Original Article
The change tendency of endoplasmic reticulum stress associated proteins in rats with spinal cord injury

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Abstract: To investigate endoplasmic reticulum (ER) stress reactions in spinal cord injury rats by evaluating the expression of the glucose-regulated protein 78 (GRP78), C/EBP homologous transcription factor protein (CHOP), X-box binding protein 1 (XBP1), Eif-2α and Bad. SCI models were established using adult female mice. After SCI, the expression of endoplasmic reticulum stress-induced apoptosis proteins were examined in the mice at specific time points using immunohistochemistry and western blot. The results of immunohistochemistry showed that in spinal cord gray matter, Chop, Grp78, XBP1, Eif-2α and Bad were specifically detected in the cytoplasm of the cell. Compare with the SCI group, there was little expression in normal group and sham group. The expression of ER stress-induced apoptosis proteins were significantly increased after spinal cord injury, and the absolute expression was higher than normal group (P < 0.05). Western-Blot results showed that compare with the SCI group, there were little expression of ER stress-induced apoptosis proteins in normal group and sham group. The expression of ER stress-induced apoptosis proteins were significantly increased after spinal cord injury, and the absolute expression was higher than normal group (P < 0.05). These results suggest that some ER stress-induced apoptosis proteins, such as Chop, Grp78, XBP1, Eif-2α and Bad, were activated after spinal cord injury, but the precise regulatory mechanisms remain unclear. In the future, understanding of the precise mechanism of ER stress-mediated apoptosis in SCI may lead to the development of novel treatment strategies.

Keywords: Spinal cord injury, Chop, Grp78, Eif-2α, endoplasmic reticulum stress-induced apoptosis

Introduction

Spinal cord injury (SCI) is a confused problem which represents huge healthy problem in modern society. The pathogenetic pathway of spinal cord injury include primary and secondary circulatory disorders and mechanical damage [1, 2]. The primary spinal cord injury is caused by external force, thus the injured part is function imbalanced [3]. The secondary injury, which is generated from the primary injury, is caused by potential complications, such as cardiac and respiratory dysfunctions, frequent infections in the bladder and kidneys, bowel problems, environment changes and improper nursing care [4]. Although the exact mechanism underlying secondary spinal cord damage remain unclear, a vast cascade of pathological events proceed during the secondary injury, such as neuronal apoptosis, ischemia, anoxia, edema, oxidative stress, vascular dysfunction and neurotoxicity. Several studies have demonstrated that these events may occur hours and days after the injury, and play an important role in mediating progressive spinal cord degeneration [5]. Some studies also showed that neuronal apoptosis occurs in the early stage of SCI, which can inhibit the neural axon regeneration, and is very important to the recovery of spinal cord. However, how to inhibit or slow down the neuronal apoptosis has not been fully clarified.

Endoplasmic reticulum (ER) is an intracellular organelle for the synthesis and folding of secret-
ED and membrane-bound proteins [6]. Endoplasmic reticulum stress (ERS) is one of many pathways which can induce apoptosis. Several apoptosis mediators are implicated in ER stress-associated cell death, such as glucose-regulated protein 78 (GRP78), the transcription activation of the C/EBP homologous transcrip-
tion factor (CHOP), the, the, and the activation of ER-associated caspase-12 [7]. When SCI happened, continuous ER stress eventually can induce neural apoptosis and the accumulated unfolded proteins can inhibit the protein synthesis depletion of Ca^{2+} from ER stores [8]. Although it has been confirmed that ER stress played an important role in SCI, some study had confirmed that the deletion of CHOP can't improve the condition of spinal cord injury [9] and the relative mechanism should be fur-
ther investigated.

In this study, we investigated the expression of some ER stress-associated proteins, such as Chop, Grp78, XBP1 and Elf-2α, in different time after SCI. Our results indicated that the ER stress-associated proteins increased after SCI, CHOP, GRP78 XBP1 and Elf-2α were involved in the early stage of SCI. These results may cer-
tainly help in understanding the basic events involved in ER stress-mediated cell survival/death signaling pathways and the molecular mechanism in the recovery of SCI.

Material and methods

Ethics statement

All experimental procedures confirmed with institutional guidelines for the care and use of laboratory animals in accordance with the NIH guidelines (NIH. Pub. No. 85-23, revised 1996) and approved by the Medical Ethics Committee of Shandong University.

Experimental animals

Healthy female Sprague-Dawley (SD) rats (wei-
ght, 200-220 g, 8-12 weeks old) were pur-
chased from the experimental animal center of Binzhou Medical University. Rats were main-
ER stress proteins and Spinal cord injury

established in the animal experimental center and four animals were housed per cage with a 12 h light/dark cycle. Room temperature was maintained at 23 ± 1°C, and all rats had free access to food and water.

Establishment of the SD rats spinal cord injury model

SD rats were randomly divided into three groups: Normal, Sham, and SCI group. The surgical procedure was carried out as previously described [3, 10]. Briefly, the Normal group was not treated. The rats in the SCI group were anesthetized by intraperitoneal injection with 4% chloral hydrate (1 mL/100 g) and laminectomy was performed at the T9-T10 spinal vertebrae, then, a 2 mm segment of spinal cord was removed using micro-scissors, and the completed transection was confirmed by visual verification. The rats in the sham group received the same surgical procedure except remove 2 mm segment of spinal cord.

Immunohistochemistry

Rats in sham and SCI group were anaesthetized with 3.5% chloral hydrate (1 ml/100 g) in different time after the operation. Then all rats were perfused with normal saline, followed by 4% PFA. Spinal cords were harvested, fixed overnight in 4% PFA at 4°C. Following this, 1.5 cm sections of the spinal cord, including the lesion/graft site, were embedded in paraffin, sectioned at a thickness of 5 μm. Then the paraffin section of different groups were incubated with primary mouse anti-GRP78 antibody (1:200 diluted by PBS, CST, USA), anti-CHOP antibody (1:200 diluted by PBS, CST, USA), anti-Eif-2α antibody (1:200 diluted by PBS, CST, USA), anti-XBP1 antibody (1:200 diluted by PBS, CST, USA), and anti-Bad antibody (1:200 diluted by PBS, CST, USA) at 37°C for 2 h, washed with PBS 5 times (3 min each time) at 4°C. After washing the sections, secondary antibody (1:400, Abcam, USA) were applied and incubated at 37°C for 1 h. Images were recorded using an microscope.

Western blot

The spinal cords in sham and SCI group were harvested in different time after the operation. The tissues were rinsed twice with ice-cold PBS and then lysed in RIPA lysis buffer containing

Figure 2. Overexpression of Grp78 after SCI. A. Spinal cord transected for different times were detected by immunostaining. Higher magnification view from the cells showed that Grp78 expressed in cells with a remarkable neuronal phenotype in SCI group than the normal and sham group, and it was mainly expressed in the cytoplasm of the cell. Scale bar: 200 μm, 100 μm, 50 μm. B. Analysis of the positive cells of the immunohistochemistry results. *Represents P < 0.05 versus normal group, **P < 0.01 versus normal group. Data are the mean values ± SEM, n = 4.
protease and phosphatase inhibitors. Tissue debris was cleared by centrifugation at 12,000 g for 10 min at 4°C. After concentration, total protein was detected using the BCA method (Beyotime, China), and protein extracts were boiled with SDS sample buffer (Beyotime). Western blotting analysis was carried out as described previously [5, zhangyuqiang]. Briefly, 25 μg of protein was subjected to SDS-PAGE and electro-transferred to PVDF membranes. After blocking with nonfat dry milk, the membranes were incubated with primary anti-GRP78 antibody (1:1000 diluted by TBST, CST, USA), anti-CHOP antibody (1:1000 diluted by TBST, CST, USA), anti-Eif-2α antibody (1:1000 diluted by TBST, CST, USA), anti-p-Eif-2α antibody (1:1000 diluted by TBST, CST, USA), anti-XBP1 antibody (1:1000 diluted by TBST, CST, USA), anti-Bad antibody (1:1000 diluted by TBST, CST, USA), anti-p-Bad antibody (1:1000 diluted by TBST, CST, USA) and anti-GAPDH antibody (1:1000 diluted by TBST, CST, USA) overnight at 4°C. The membranes were then incubated with secondary antibody (1:5000, diluted by TBST, Abcam, USA) after three rinses. The blot signal was detected using an ECL detection kit (Millipore, USA) and analyzed with Image J software.

**Statistical analysis**

Data were analyzed with SPSS 13.0 statistical software. All data in the figures were presented as mean ± SEM. Two-group comparisons were estimated by the Student’s T test. Multiple group comparisons were tested via one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test for a pairwise comparison, and the significance level was set at P < 0.05.

**Result**

*The expression of endoplasmic reticulum stress-induced apoptosis proteins at different group and different time after the spinal cord injury by immunohistochemistry*

To characterize whether ER stress-induced apoptosis proteins are expressed in SCI, we used immunohistochemistry to test their expres-
Expression in normal, sham and SCI rats (injured for 1 day, 3 day, 7 days, 14 days, 28 days). In spinal cord gray matter, Chop, Grp78, XBP1, Eif-2α and Bad were specifically detected in the cytoplasm of the cell. Compare with the SCI group, there was little expression in normal group and sham group. The expression of ER stress-induced apoptosis proteins were significantly increased after spinal cord injury, and the absolute expression was higher than normal group (P < 0.05) (Figures 1-5).

The expression of endoplasmic reticulum stress-induced apoptosis proteins at different group and different time after the spinal cord injury by Western Blot

According to the result of Western Blot, compare with the SCI group, there were little expression of ER stress-induced apoptosis proteins in normal group and sham group. The expression of ER stress-induced apoptosis proteins were significantly increased after spinal cord injury, and the absolute expression was higher than normal group (P < 0.05) (Figures 6-12).

Discussion

In this study, our results showed that the expression of ER stress-induced apoptosis proteins, such as Chop, Grp78, XBP1, Eif-2α and Bad, were significantly increased after spinal cord injury, and the absolute expression was higher than the normal group and the sham group. This finding demonstrated the relationship between endoplasmic reticulum stress and spinal cord injury.

When spinal cord injury occurred, the blood-brain barrier was disrupted, this reaction can induce a reactive process of secondary injury [11, 12]. The secondary damage will last for days and weeks, and leads to exacerbate the neurological dysfunction [13]. The apoptotic cells in the injured spinal cord play an important role in the early stage after SCI [14]. In our body, there were many stringent quality control systems, which employed to ensure the normal metabolism. The endoplasmic reticulum was responsible for the folding and maturation of newly synthesized transmembrane proteins.
and secretory proteins [15]. Recently, there has been growing interest in protein-modification disorders related to endoplasmic reticulum (ER) stress during delayed neuronal cell death

Figure 5. Overexpression of Bad after SCI. A. Spinal cord transected for different times were detected by immunostaining. Higher magnification view from the cells showed that Bad expressed in cells with a remarkable neuronal phenotype in SCI group than the normal and sham group, and it was mainly expressed in the cytoplasm of the cell. Scale bar: 200 μm, 100 μm, 50 μm. B. Analysis of the positive cells of the immunohistochemistry results. * Represents P < 0.05 versus normal group, **P < 0.01 versus normal group. Data are the mean values ± SEM, n = 4.

Figure 6. The expression of CHOP in different group by Western Blot. The expression of CHOP was significantly increased after spinal cord injury, and 1 day group was the highest expression, and the normal is the lowest. GAPDH was used as an internal loading control. Bar chart is the quantitation of relative expression of CHOP to GAPDH. * Represents P < 0.05 versus the normal group, **P < 0.01 versus the normal group. Data are the mean values ± SEM, n = 4.
ER stress proteins and Spinal cord injury

Figure 7. The expression of Grp78 in different group by Western Blot. The expression of Grp78 was significantly increased after spinal cord injury, and 7 day group was the highest expression, and the normal is the lowest. GAPDH was used as an internal loading control. Bar chart is the quantitation of relative expression of CHOP to GAPDH. *Represents $P < 0.05$ versus the normal group, **$P < 0.01$ versus the normal group. Data are the mean values ± SEM, $n = 4$.

Figure 8. The expression of XBP1 in different group by Western Blot. The expression of XBP1 was significantly increased after spinal cord injury, and 1 day group was the highest expression, and the normal is the lowest. GAPDH was used as an internal loading control. Bar chart is the quantitation of relative expression of CHOP to GAPDH. *Represents $P < 0.05$ versus the normal group, **$P < 0.01$ versus the normal group. Data are the mean values ± SEM, $n = 4$.

Figure 9. The expression of Eif-2α in different group by Western Blot. The expression of Eif-2α was significantly increased after spinal cord injury, and 1 day group was the highest expression, and the normal is the lowest. GAPDH was used as an internal loading control. Bar chart is the quantitation of relative expression of CHOP to GAPDH. *Represents $P < 0.05$ versus the normal group, **$P < 0.01$ versus the normal group. Data are the mean values ± SEM, $n = 4$. 
ER stress proteins and Spinal cord injury

Figure 10. The expression of p-Eif-2α in different group by Western Blot. The expression of p-Eif-2α was significantly increased after spinal cord injury, and 7 day group was the highest expression, and the normal is the lowest. GAPDH was used as an internal loading control. Bar chart is the quantitation of relative expression of CHOP to GAPDH. *Represents $P < 0.05$ versus the normal group, **$P < 0.01$ versus the normal group. Data are the mean values ± SEM, $n = 4$.

Figure 11. The expression of Bad in different group by Western Blot. The expression of Bad was significantly increased after spinal cord injury, and 7 day group was the highest expression, and the normal is the lowest. GAPDH was used as an internal loading control. Bar chart is the quantitation of relative expression of CHOP to GAPDH. *Represents $P < 0.05$ versus the normal group, **$P < 0.01$ versus the normal group. Data are the mean values ± SEM, $n = 4$.

Figure 12. The expression of p-Bad in different group by Western Blot. The expression of p-Bad was significantly increased after spinal cord injury, and 3 day group was the highest expression, and the normal is the lowest. GAPDH was used as an internal loading control. Bar chart is the quantitation of relative expression of CHOP to GAPDH. *Represents $P < 0.05$ versus the normal group, **$P < 0.01$ versus the normal group. Data are the mean values ± SEM, $n = 4$.
in various central nervous system conditions, such as cerebral ischemia, Alzheimer’s disease, Parkinson’s disease, brain injury and SCI [16, 17]. When ER stress occur, response unfolded proteins were eliminated via three pathways, many factors such as protein kinase-like ER kinase, activating transcription factor 6, Chop, Grp78 and inositol-requiring kinase, were involved in this reaction [18].

Chop is extensively existed in mammalian cells, and can be found in most mammalian cells and tissues. It has quite close relationship with cell proliferation, differentiation and apoptosis [19]. Grp78, the molecular chaperone, which was also called as BIP, were mainly located in the endoplasmic reticulum and can maintain the stable and balance of the endoplasmic reticulum [20]. Results from the our research demonstrated that the expression of Chop was significantly increased after spinal cord injury, some related factors including IRE1, PERK and ATF6, can activate Chop [21]. At the same time, Grp78 was also induced via the activating transcription factor 6 and IRE1α pathways to break down and eliminate unfolded proteins [22, 23]. When ER homeostasis was perturbed by intraluminal calcium, misfolded proteins accumulate, the Bip/Grp78s were released from the three sensors. Since the accumulation of unfolded proteins leads to apoptosis, the enhancement of Grp78 was a possible strategy to inhibit apoptotic cell death following neurotrauma and neurodegenerative disease [24].

Generally, when ERS happens, the oligomerization will happen on the endoplasmic reticulum and the phosphorylation would lead to the phosphorylation of Eif-2α, thus the p-eIF2α pathway would be formed which will inhibit and restrain the transcription of the mRNA, and the accumulated protein would be decreased [25]. In our study, the expression of Eif-2α and p-eIF2α were also significantly increased after spinal cord injury, which also indicated it was involved in the spinal cord injury. In addition to Eif-2α, XBP1 (X-box binding protein 1) were also activated in the transcription of ER chaperone genes. This is followed by the binding of the general transcription factor, nuclear factor Y, to the CCAAT part of the ERSE, leading to the activation of ER chaperone gene transcription [26].

In conclusion, we have shown that some ER stress-induced apoptosis proteins, such as Chop, Grp78, XBP1, Eif-2α and Bad, were activated after spinal cord injury, to re-establish cellular homeostasis, UPRs were activated at different levels. They also serve as apoptotic inducers that denote normal cells destined for death during persistent ER stress. However, the precise regulatory mechanisms remain unclear. In the future, understanding of the precise mechanism of ER stress-mediated apoptosis in SCI may lead to the development of novel treatment strategies.

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Disclosure of conflict of interest

None.

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