Inhibition of Caspase-1 by VX-765 Reduces Pyroptosis and Attenuates Neuroinflammation After Spinal Cord Injury

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Research

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Abstract

Background: Excessive inflammatory response, neuronal loss, and demyelination mediate the pathogenesis of spinal cord injury (SCI). VX-765, a highly selective caspase-1 inhibitor, is recently revealed to control pyroptosis in some disease models. However, the effects and underlying mechanisms of VX-765 in SCI remains unclear.

Methods: Functional recovery was assessed using Basso Mouse Scale (BMS) and BMS subscore. Proinflammatory cytokines levels was evaluated by western blot or enzyme-linked immunosorbent assay. The number of neutrophils was assessed by flow cytometry. Neuronal loss was evaluated by immunochemistry staining of NeuN. Demyelination was evaluated using immunofluorescence staining of myelin basic protein.

Results: Here we report that VX-765 led to a significant recovery of hindlimb locomotor function after SCI in mice. Moreover, VX-765 prevented caspase-1 activation, inhibited pyroptosis of neuron and oligodendrocyte, reduced proinflammatory cytokines levels and loss of neuron and myelin, and declines the number of neutrophils in mice. Furthermore, VX-765 suppressed caspase-1 activation, decreased proinflammatory cytokine levels, diminished pyroptosis and promoted the survival of neurons in oxygen-glucose deprivation oligodendrocytes.

Conclusions: Together, our data indicate that VX-765 provide a novel potential therapy strategy to improve recovery after SCI.

Background

Spinal cord injury (SCI) is a severe disease and characterized by long-lasting neurological disability[1–3]. Accumulating evidence has suggested that primary injury and secondary injury constitute the pathology of SCI[4, 5]. Previous studies have revealed that excessive inflammation [6, 7], neuronal loss[8, 9],demyelination[10], and oxidative stress[11] play an important role in the pathogenesis of SCI. Excessive inflammatory response[8],neuronal loss[12], and demyelination[13] can result in persistent damage and progressive degeneration after SCI. Previous evidence has also suggested that alleviation of neuroinflammation[14], secondary neuronal loss[15], and demyelination[16] are helpful for improving functional recovery after SCI.

Pyroptosis belongs to a newly discovered pattern of programmed cell death associated with inflammation, which is dependent on the classic pathway of caspase-1 and the non-canonical pathway of caspase-4/-5 in humans or caspase-11 in mice[17]. After being cleaved by caspase, gasdermin D (GSDMD) produces a N-terminal fragment of GSDMD (GSDMD-NT) to trigger pyroptosis with membrane rupture and release of inflammatory cellular contents including pro-inflammatory cytokine IL-1β and IL-18 [18–20]. In addition, mounting evidences suggest that pyroptosis is involved in neuroinflammation and neuronal loss in SCI[21]and traumatic brain injury[22], and inflammation and demyelination in multiple sclerosis[23].
VX-765 is a highly selective caspase-1 inhibitor, which controls the generation and release of proinflammatory cytokine IL-1β and IL-18[24]. VX-765 was able to attenuate myocardial infarction in a model of ischaemia-reperfusion injury[25]. VX-765 administration at reperfusion in P2Y 12 receptor antagonist-treated rats provides long-term reduction in myocardial infarct size and preservation of ventricular function[26]. VX-765 attenuates atherosclerosis in ApoE deficient mice through modulating VSMCs pyroptosis[27]. Application of VX-765 in the acute phase of SCI can improve the local microenvironment of SCI[28]. However, whether VX-765 can attenuate neuroinflammation and improve functional recovery and can be used as a therapeutic strategy for SCI requires further exploration.

Here we examined whether VX-765 can provide significant amelioration of neuroinflammation, loss of neuron and myelin, and convey beneficial effects on histological outcomes and functional recovery post-SCI. We also examined the effects of VX-765 on pyroptosis in mice and in in the primary cultured neurons after SCI. Our data demonstrated VX-765 alleviated neuroinflammation, neuronal loss and demyelination, and improved motor coordination. Together, our findings suggest that VX-765 might be a potential therapy strategy to improve recovery after SCI.

**Methods**

**Animal models and surgical procedure.** All surgical procedures and animal care were approved by the Hangzhou First People's Hospital's Ethics Committee and followed the Animal Care guidelines of China. Adult female C57BL/6 mice (8–10 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd and anesthetized with 1% sodium pentobarbital (75mg/kg). After performing the laminectomy of T10-T11 vertebrae laminae, the exposed spinal cord was compressed using the vascular clamp (30 g force) for 1 min based on previous studies[29,30]. Mice in sham group only underwent laminectomy without crush injury to the spinal cord. After operation, manual urinary bladder emptying (twice a day until recovery) was performed. Immediately after SCI, VX-765(50mg/kg, Sigma) or vehicle (saline) was injected intraperitoneally and then repeated every day for 3 days (totally 3 times). VX-765 dosage was chosen accordingly to the level of VX-765 in a mouse model of multiple sclerosis[21].

**Functional assessment**

Locomotor functions was assessed using the Basso mouse scale (BMS) by two blind observers[31,32]. Consensus scores for each mouse based on hindlimb movements were averaged for a maximum of 9 points for the BMS score and 11 points for the BMS subscore. The BMS was evaluated at 1, 3, 7, 14, 21, and 28 d postinjury with 9 mice in each group.

**Tissue collection**

The experimental mice underwent euthanasia with intraperitoneal injection of an overdose of sodium pentobarbital 3 days or 28 days post-SCI. At 3 days after injury, the spinal cord sample was removed for western blot (10 mm, centered at the lesion epicenter) and flow cytometry (5 mm, centered at the lesion epicenter). At 3 days and 28 days after injury, the spinal cord sample (5 mm length centered at the lesion epicenter)
epicenter) was fixed in formaldehyde solution and transversely sliced to 4-μm-thick sections for immunostaining and hematoxylin and eosin (HE) staining. Nine series of sections at 200 μm intervals rostral and caudal to the injury epicenter were picked up on glass slides.

**Histological analyses**

For hematoxylin and eosin (HE) staining, sections were stained with HE reagent. The evaluation of histopathologic changes was performed using a 6-point scale determined by injury neurons in the gray matter and damage of tissue structure as described previously[33]. The score ranged from 0 (no lesion observed) to 6 (large infarction, more than half of the gray matter area). Sections were scored in a blinded manner. The histologic score from four optical fields in the each section(n=9 sections/mice) were averaged to get a final score by (n=5 mice/group).

**Immunostaining**

For immunofluorescence staining of myelin basic protein (MBP), sections of spinal cord were washed with PBS containing 0.3% Triton X-100 for 2 h, and incubated with anti-MBP (1:100, Abcam, catalog # ab40390) at 4°C overnight. Subsequently, sections were washed in PBS, incubated in the secondary antibody (1:400, Life technologies, catalog # A21207) at room temperature for 2 h, and then incubated with DAPI for 3 min. Sections were visualized with a confocal microscope (Olympus Inc., Tokyo, Japan) for immunofluorescence staining. The fluorescence area from the optical field in the section(n=9 sections/mice) were evaluated and analyzed (n=5 mice/group).

For immunochemistry staining, sections were incubated with 0.3% hydrogen peroxide for 10 min, washed in PBS, treated with EDTA antigen retrieval solution, and then incubated with anti-NeuN (1:100, Abcam, catalog # ab177487) at 4°C overnight. After washing in PBS, sections were added appropriate amount of enzyme labeled sheep anti-mouse IgG polymer (Beijing Zhongshu Jinqiao Biotechnology Co., Ltd., Beijing, China), incubate at room temperature for 20 minutes, and then added appropriate amount of freshly prepared chromogenic solution. Sections were visualized with a fluorescence microscope (Olympus Inc., Tokyo, Japan) for immunochemistry staining. To obtain quantitative measurements of positive cells in the ventral horn, the number of positive cells from the optical field in the section(n=9 sections/mice) were analyzed (n=5 mice/group).

**Primary oligodendrocyte culture and injury models**

Primary oligodendrocyte was cultured as following. Briefly, cerebral cortices from embryonic day 2 mouse were minced, removed of other substances, dissociated with 0.25 % trypsin (Invitrogen, Carlsbad, CA, USA), and then passed through cell strainer. Cells were plated on poly-L-lysine-coated dishes at a density of 1 × 10^6/ml and subsequently maintained in DMEM medium in a humidified incubator (5% CO_2 and 95% air) at 37°C for 1 h, and then transferred to T25 culture bottle. The bottle was sealed with a sealing film, placed in 37°C constant temperature shaking bed to shake, suck out the medium to remove microglial and other hybrid cells, and then sited in incubator for 1 h to remove astrocytes. When density of
cells reached 80% density, cells were transferred to induced differentiation medium DMEM/F12 and performed cultivation and passage.

For oxygen-glucose deprivation (OGD), the culture medium was replaced to glucose-free Dulbecco's Modified Eagle Medium(Gibco, Waltham, MA, USA), and then cultured in a hypoxic chamber(5% CO$_2$, 0.02%O$_2$ and 94.98%N$_2$) for 6 h at 37 °C. Oligodendrocytes were was pretreated with VX-765 (50 uM) for 4 h prior to OGD based on previous study[21].

**Cytokines assay**

Levels of IL-1β and IL-18 in in supernatant were detected by enzyme-linked immunosorbent assay (ELISA) kits of IL-1β (catalog # BPE30552H) and IL-18 (catalog #SEA064Mu) following the manufacturer's instructions (Wuhan Youersheng, Wuhan, Hubei, China).

**Flow cytometry**

Spinal cord tissue (5 mm, centered at the lesion epicenter) was harvested, cut, and digested with collagenase. Subsequently, the collected cells were filtered with screen mesh, suspended and incubated with anti-GSDMD (1:100, Abcam, catalog # ab219800) and anti-NeuN (1:100, Abcam, catalog # ab177487), or anti-GSDMD (1:100, Abcam, catalog # ab219800) and anti-MBP (1:100,Abcam, catalog # ab40390). In addition, combination of markers, CD45$^+$ (1:100, eBioscience, catalog #11-0112-85), CD11b$^+$ (1:100, eBioscience, catalog #45-5931-80), F4/80$^-$ (1:100, BioLegend, catalog #103111) and Gr1$^+$ (1:100, eBioscience, catalog #12-4801-80), was used to identify for neutrophils. To obtain quantitative measurements of double positive cells, a flow cytometer (BD Biosciences, San Jose, CA, USA) was used for examination and analysis (n=15 mice/group).

The oligodendrocytes were harvested and incubated with GSDMD (1:100, Abcam, catalog # ab219800) for 24 h. Subsequently, the cells were incubated with SYTOX® Blue dead cell stain (a high-affinity nucleic acid which only penetrates compromised plasma membranes) (Molecular Probes, Eugene, OR, USA) for 10 min at room temperature to measure the formation of membrane pore[34], and then examined and analyzed by a flow cytometer (BD Biosciences, San Jose, CA, USA).

**Cell viability and cytotoxicity assays**

Cell viability was determined using Cell Counting Kit-8(CCK-8) Detection Assay Kit (Lianke, catalog # 70-CCK805). Cells were seeded in plates, pretreated VX-765 (50 uM) for 4h, and then received OGD as described above. Subsequently, CCK-8 solution was added to the culture. Next, the absorbance at 490 nm was measured.

Lactate dehydrogenase (LDH) release detection was determined for cytotoxicity using a detection assay kit (Jiancheng, Nanjing, Jiangsu, China).
The supernatant in serum-free media was filtered and then transferred to 96-well plates, followed by incubation with the reaction mixture. Measurement of absorbance at 490 nm was analysed for quantification of LDH concentration.

**Western blotting**

The protein samples of spinal cord tissue as well as cells were collected and extracted using RIPA lysate buffer (Beyotime, Nanjing, Jiangsu, China), followed by measurement with BCA Protein Assay Kit (Beyotime). The collected protein samples were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein sample buffer, subsequently transferred to polyvinylidene difluoride membranes. The membranes were washed, blocked with non-fat dry milk and incubated with primary antibodies against NLRP3 (1:1000, Cell Signaling Technology, catalog #15101), ASC (1:1000, Cell Signaling Technology, catalog # 67824), caspase-1 (1:1000, Abcam, catalog # ab138483), GSDMD (1:1000, Abcam, catalog # ab219800), IL-1β (1:1000, Cell Signaling Technology, catalog # 12507) and IL-18 (1:500, Abcam, catalog # ab71495), followed by incubated with secondary antibody(1:5000, Lianke, catalog # Goat-anti Mouse GAM007; Goat-anti Rabbit GAR0072). Next, the intensity of these bands was measured with an enhanced chemiluminescence reagents (Perkin Elmer Life Sciences, Waltham, MA, USA) and analyzed with MultiGauge image analysis software (Fujifilm Holdings Corporation, Tokyo, Japan).

**Results**

**VX-765 improves behavioral recovery in mice**

We first evaluated locomotor recovery using the BMS score and BMS subscore in the C57BL/6 mouse on days 1, 3, 7, 14, 21, and 28 after SCI or sham surgery. At 1, 3 and 7 days postinjury, no difference was observed in the BMS score between the SCI and VX-765 groups. By contrast, at 14, 21, and 28 days post-injury, BMS score of the VX-765 group was higher than that of the sham group(Fig. 1A). In addition, the BMS subscore of mice in the VX-765-treated group was significantly higher compared to that of mice in the vehicle-treated group at 14, 21, and 28 days post-injury (Fig. 1B).

**VX-765 alleviates the severity of histological damage in mice**

The effects of VX-765 on histological outcomes in the injury spinal cord of mice were examined using HE staining 3 days (Fig. 2A-2C) and 28 days (Fig. 2D-2F) after SCI or sham surgery. In the SCI group, obvious edema and detrimental alterations in the white matter were observed. In the VX-765 group, VX-765 treatment attenuates SCI-mediated histopathological alterations (3 days: p=0.023; 28 days: p=0.037).

**VX-765 reduces neuronal loss and demyelination in mice**

To examine the effects of VX-765 on neuronal loss in the SCI mice, the expression of NeuN was measured using immunohistochemistry analysis on days 28 after SCI or sham surgery. Intraperitoneal injections of VX-765 significantly increased the number of NeuN positive cells in the VX-765 mice.
compared with those of SCI mice (p=0.018, Fig.3A). The data indicates that VX-765 can decrease neuronal loss in the injured spinal cord.

Next, the effects of VX-765 on demyelination in the injury spinal cord of mice were examined using immunofluorescence staining of MBP on days 28 after SCI or sham surgery. Application of VX-765 increased MBP immunoreactivity in the VX-765 group compared with SCI group (p=0.003, Fig.3B). Together, the data suggests that VX-765 can alleviate myelin loss in mice after SCI.

**VX-765 declines the number of neutrophils in mice**

To assess whether resolution of neutrophil inflammation after SCI is linked to VX-765 treatment, we investigated the neutrophil counts in the injured spinal cord at day 3. SCI induced a significant higher number of neutrophil counts in the SCI group compared with sham group (p=0.005, Fig.4). However, VX-765 reduced the neutrophil counts and accelerated the clearance of neutrophils from the contused spinal cord (p=0.038, Fig.4).

**VX-765 inhibits pyroptosis, reduces proinflammatory cytokine levels, and controls the activation of caspase-1 activation in mice**

To assess the effects of VX-765 on pyroptosis of neuron and oligodendrocyte in SCI mice, flow cytometry of NeuN and GSDMD (p<0.001, Fig.5A,B), and MBP and GSDMD (p=0.027, Fig.5C,D) were performed at 3 days after the SCI or sham surgery. The number of double positive cells in the SCI group were significantly more than those in the sham group (p<0.001 for NeuN and GSDMD, Fig.5A,B; p=0.001 for MBP and GSDMD, Fig.5C,D). Nevertheless, the increased double positive cells observed in the SCI group were significantly reduced by application of VX-765 (p=0.027 for NeuN and GSDMD, Fig.5A,B; p=0.011 for MBP and GSDMD, Fig.5C,D).

As reported previously, the protein level of caspase-1 reach the peak 3 days after SCI[35,36]. There was an increase in the protein levels of caspase-1, GSDMD, IL-1β and IL-18 in the SCI group compared to those in the sham group (p=0.009 for caspase-1, p=0.002 for GSDMD, p<0.001 for IL-1β, p=0.009 for IL-18, Fig.5E,F). VX-765 treatment significantly prevented SCI-mediated rise of caspase-1, GSDMD, IL-1β and IL-18 (p=0.029 for caspase-1, p=0.049 for GSDMD, p=0.008 for IL-1β, p=0.037 for IL-18, Fig.5E,F). These results, therefore, suggest that VX-765 suppresses caspase-1 activation, pyroptosis of neuron and oligodendrocyte and neuroinflammation after SCI.

**VX-765 inhibits caspase-1 activation and pyroptosis, reduces proinflammatory cytokine levels and promotes cell survival in the primary cultured neurons**

To further confirm the role of VX-765 in pyroptosis and survival of cultured oligodendrocytes after SCI, the condition of OGD was used to mimic SCI model in the primary cultured oligodendrocytes.

Western blot analysis revealed that OGD led to significant increases in the expression of caspase-1 and GSDMD, relative to control cells (p=0.018 for caspase-1, p=0.020 for GSDMD, Fig.6A,B). Nevertheless, VX-
765 significantly inhibited OGD-mediated increase of caspase-1 and GSDMD level (p=0.031 for caspase-1, p=0.025 for GSDMD, Fig.6A,B). Furthermore, we measured the levels of IL-1β and IL-18 in the culture supernatant. We found that the levels of IL-1β and IL-18 in the OGD group were significantly higher than those in the control group (p=0.003 for IL-1β, p=0.005 for IL-18, Fig.6C,D). However, intraperitoneal injections of VX-765 significantly decreased the expressions of IL-1β and IL-18 compared with OGD group (p=0.035 for IL-1β, p=0.038 for IL-18, Fig.6C,D).

To further detect the pyroptosis cells in the primary cultured oligodendrocytes, flow cytometry with incubation of GSDMD and SYTOX® Blue dead cell stain was used. As shown in Figure 6E,F, double positive cells of GSDMD and SYTOX Blue staining were significantly increased in OGD group in comparison with that in the control group (p<0.001, Fig.6E,F), whereas the double positive cells exhibit a significant decrease in VX-765 -treated group (p=0.034, Fig.6E,F).

Next, CCK-8 and LDH assay were performed to investigate the effects of mt-ROS inhibition on cell survival rate and neurotoxicity in the primary cultured neurons, respectively. We found that OGD caused a higher LDH activity (p<0.001, Fig.6G) and a lower cell survival rate (p=0.041, Fig.6H) in the culture supernatant in the OGD group compared to control group. Nevertheless, SS-31 induced significantly higher cell survival rate value (p=0.041, Fig.6G) and lower LDH activity (p=0.015, Fig.6H) in the SS-31 group compared to OGD group.

Discussion

The experiments performed in this study demonstrated that VX-765 treatment attenuated tissue impairment and improved functional recovery in mice after SCI. Moreover, VX-765 controlled neuroinflammation and reduced the loss of neuron and myelin. Further in vitro and in vivo data confirmed that VX-765 treatment inhibited NLRP3 inflammasome activation and pyroptosis. Together, our findings suggest that VX-765 may provide an approach to provide therapeutic benefits for treating SCI.

IL-1β and IL-18 are known to the important proinflammatory intracellular contents after pyroptosis[37]. IL-1β contributes strongly to detrimental effects on lesion development (in terms of glia activation and size) and the plasticity of axons after SCI[38]. Furthermore, IL-1β may be implicated in the prognosis of SCI[39]. IL-1β is reported to dampen functional recovery after neural stem cell transplant therapy in SCI rats[40]. Importantly, reduction of IL-1β expression have been shown to be exhibit potential beneficial effects for mitigation of tissue damage and ameliorating the outcomes [41]. In addition, IL-18 is closely related with the severity of SCI[42]. In this study, we found that VX-765 treatment reduced the protein levels of IL-1β and IL-18 in vivo and in vitro after SCI. Our present findings suggest that VX-765 can suppress SCI-mediated neuroinflammation.

Together with increase of proinflammatory cytokines, we found that the loss of neuron and myelin was significantly after SCI. It was reported previously that neuronal loss and demyelination play vital roles in the secondary injury after SCI and may be potential therapy targets to improve functional recovery[43, 44]. Moreover, neuronal loss is implicated in the progression of SCI and failure of regeneration in neurons.
contribute to paralysis following SCI[45]. Furthermore, improving motor neurons survival can promote locomotor recovery after traumatic SCI[46]. In addition, inhibition of demyelination can improve recovery in a mouse model of SCI[47]. Here we found that the administration of VX-765 significantly reduced neuronal loss. Furthermore, the present work showed that VX-765 alleviated demyelination. All these data collectively suggest that VX-765 attenuates SCI-mediated tissue damage.

To clarify the mechanisms of neuronal loss, demyelination and inflammatory cytokines after SCI, we studied pyroptosis in primary cultured neurons and in the injury spinal cord of mice. Pyroptosis, which is a new identified mode of cell death, is concomitant with the release of proinflammatory cytokines[48]. Earlier studies have shown that zinc exposure may induce olfactory neuron pyroptosis by inflammasome[49], and pyroptosis is implicated in neuronal loss[50]. Moreover, 27-Hydroxycholesterol is involved in the pathogenesis of neuronal loss through inducing pyroptosis[50]. In addition, valproic acid mitigates ischemic/reperfusion injury-induced neuronal damage through inhibition of pyroptosis[51]. In this study, we found that SCI caused pyroptosis of neuron and oligodendrocyte in mice after SCI. Nevertheless, VX-765 administration significantly suppressed pyroptosis occurrence of neuron and oligodendrocyte in mice. In addition, VX-765 prominently inhibited oligodendrocyte pyroptosis and improved oligodendrocyte survival after OGD in primary cultured oligodendrocytes. This is the first study suggesting that VX-765 can control pyroptosis and reduce neuronal loss and demyelination.

Conclusion

In summary, this is the first report demonstrating that VX-765 inhibits pyroptosis and controls neuroinflammation in vitro and in vivo after SCI. Moreover, VX-765 reduces neuronal loss and demyelination, attenuates pathophysiological changes and improving locomotor recovery after SCI in mice. Together, our data indicate that VX-765 may provide a novel approach for promoting recovery after SCI.

Abbreviations

SCI: Spinal cord injury; GSDMD: gasdermin D; IL: interleukin; NLRP3: nucleotide-binding oligomerization domain-like receptor 3; BMS: Basso Mouse Scale; H&E: hematoxylin and eosin; MBP: myelin basic protein; EDTA: ethylenediaminetetraacetic acid; OGD: oxygen-glucose deprivation; ELISA: enzyme-linked immunosorbent assay; CCK-8: Cell Counting Kit-8; LDH: lactate dehydrogenase; ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain.

Declarations

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Author's contributions

LLZ and WJ wrote the paper. LLZ, MQL, WJ and FH performed the experiments. GMD analyzed the data. WJ designed the research and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed in this study are included in this published article and its additional files.

Ethics approval and consent to participate

All protocols were approved by the Animal Care and Use Committee of Hangzhou First People’s Hospital and Zhejiang University, and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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