkine) and its receptor CX3CR1 in traumatic brain and spinal cord injury: insight into recent advances in actions of neurochemokine agents, Mol. Neurobiol. 54 (2017) 2167–2188, https://doi.org/10.1007/s12035-016-9787-4.
[6] K. Mortezaee, N. Khanlarkhani, C. Beyer, A. Zendedel, Inflammasome: its role in traumatic brain and spinal cord injury, J. Cell. Physiol. 233 (2018) 5160–5169, https://doi.org/10.1002/jcp.26287.
[7] S. Okada, The pathophysiological role of acute inflammation after spinal cord injury, Inflamm. Regen. 36 (20) (2016), https://doi.org/10.1186/s41232-016-0026-1.
[8] J. Shi, W. Gao, F. Shao, Pyroptosis: gasdermin-mediated programmed necrotic cell death, Trends Biochem. Sci. 42 (2017) 245–254, https://doi.org/10.1016/j.tibs.2016.10.004.
[9] L. Vande Walle, M. Lamkanfi, Pyroptosis, Curr. Biol. 26 (2016) R568–R572, https://doi.org/10.1016/j.cub.2016.02.019.
[10] G.A. Grabowski, Overview of inflammation in neurometabolic diseases, Semin.

Pediatr. Neurol. 24 (2017) 207–213, https://doi.org/10.1016/j.spen.2017.08.005.
[11] S.W. Lee, S. Gajavelli, M.S. Spurlock, et al., Microglial inflammasome activation in penetrating ballistic-like brain injury, J. Neurotrauma (2018) 1–39, https://doi.org/10.1089/neu.2017.5530.
[12] J. Pirhonen, T. Sareneva, M. Kurimoto, et al., Virus infection activates IL-1 beta and IL-18 production in human macrophages by a caspase-1-dependent pathway, J. Immunol. 162 (1999) 7322–7329.
[13] T.D. Kanneganti, M. Body-Malapel, A. Amer, et al., Critical role for cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA, J. Biol. Chem. 281 (2006) 36560–36568, https://doi.org/10.1074/jbc.M607594200.
[14] J. Shi, Y. Zhao, K. Wang, et al., Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death, Nature 526 (2015) 660–665, https://doi.org/10.1038/nature15514.
[15] W. Liu, J. Liu, W. Wang, et al., NLRP6 induces pyroptosis by activation of caspase-1 in gingival fibroblasts, J. Dent. Res. (2018), https://doi.org/10.1177/0022034518775036 22034518775036.
[16] J. Yuan, A. Najafov, B.F. Py, Roles of caspases in necrotic cell death, Cell 167 (2016) 1693–1704, https://doi.org/10.1016/j.cell.2016.11.047.
[17] D.J. DiSabato, N. Quan, J.P. Godbout, Neuroinflammation: the devil is in the details, J. Neurochem. 139 (Suppl) (2016) 136–153, https://doi.org/10.1111/jnc.13607.
[18] E. Letellier, S. Kumar, I. Sancho-Martinez, et al., CD95-ligand on peripheral myeloid cells activates Syk kinase to trigger their recruitment to the inflammatory site, Immunity 32 (2010) 240–252, https://doi.org/10.1016/j.immuni.2010.01.011.
[19] A.D. Gaudet, L.K. Fonken, Glial cells shape pathology and repair after spinal cord injury, Neurotherapeutics (2018), https://doi.org/10.1007/s13311-018-0630-7.
[20] L.J. Noble, J.R. Wrathall, Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat, Exp. Neurol. 103 (1989) 34–40.
[21] R. Kannaiyan, K.A. Manu, L. Chen, et al., Celastrol inhibits tumor cell proliferation and promotes apoptosis through the activation of c-Jun N-terminal kinase and suppression of PI3 K/Akt signaling pathways, Apoptosis 16 (2011) 1028–1041, https://doi.org/10.1007/s10495-011-0629-6.
[22] H.Y. Li, J. Zhang, L.L. Sun, et al., Celastrol induces apoptosis and autophagy via the ROS/JNK signaling pathway in human osteosarcoma cells: an in vitro and in vivo study, Cell Death Dis. 6 (2015) e1604, https://doi.org/10.1038/cddis.2014.543.
[23] J. Liu, J. Lee, M.A. Salazar Hernandez, et al., Treatment of obesity with celastrol, Cell 161 (2015) 999–1011, https://doi.org/10.1016/j.cell.2015.05.011.
[24] A. Salminen, M. Lehtonen, T. Paimela, K. Kaarniranta, Celastrol: molecular targets of thunder god vine, Biochem. Biophys. Res. Commun. 394 (2010) 439–442, https://doi.org/10.1016/j.bbrc.2010.03.050.
[25] Y. Li, D. He, X. Zhang, et al., Protective effect of celastrol in rat cerebral ischemia model: down-regulating p-JNK, p-c-Jun and NF-kappaB, Brain Res. 1464 (2012) 8–13, https://doi.org/10.1016/j.brainres.2012.04.054.
[26] J. Konieczny, D. Jantas, T. Lenda, et al., Lack of neuroprotective effect of celastrol under conditions of proteasome inhibition by lactacystin in in vitro and in vivo studies: implications for Parkinson's disease, Neurotox. Res. 26 (2014) 255–273, https://doi.org/10.1007/s12640-014-9477-9.
[27] A.S. Rivlin, C.H. Tator, Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat, Surg. Neurol. 10 (1978) 38–43.
[28] H. Yang, C. Liu, J. Jiang, et al., Celastrol attenuates multiple sclerosis and optic neuritis in an experimental autoimmune encephalomyelitis model, Front. Pharmacol. 8 (44) (2017), https://doi.org/10.3389/fphar.2017.00044.
[29] W. Wang, C. Ha, T. Lin, et al., Celastrol attenuates pain and cartilage damage via SDF-1/CXCR4 signalling pathway in osteoarthritis rats, J. Pharm. Pharmacol. 70 (2018) 81–88, https://doi.org/10.1111/jphp.12835.
[30] C.W. Lin, B. Chen, K.L. Huang, et al., Inhibition of autophagy by estradiol promotes locomotor recovery after spinal cord injury in rats, Neurosci. Bull. 32 (2016) 137–144, https://doi.org/10.1007/s12264-016-0017-x.
[31] A.S. Rivlin, C.H. Tator, Objective clinical assessment of motor function after experimental spinal cord injury in the rat, J. Neurosurg. 47 (1977) 577–581, https://doi.org/10.3171/jns.1977.47.4.0577.
[32] T. You, Y. Cheng, J. Zhong, et al., Roflupram, a phosphodiesterase 4 inhibitior, suppresses inflammasome activation through autophagy in microglial cells, ACS Chem. Neurosci. 8 (2017) 2381–2392, https://doi.org/10.1021/acschemneuro.7b00065.
[33] E.A. Kim, C.H. Cho, J. Kim, et al., The azetidine derivative, KHG26792 protects against ATP-induced activation of NFAT and MAPK pathways through P2X7 receptor in microglia, Neurotoxicology 51 (2015) 198–206, https://doi.org/10.1016/j.neuro.2015.10.013.
[34] H.W. Jung, Y.S. Chung, Y.S. Kim, Y.K. Park, Celastrol inhibits production of nitric oxide and proinflammatory cytokines through MAPK signal transduction and NF-kappaB in LPS-stimulated BV-2 microglial cells, Exp. Mol. Med. 39 (2007) 715–721, https://doi.org/10.1038/emm.2007.78.
[35] M.M. Mortazavi, K. Verma, O.A. Harmon, et al., The microanatomy of spinal cord injury: a review, Clin. Anat. 28 (2015) 27–36, https://doi.org/10.1002/ca.22432.
[36] S. Xu, W. Zhu, M. Shao, et al., Ecto-5′-nucleotidase (CD73) attenuates inflammation after spinal cord injury by promoting macrophages/microglia M2 polarization in mice, J. Neuroinflammation 15 (2018) 155, https://doi.org/10.1186/s12974-018-1183-8.
[37] K. Faust, S. Gehrke, Y. Yang, et al., Neuroprotective effects of compounds with antioxidant and anti-inflammatory properties in a Drosophila model of Parkinson's disease, BMC Neurosci. 10 (2009) 109, https://doi.org/10.1186/1471-2202-10-109.
[38] D. Paris, N.J. Ganey, V. Laporte, et al., Reduction of beta-amyloid pathology by celastrol in a transgenic mouse model of Alzheimer's disease, J. Neuroinflammation 7 (2010) 17, https://doi.org/10.1186/1742-2094-7-17.
[39] R. Cascao, B. Vidal, H. Raquel, et al., Effective treatment of rat adjuvant-induced arthritis by celastrol, Autoimmun. Rev. 11 (2012) 856–862, https://doi.org/10.1016/j.autrev.2012.02.022.
[40] G.F. Pinna, M. Fiorucci, J.M. Reimund, et al., Celastrol inhibits pro-inflammatory cytokine secretion in Crohn's disease biopsies, Biochem. Biophys. Res. Commun. 322 (2004) 778–786, https://doi.org/10.1016/j.bbrc.2004.07.186.
[41] K.G. Witcher, D.S. Eiferman, J.P. Godbout, Priming the inflammatory pump of the CNS after traumatic brain injury, Trends Neurosci. 38 (2015) 609–620, https://doi.org/10.1016/j.tins.2015.08.002.
[42] S. Chen, J. Ye, X. Chen, et al., Valproic Acid Attenuates Traumatic Spinal Cord Injury-Induced Inflammation Via STAT1 and NF-κ B Pathway Dependent of HDAC3, (2018), pp. 1–14.
[43] A.L. Bowes, P.K. Yip, Modulating inflammatory cell responses to spinal cord injury: all in good time, J. Neurotrauma 31 (2014) 1753–1766, https://doi.org/10.1089/neu.2014.3429.
[44] E.A. Miao, J.V. Rajan, A. Aderem, Caspase-1-induced pyroptotic cell death, Immunol. Rev. 243 (2011) 206–214, https://doi.org/10.1111/j.1600-065X.2011.01044.x.
[45] T. Bergsbaken, S.L. Fink, B.T. Cookson, Pyroptosis: host cell death and inflammation, Nat. Rev. Microbiol. 7 (2009) 99–109, https://doi.org/10.1038/nrmicro2070.
[46] D. Yang, Y. He, R. Munoz-Planillo, et al., Caspase-11 requires the pannexin-1 channel and the purinergic P2X7 pore to mediate pyroptosis and endotoxic shock, Immunity 43 (2015) 923–932, https://doi.org/10.1016/j.immuni.2015.10.009.
[47] J.G. Walsh, D.A. Muruve, C. Power, Inflammasomes in the CNS, Nat. Rev. Neurosci. 15 (2014) 84–97, https://doi.org/10.1038/nrn3638.
[48] H. Guo, J.B. Callaway, J.P. Ting, Inflammasomes: mechanism of action, role in disease, and therapeutics, Nat. Med. 21 (2015) 677–687, https://doi.org/10.1038/nm.3893.
[49] F. Shi, Y. Yang, M. Kouadir, et al., Inhibition of phagocytosis and lysosomal acidification suppresses neurotoxic prion peptide-induced NALP3 inflammasome activation in BV2 microglia, J. Neuroimmunol. 260 (2013) 121–125, https://doi.org/10.1016/j.jneuroim.2013.04.016.
[50] M. Lamkanfi, V.M. Dixit, Mechanisms and functions of inflammasomes, Cell 157 (2014) 1013–1022, https://doi.org/10.1016/j.cell.2014.04.007.
[51] S.W. Lee, S. Gajavelli, M.S. Spurlock, et al., Microglial inflammasome activation in penetrating ballistic-like brain injury, J. Neurotrauma (2018), https://doi.org/10.1089/neu.2017.5530.
[52] W. Liu, Y. Chen, J. Meng, et al., Ablation of caspase-1 protects against TBI-induced pyroptosis in vitro and in vivo, J. Neuroinflammation 15 (48) (2018), https://doi.org/10.1186/s12974-018-1083-y.
[53] A. Slowik, L. Lammerding, A. Zendedel, et al., Impact of steroid hormones E2 and P on the NLRP3/ASC/Casp1 axis in primary mouse astroglia and BV-2 cells after in vitro hypoxia, J. Steroid Biochem. Mol. Biol. (2018), https://doi.org/10.1016/j.jsbmb.2018.05.003.