Necrostatin-1 Mitigates Endoplasmic Reticulum Stress After Spinal Cord Injury

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Abstract  Necrostatin-1 (Nec-1) has been shown to inhibit necroptosis and convey a significant protective effect after spinal cord injury (SCI). This small molecule inhibitor may reduce tissue damage and restore neurological function by lessening mitochondrial injury after SCI and preserving energy homeostasis. However, the effects of Nec-1 on endoplasmic reticulum stress (ERS)—an important pathological consequence of SCI—are still not clear. The present study investigates the relationship between necroptosis and ERS in a rat model of SCI. Electron microscopy was employed to observe ultra-structural changes in the endoplasmic reticulum and mitochondria after lesioning. Real-time quantitative PCR was used to measure the mRNA levels of ERS-related pro-apoptotic molecules such as C/EBP homologous protein (CHOP), immunoglobulin-binding protein (BiP/GRP78) and X box-binding protein-1 (XBP-1). Western blot and immunofluorescence were conducted to analyze CHOP, GRP78 and XBP-1 protein expression after lesioning. Results demonstrated that applying Nec-1 in SCI reduces ultra-structural damage to the endoplasmic reticulum and mitochondria and inhibits expression of ERS-related genes and proteins after lesioning. Immunofluorescence also shows ERS-related proteins mainly expressed in the cytoplasm of nerve cells. Taken together, these results demonstrate that Nec-1 has protective effect on the endoplasmic reticulum and mitochondria and alleviates ERS after SCI.

Keywords  Necroptosis · Necrostatin-1 · Endoplasmic reticulum stress · XBP-1 · CHOP · BiP/GRP78

Introduction

The World Health Organization (WHO) reports that there are 500,000 new cases of SCI every year. Current SCI treatments do not improve neurological function, making SCI permanently crippling and a severe worldwide medical problem. Necrosis, apoptosis and necroptosis are common types of cell death caused by ischemia–reperfusion injury in SCI [1, 2]. Inhibition of apoptosis has a known protective effect for SCI [3, 4], and the protective effect of inhibiting necroptosis has also been confirmed in the last decade [5, 6]. Nec-1 is a small molecule inhibitor of necroptosis targeting receptor-interacting protein kinase 1 (RIPK1). RIPK1 is a component of the necroptosome, which also consists of receptor-interacting protein kinase 3 (RIPK3) and mixed-lineage kinase like domain (MLKL) [7]. Previous studies have shown that Nec-1 can mitigate cellular oxidative stress brought on by ischemia, glucose deprivation, and disruption of intracellular ion signaling [8, 9], which are inherent pathological changes of endoplasmic reticulum stress (ERS) [10, 11]. Recent studies also indicate that Nec-1 may reduce astrocyte death and rescue neurotrophic function of reactive astrocytes, thereby promoting adjacent neuron survival [12].

ERS is one of the key pathological changes after SCI contributing to cell death and neuro-dysfunction [13]. High levels of ERS result in suspension of protein synthesis and cause protein unfolding or misfolding, as well

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as swelling and shedding of ribosomes. Significant misfolded or unfolded protein accumulated in ER results in an unfolded protein response (UPR); therefore, UPR serves as an indicator of ERS. PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor 6 (ATF6) are important signaling molecules of three ERS pathways [14]. In non-ERS condition, immunoglobulin-binding protein (BiP/GRP78) binds with these three molecules and maintains the inactive state of these pathways. As part of the UPR, activated inositol-requiring enzyme-1α (IRE1α) cleaves 26 base pairs from X box-binding protein-1 (XBP-1) mRNA, and the translation product of truncated XBP-1 up-regulates BiP/GRP78 expression to further reduce ERS [14]. Under ERS conditions, cells may express pro-apoptotic molecules such as C/EBP homologous protein (CHOP) and caspase-12 or pro-survival molecules such as growth arrest and DNA damage inducible gene 34 (GADD34) and BiP/GRP78, thus determining cellular fate of death or survival. In this context, BiP/GRP78, XBP-1 and CHOP also serve as sensors of ERS [15].

Recent research shows that the necroptosis proceeds by transportation of the necroptosome to the mitochondrial membrane, thus committing to cell death by membrane perforation and mitochondrial leakage [16, 17]. In addition, Wang et al. have confirmed [18] that Nec-1 extenuates mitochondrial injury after SCI. Nec-1 may promote energy production of mitochondria, preserve the mitochondrial membrane potential and Ca2+ gradient, inhibit the release of cytochrome c and help maintain normal mitochondrial ultra-structure after SCI. Another study demonstrated the presence of necroptosome proteins such as RIPK3 and MLKL on ER and mitochondria membranes, but more particularly on the ER membrane [16]. The effect of Nec-1 on mitochondria has been verified, any effects on the ER have not been determined.

In the present study, a rat SCI model was utilized to investigate the effect of Nec-1 on ERS and further explore the mechanisms which convey the protective effects of Nec-1 after SCI.

Methods

Animal Care

All animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). The study protocols were designed to minimize animal suffering and approved by the Xiamen University Animal Experimentation Ethics Committee.

Disease Model

All procedures were performed under protocols approved by Xiamen University’s Animal Care and Use Committee. Specific pathogen-free (SPF) level male Sprague Dawley rats (10–12 weeks, weighing 220 ± 30 g) were purchased from the Animal research center of Xiamen University and used to generate the spinal cord injury (SCI) model. A total of 60 rats were randomly divided into four groups: Sham (only subjected to laminectomy), Sham+Necrostatin-1 (laminectomy and subdural Necrostatin-1 injection), SCI+Necrostatin-1 (subdural Necrostatin-1 injection 30 min before injury) and SCI only (untreated SCI). Each group consisted of 15 animals (n = 15).

Rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium (Sigma, USA) at a dosage of 30 mg/kg. For each animal, an incision was made at the Th10 vertebra (~4 cm below the base of the skull), first slitting skin and then the subcutaneous tissues in turn. The paraspinal muscles were separated to expose the lamina, then the spinous process and pedicle of Th9 and Th10 were removed to expose 8 mm of spinal cord. In the SCI only and SCI+Necrostatin-1 groups, the spinal cord was contused at Th10 with a spinal cord impactor, model III (W.M.Keck, USA). Spasmodic tail swings, lower limb flutters, and body/lower limb flaccid paralysis indicate successful generation of the SCI. Finally, subcutaneous tissue and skin were sutured. In the Sham+Necrostatin-1 and SCI+Necrotatin-1 groups, 2 μL of Necrostatin-1 dissolved in DMSO (Sigma, USA) at a concentration of 25 μg/μL was injected into the Th10 spinal cord 30 min before injury. After the operation, rats were kept in a warm environment (24–28 °C) and given fresh water and food. Penicillin was administered intra-peritoneally once after lesioning (40,000 U). The animals underwent bladder emptying by manual compression three times a day.

RT-qPCR Quantification of CHOP and GRP78 mRNA

Real-time qPCR was used for quantification of CHOP and GRP78 mRNA. Tissue for RNA extraction was harvested at 6 and 12 h after lesioning. Trizol (Invitrogen, USA) was used to extract total RNA according to the manufacturer protocol. For each sample, 1 μg of total RNA was converted to cDNA using the First Strand cDNA Synthesis Kit (Takara Bio, Dalian, China). Genes measured were: CHOP (Forward: TGG AAG CCT GGT ATG AGG ATC TG, Reverse: GAG GTG CTT GTG ACC TCT GCT G), GRP78 (Forward: TCA GCC CAC CGT AAC AAT CAA G, Reverse: TCA GCC CAC CGT AAC AAT CAA G) and β-actin (Forward:

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GGA GAT TAC TGC CCT GGC TCC TA, Reverse: GAC TCA TCG TAC TCC TGC TTG CTG CTG). All primers were designed and validated by the Takara Biological Company. Template cDNA (10 ng/rxn), SYBR Green fluorescent reagent (Tli RNaseH Plus, Takara Bio, Dalian, China) and Roche LightCycler® 480 II (Roche, Switzerland) were utilized for real-time qPCR analysis. The reaction condition as follow: initial denaturation 95 °C for 5 min, denaturation 95 °C for 30 s, annealing 58 °C for 30 s, extension 72 °C for 30 s, total 45 cycles. Relative gene expression was calculated using the 2−△△CT method with β-actin as the endogenous control gene.

RT-qPCR Quantification of XBP1 mRNA

Harvest of tissue was the same as for quantification of CHOP and GRP78 mRNA. Total RNA extraction to determine XBP1 mRNA levels was performed using the Eastep® Super Total Extraction Kit (Takara Bio, Dalian, China). Reverse transcription utilized the PrimeScript™ RT-PCR Kit. RT-PCR was performed according to the manufacturer protocols for each reagent. Genes in this analysis included: XBP1 (Forward: AAA CAG AGT AGC AGC ACA GAC TG, Reverse: GGA TCT CTA AGA CTA GAG GCT TGG TG) and β-actin (as above). The reaction condition as follow: initial denaturation 98 °C for 10 min, denaturation 98 °C for 30 s, annealing 58 °C for 30 s, extension 72 °C for 1 min, total 35 cycles. Two XBP1 cDNA products were isolated by 2% agarose gel electrophoresis and purified using the Universal DNA Purification Kit (Tiangen Biotech, Beijing, China): un-spliced XBP1 mRNA (XBP1u) and spliced XBP1 mRNA (XBP1s). The XBP1s mRNA encodes the XBP1 transcription factor and is missing a 26 bp intron. XBP1u mRNA can be identified by the presence of a unique Pst I endonuclease (Thermo Scientific, USA) restriction site within this intron by digesting with the Pst I endonuclease at 37 °C for 16 h. The digestion products were then separated and relatively quantified via electrophoresis using the formula: XBP1s mRNA = XBP1s mRNA/(XBP1s mRNA + XBP1u mRNA). β-actin cDNA served as a loading control.

Western Blot and Immunofluorescence Analysis

Western blot (WB) was used to analyze the levels of proteins related to ERS. Tissues were collected at 12 and 24 h after lesioning. Total protein was extracted from the spinal cord using RIPA lysis buffer (Beyotime, China), boiled with 6x loading buffer for 15 min, then loaded into 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After electrophoresis (1.5 h at 4 °C), protein was transferred onto a PDVF membrane (Invitrogen, USA). After blocking with 5% fat-free milk at 4 °C for an hour, the membrane was immersed in the primary antibody solution overnight at 4 °C. Antibodies included: anti-CHOP (1:400, Santa Cruz Biotechnology, USA), anti-GRP78 (1:2500, Abcam, United Kingdom), and anti-XBP1 (1:1200, Abcam, United Kingdom). After incubation with the primary antibody and washing with 1× Tris buffered saline and Tween (TBST) three times for 5 min, the membrane was incubated in the secondary antibody solution (1:10,000, Solarbio, China) at room temperature for 1 h. Film was applied to record the optical signal from each protein band after washing with 1× TBST 5 min for three times. A digital camera (SX720, Canon, Japan) and ImageJ software (National Institutes of Health, Bethesda, MD) were used to obtain images and calculate the optical density of each protein band.

Immunofluorescence (IF) analysis was used to determine the cellular locations of ERS-related proteins. After fixation with 4% paraformaldehyde (Sigma, USA) and dehydration with 30% sucrose (Sigma, USA) solution, spinal cord tissue was embedded and frozen in optimal cutting temperature compound (OCT) to produce 15 µm sections. The sections were permeated using phosphate buffer saline (PBS) with 0.5% TritonX-100, blocked with 10% donkey serum (Solarbio, China) for 1 h at room temperature, then incubated with a primary antibody for 12 h at 4 °C. Primary antibodies included: anti-CHOP (1:100, Santa Cruz Biotechnology, USA), anti-GRP78 (1:500, Abcam, United Kingdom), anti-XBP1 (1:170, Abcam, United Kingdom), anti-GFAP (1:800, Abcam, United Kingdom), anti-TUJ1 (1:1000, Abcam, United Kingdom) and anti-iba-1 (1:500, Abcam, United Kingdom). After washing three times with 0.1% PBST, the sections were incubated at room temperature in the dark with secondary antibodies: Alexa Fluor 488 (Green, 1:500, Abcam, United Kingdom), Alexa Fluor 594 (Red, 1:500, Abcam, United Kingdom), sections were quenched and washed three times using 0.1% PBST, then mounting medium with DAPI was added and sections were cover-slipped and sealed. Images were acquired using a laser scanning confocal fluorescence microscope (Olypus fv1000, Japan). All the frozen sections was made within 2 mm around the injury center, and the thickness of sections was set as 15 µm.

Ultrastructure of Endoplasmic Reticulum

Tissue samples to be analyzed by transmission electron microscope (TEM) were harvested at 24 h after molding around the injury center within 2 mm (anterior horn of the spinal cord and approximate volumes of 1 mm×1 mm×2 mm). Tissues were immersed into 2.5%
glutaraldehyde for 4 h immediately after harvest, washed with 0.1 M PBS three times for 15 min, blocked with 1% osmium tetroxide, and then dehydrated in gradient alcohol solutions and 100% acetone (washed three times for 20 min). Processed tissues were embedded with Epikoto 812 and sectioned at 50 nm. Finally, sections were stained with 3% uranium citrate and lead acetate and imaged by TEM (JEM-2100, Japan). Each group contained 3 rats and each rat was imaged 6 sections within 3 mm around lesion and each section detected 8 areas including neuron and glial cell.

**Statistical Analysis**

All data are presented as mean ± standard deviation (SD) and analyzed using GraphPad Prism 5.01 software (GraphPad Software, USA). Statistical significance was determined using 1-way ANOVA tests and relative expression of proteins was analyzed using the Student’s t test. p < 0.05 was considered as statistically significant difference.

**Results**

**Nec-1 Reduces Expression of ERS-Related Genes**

Significant ERS occurs after lesioning because of complicated pathophysiological changes in the affected tissues. Real-time quantitative PCR was used to determine ERS-related gene expression at 6 and 12 h after lesioning (Figs. 1, 2, n = 3). CHOP mRNA levels increased significantly in the control SCI group at both 6 and 12 h and decreased with treatment in the SCI+Nec-1 group (6 h, p < 0.001. 12 h, p < 0.05). CHOP mRNA level was also higher in the SCI+Nec-1 group than the sham+Nec-1 group at both time points (6 h, p < 0.01. 12 h, p < 0.01) (Fig. 1a, b). GRP78 mRNA also decreased when SCI was treated with Nec-1 (6 h, p < 0.01; 12 h, p < 0.01). Comparing the SCI+Nec-1 group with the sham+Nec-1 group, there was a significant difference at 12 h after lesioning (p < 0.001), but no significant difference at 6 h (p > 0.05) (Fig. 1c, d). The XBP1s mRNA level in the SCI group was higher than in SCI+Nec-1 group at both 6 and 12 h after lesioning (6 h, p < 0.05. 12 h, p < 0.05). There was a significant difference in XBP1s...

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Fig. 1  a–d show CHOP mRNA and GRP78 mRNA expression level at 6 and 12 h after lesioning in four groups. Results are expressed as mean ± SD. CHOP and GRP78 mRNA expression level increased after lesioning at 6 and 12 h in SCI group, but in SCI+Nec-1 group, Nec-1 resulted in attenuation of CHOP and GRP78 mRNA expression level comparing with SCI group (p < 0.05). Sham groups versus sham+Nec-1 group, Nec-1 did not affect the baseline CHOP and GRP78 mRNA expression level (p = NS)
mRNA levels between SCI+Nec-1 and sham+Nec-1 groups at 12 h after lesioning (p < 0.05), but no significant difference at 6 h (p > 0.05) (Fig. 2a–c).

**Expression and Localization of ERS-Related Proteins**

Western blot (WB) was used for the detection of ERS-related proteins at 12 and 24 h after lesioning (Fig. 3, n = 3). As shown in Fig. 3b–g, three ERS related protein expression in the SCI+Nec-1 group decreased relative to the SCI group at both 12 and 24 h (p < 0.05), but no statistical differences between sham and sham+Nec-1 group (p < 0.05). Comparing SCI group with sham+Nec-1 group, there was significant difference at 12 and 24 h after lesioning (p < 0.05). Comparing SCI+Nec-1 group with sham+Nec-1 group, there was no significant difference at 12 and 24 h after lesioning (p > 0.05). Figure 3a show the band of ERS related protein.

Immunofluorescence (IF) was used for localization analysis of ERS-related proteins at 24 h after lesioning (Figs. 4, 5, 6). IF images (Fig. 4a–i) show that GRP78 protein was mass expression in neuron and astrocyte cytoplasm, but in microglia was much fewer than neuron and astrocyte. IF images (Fig. 5a–i) reveal that CHOP protein was expression in neuron and astrocyte cytoplasm, but in microglia was fewer than in neuron and astrocyte. IF images (Fig. 6a–i) display that XBP1 protein was mass expression in neuron and astrocyte cytoplasm, but in microglia was fewer than in neuron and astrocyte.

**Nec-1 Preserves ER and Mitochondrial Ultra-Structure**

After lesioning, stress responses in the injured cells cause organelles to undergo changes in shape and function. In the sham and sham+Nec-1 groups, the neuron...
ER showed normal ultrastructure characterized by orderly rows with large numbers of ribosomes attached on membrane. However, in the SCI group, the neuron ER changed to a spherical shape with numerous shed ribosomes distributed around the membrane. The shape of the neuron ER in SCI+Nec-1 group was only slightly spherical and ribosome shedding was less significant than in the SCI group (Fig. 7a–d). ER of glial cells showed the same tendency as neurons (Fig. 7e–h). Each rat made 6 sections within 3 mm around lesion and each section detected 8 areas including neuron and glial cell. We find that both neuron and glial cell appear ERS and the extent of ERS between neuron and glial cell are no discrepancy.

The ultrastructure of mitochondria appeared normal in the sham and sham+Nec-1 groups, with a clear bilayer membrane, orderly rows of cristae and no swelling. In the SCI group, mitochondria structure was seriously damaged, as

Fig. 3  a–g show ERS-related protein (GRP78, CHOP and XBP1) expression levels. Results are expressed as mean ± SD. a shows western blot of three ERS-related proteins. b–g show administration of Nec-1, with all three ERS-related proteins reduced in the SCI+Nec-1 group relative to the SCI group at 12 and 24 h after lesioning (p < 0.05). Sham groups versus sham+Nec-1 group, Nec-1 did not affect the baseline ERS-related protein expression level (p = NS)
evidenced by the unclear appearance of the bilayer membrane and almost complete obliteration of the cristae. However, in the SCI+Nec-1 group, the mitochondria appeared almost normal, with a clear bilayer membrane and only minor cristae damage (Fig. 7i–l).

**Discussion**

Necroptosis has been researched in ischemia–reperfusion injury, transplantation immunology, degenerative diseases and some viral infection diseases [5, 19, 20]. Nec-1 is a small molecule inhibitor of necroptosis and therefore a candidate drug for the treatment of SCI [21, 22].

Although many studies have investigated the mechanism of the protective effect of Nec-1 after SCI, the relationship between Nec-1 and ERS has not been investigated. The present study has elucidated this relationship by detection of ERS-related genes and proteins and observation of the ultrastructural changes to the ER after SCI. Study have confirmed that ERS related gene (CHOP mRNA, GRP78 mRNA and XBP1 mRNA) expression raised at 6 h after SCI, reach the peak 12 h after lesioning and declined within 24 h [23]. In our study, we use Real-time PCR and RT-PCR

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**Fig. 4** a–i GRP78 localization in injury site. Double immunofluorescent staining of GRP78 (a, d, g), GFAP to label astrocytes (b), Iba-1 to label microglia (e), Tuj1 to label neurons (h). The merged images are presented in c, f and i. Anti-GRP78 was labeled with secondary antibodies coupled with Alexa Fluor 488 (green) and GFAP, Tuj1 and Iba-1 antibodies were detected using secondary antibodies coupled to Alexa Fluor 594 (red). The nuclei were visualized using DAPI in blue. White arrows show co-staining cells. Scale bar = 40 μm for all panels. (Color figure online)
to detection ERS related gene expression level at 6 and 12 h after lesioning. Our result show that Nec-1 take part in reg-ulation of ERS at early stage after lesioning and do not lead up-regulation of ERS gene in normal spinal cord and also could effective decline ERS related gene expression. We conducted WB to determine the ERS related protein (CHOP, GRP78 and XBP1) at 12 and 24 h after lesioning [24]. The result of WB show the ERS related protein have relation ship with necroptosis and the same as others study [25, 26]. Our study discover that Nec-1 could decline ERS related protein expression level. Another experimental also demonstrated that GRP78 declined after administrating Nec-1 in mouse ischemic brain injury model [16]. In our IF research, we found that the ERS related protein mostly expressed in neuron and astrocyte, but seldom expressed in microglia, the result was consistent with Matsuyama et al. [27]. TEM analysis of nerve cells revealed ultrastructural changes to the ER and mitochondria caused by SCI, but treatment with Nec-1 rescued the appearance of these organelles. The protective effect of Nec-1 for neuron ER ultrastructure matches the results obtained for mitochondria by Wang et al. [28].

![Fig. 5 a–i CHOP localization in injury site. Double immunofluorescent staining of CHOP (a, d, g), GFAP to label astrocytes (b), Iba-1 to label microglia (e), Tuj1 to label neurons (h). The merged images are presented in c, f and i. Anti-CHOP was labeled with secondary antibodies coupled with Alexa Fluor 488 (green) and GFAP, Tuj1 and Iba-1 antibodies were detected using secondary antibodies coupled to Alexa Fluor 594 (red). The nuclei were visualized using DAPI in blue. White arrows show co-staining cells. Scale bar = 40 μm for all panels. (Color figure online)
This work indicates that Nec-1 reduces ERS in neuron, astrocyte and microglia and protects the neuron ultra-structure of both the ER and mitochondria after rat SCI. Nec-1 is a promising candidate drug for treatment of SCI and warrants further study. Although this work has confirmed the protective effect of Nec-1 on ERS in neuron, astrocyte and oligodendrocyte, the detailed molecular mechanism connecting the necroptosome and three ERS pathways has not yet been elucidated. ER is calcium storage organelle, and previous studies have shown that Nec-1 helps maintain the normal mitochondrial calcium gradient [28]. It is unknown whether Nec-1 could restore the calcium concentration of ER after SCI. Future studies must investigate the relationship between necroptosome and calcium channel proteins and comprehensively determine the effectiveness of Nec-1 for treating SCI.

**Fig. 6**  
XBP1 localization in injury site. Double immunofluorescent staining of XBP1 (a, d, g), GFAP to label astrocytes (b), Iba-1 to label microglia (e), Tuj1 to label neurons (h). The merged images are presented in panels c, f and i. Anti-XBP1 was labeled with secondary antibodies coupled with Alexa Fluor 488 (green) and GFAP, Tuj1 and Iba-1 antibodies were detected using secondary antibodies coupled to Alexa Fluor 594 (red). The nuclei were visualized using DAPI in blue. White arrows show co-staining cells. Scale bar=40 μm for all panels. (Color figure online)
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References


Fig. 7  a–h neuron and glial cell ultrastructure changes of ER at 24 h after lesioning. a, b, e, f show Nec-1 has no influence on normal neuron and glial cell ER ultrastructure, while c, d, g, h show Nec-1 could mitigate neuron and glial cell ER ultrastructure injury after lesioning. Nec-1 could mitigate ER vacuole-like changes and ribosome shedding. i–l show neuron mitochondria ultrastructure changes at 24 h after lesioning. Comparing k and l find that application of Nec-1 restores the appearance of neuron mitochondria to nearly normal, with the bilayer membrane and cristae only slightly injured comparing with SCI group. Black scale bar = 500 nm, white scale bar = 200 nm.