Original Paper

Ryanodine Receptor 2 Plays a Critical Role in Spinal Cord Injury via Induction of Oxidative Stress

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Key Words

Spinal cord injury • Ryanodine receptor 2 • Inflammation • Oxidative stress • NADPH oxidase 2

Abstract

Background/Aims: Spinal cord injury (SCI) is a severe health problem worldwide. Ryanodine receptors (RyRs) are a class of intracellular calcium channels in various excitable tissues such as muscles and nervous tissues. The current study was designed to investigate the possible role of RyR2 upregulation in SCI and to elucidate the possible molecular mechanisms. **Methods:** Rats were injected with LVshRNAi- RyR2 and then exposed to spinal cord contusion injury. **Results:** The results showed that knockdown of RyR2 significantly promoted the recovery of structural and functional injury in spinal cord, as evidenced by reduction of lesion volume and increase of Basso, Beattie and Bresnahan (BBB) and combined behavioral score (CBS) scores. Knockdown of RyR2 inhibited the increase of proinflammatory cytokines, including IL-1 β and TNF α . Moreover, downregulation of RyR2 increased oxygen consumption rate and decreased the expression of glucose-regulated protein 78 (GRP78), activating transcription factor 3 (ATF3) and ATF6, indicating the improvement of mitochondrial dysfunction and endoplasmic reticulum stress after SCI. Furthermore, silence of RyR2 reduced oxidative stress, as reflected by decrease of TBARS and GSSG content and increase of GSH level. The expression of NADPH oxidase 2 (NOX2), NOX4 and p66^{shc} were increased in SCI rats. Knockdown of RyR2 significantly decreased NOX2 expression, but had no evident effect on NOX4 and p66^{shc} expression. These results indicated NOX2 may be involved in RyR2-induced ROS generation which mediated contusion-induced spinal cord injury. **Conclusion:** The data provide novel insights into the mechanism of RyR2-mediated injury and the potential therapeutic targets for injury in spinal cord.

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Introduction

Spinal cord injury (SCI) is a severe health problem worldwide [1]. SCI could severely impair neural structure and function, resulting in reduction of quality of life in patients [2-5]. SCI is a biphasic injury process including primary and secondary injury. The primary injury, caused by initial physical impact, is characterized by acute bleeding and ischemia. After primary injury, secondary injury can be triggered by several mechanisms, including inflammation, oxidative stress, apoptosis and degeneration of neurons [6, 7]. To date, loss of neuron resulted by primary injury could not be reversed. Therefore, most of the attention has been paid on molecular mechanism and therapeutic strategy of the secondary injury. However, the molecular mechanism of secondary damage after SCI is still largely unknown.

Ryanodine receptors (RyRs) are a class of intracellular calcium channels in various excitable tissues such as muscles and nervous tissues [8-10]. RyRs consist of three major isoforms (RyR1, 2, and 3), which are involved in calcium release from different intracellular organelles via differential signaling pathways [11]. RyRs mediate the release of calcium ions from the sarcoplasmic reticulum and endoplasmic reticulum [12]. Calcium influx and elevation of intracellular free calcium activate degenerative enzymes, causing cell injury and death after trauma. Blockade of calcium release through RyRs confers significant improvement of SCI [13]. Moreover, RyR2 expression in astrocyte and axons is markedly increased after hypoxic injury of spinal cord dorsal column [14]. However, mechanisms underlying RyR2-associated development of SCI are not clear.

The current study was designed to investigate role of RyR2 upregulation in SCI and to elucidate the possible molecular mechanisms. We found that knockdown of RyR2 decreased NADPH oxidase 2 (NOX2) expression, reduced oxidative stress, improved mitochondrial dysfunction and endoplasmic reticulum (ER) stress, prohibited inflammation, resulting improved recovery of SCI. We suggest that RyR2 play an important role in SCI through regulation of redox state and thus mitochondrial and ER function and inflammation.

Materials and Methods

Chemicals and reagents

β-actin and RyR2 antibodies were obtained from Santa Cruz. GRP78, ATF3 and ATF6 antibodies were obtained from Cell Signaling Technology. Most of the other reagents were obtained from Sigma.

Animal treatment

Adult male Sprang-Dawley rats (200-250 g) were purchased from the Experimental Animals Center of Fourth Military Medical University. All animal experiments were conducted in accordance with the National Institute of Health Guide on the Care and Use of Laboratory Animals and were approved by the Laboratory Animal Users Committee at Fourth Military Medical University. All animals were housed in individual cages in a temperature and light ± dark cycle controlled environment with free access to food and water.

A midthoracic (T9) laminectomy was conducted in rats and LVshRNAi-RyR2 or LV-Ctrl was injected (3×10^7 transducing units in 1 μl) at both the rostral and caudal ends of the laminectomy site using a Hamilton syringe (Hamilton, Reno, NV, USA) with a bevelled needle as previously reported [15]. During the injection, the syringe was inserted to 1-2 mm beneath the dorsal surface of the spinal cord, and 2 μl of LV was injected at a rate of 1 μl per 15 min by a Legato 130 Syringe Pump (KD Scientific, Holliston, MA, USA). Rats in the sham group received a laminectomy without injection (n=10). After the injection, rats were monitored for 1 week to ensure viral incorporation and efficiency of knockdown of target gene. 2 days after injection, functional observation was conducted using locomotor testing to evaluate whether there was evident damage caused by the operation. After 1 week recovery, the laminectomy site was exposed and a moderate spinal cord contusion injury was established by dropping a 10-g weight from a height of 5 cm onto an NYU impactor positioned on the exposed site [16-18]. Rats were sacrificed at 1 day (n=8/group), 3 days (n=8/group), 7 days (n=8/group), 14 days (n=8/group), 21 days (n=8/group) and 28 days (n=8/group) after injury. After the treatment, a 5-mm spinal cord segment, 2.5 mm rostral and 2.5 mm caudal to the lesion epicenter, was collected and used for the following detections, respectively.

Assessment of motor function

Locomotor function of hind limb was assessed using the Basso, Beattie and Bresnahan (BBB) rating scale [19]. A combined behavioral score (CBS) was also conducted to evaluate neurological functional deficits as previously described [20, 21]. Two independent and well-trained investigators blinded to treatment conducted the experiments and scored the locomotor function and neurological function at 7, 14, 21 and 28 days after the injury [22].

Determination of proinflammatory cytokines

The spinal cord tissues were homogenated and the content of IL-1 β and TNF α in spinal cord tissue homogenates were measured using ELISA kits according to the manufacturers' instructions (Abcam).

Protein extraction and western blot

Total protein was extracted using RIPA lysis buffer according to the manufacturer's protocols. Protein concentrations in supernatants were measured using a Bradford protein assay kit (Pierce, USA). About 20 μ g of protein lysis was separated using SDS-PAGE. Then, protein was transferred to PVDF membranes (Millipore, MA, USA), which were then blocked with 5 % non-fat milk. Membranes were incubated with primary antibodies overnight at 4 °C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce, USA) at 37 °C for 1 h. Protein bands were visualized with the ECL and captured using BIORAD Imaging Systems (BIORAD, CA, USA).

Real-time PCR

Total RNA was extracted using the Trizol method, according to the manufacturer's instructions (Invitrogen, USA). After reverse transcription, quantitative real-time PCR (qRT-PCR) was analyzed in a BIORAD CFX96 Real-Time System, using SYBR Premix Ex Taq^{TM} II (Takara, Japan). The expression of target genes was normalized to β -actin and the gene expression was comparatively analyzed using the $2^{-\Delta\Delta Ct}$ method.

Oxygen consumption

The spinal cord tissues were homogenated and oxygen consumption was recorded for 10 min using a Clark Oxygen Electrode. Oxygen consumption rate was calculated and expressed as percentage of remaining oxygen in the reaction mixture.

Assessment of oxidative stress

The spinal cord tissues were homogenated and the content of TBA-reactive substances (TBARS), glutathione (GSH) and oxidized form of GSH (GSSG) levels were measured using commercial kits according to the manufacturers' instructions (Nanjing Jiancheng, China).

Statistical analysis

The results were presented as means $\pm$ SEM. Results were analyzed using GraphPad Software. For two independent group comparisons (lesion volume, BBB and CBS scores), student t-test was conducted. The statistical significance of differences between three groups (proinflammatory cytokines, oxygen consumption rate, and oxidative stress-related parameters) was analyzed via one-way analysis of variance followed by a Dunnett's t-test for multiple comparisons. A P value <0.05 was considered to be statistically significant.

Results

Knockdown of RyR2 facilitates the recovery after SCI

To investigate the role of RyR2 in SCI, rats were injected with lentivirus-mediated shRNAi before the injury operation. In Fig. 1, we showed that 1, 14 and 28 days after the injury, RyR2 expression was significantly increased, compared with that of Sham group. In LVshRNAi-RyR2 group, expression of RyR2 in spinal cord was notably prohibited (Fig. 1). The results indicated that LVshRNAi-mediated knockdown of RyR2 was significant.

Fig. 1. Effect of knockdown of RyR2 on RyR2 expression during spinal cord recovery. At 1, 14, and 28 days post-injury, western blot was conducted to determine protein expression of RyR2 in spinal cord. * $P < 0.05$, compared with Sham group. ** $P < 0.05$, compared with the LV-Ctrl group.

Fig. 2. Effect of knockdown of RyR2 on lesion volume and locomotor function recovery. (A) 28 days after the injury, stereological assessment was performed to assess lesion volume and quantitative analysis of lesion volume was showed. (B and C) At 7, 14, 21 and 28 days post-injury, the Basso-Beattie-Bresnahan (BBB) score (B) and combined behavioral score (CBS) (C) of rats were observed. * $P < 0.05$, compared with the LV-Ctrl group.

As shown in Fig. 2A, LVshRNAi-RyR2 treatment notably decreased the lesion volume of spinal cord in rats. In addition, the results of locomotor and neurological function showed that knockdown of RyR2 markedly increased the BBB (Fig. 2B) and CBS (Fig. 2C) scores, compared with LV-Ctrl groups. These results indicated that upregulation of RyR2 was involved in contusion-induced SCI and knockdown of RyR2 could facilitate the recovery after SCI.

Knockdown of RyR2 inhibits inflammation after SCI

To evaluate potential effects of RyR2 on inflammation after SCI, proinflammatory cytokines were determined. As reflected in Fig. 3, IL-1 β and TNF α levels after SCI were significantly higher than that of Sham group and knockdown of RyR2 remarkably inhibited the increase of IL-1 β and TNF α levels. The results demonstrated that upregulation of RyR2 was involved in SCI-associated inflammation.

Fig. 3. Effect of knockdown of RyR2 on inflammation in spinal cord. 28 days after the injury, the spinal cord tissues were homogenated and the content of IL-1 β (A) and TNF α (B) in spinal cord tissue homogenates were measured using ELISA kits. *P<0.05, compared with Sham group. **P<0.05, compared with the LV-Ctrl group.

Fig. 4. Effect of knockdown of RyR2 on mitochondrial and ER function in spinal cord. 28 days after the injury, the spinal cord tissues were collected. (A) Oxygen consumption rate of spinal cord tissue was evaluated using an oxygen electrode. (B) Protein expression of GRP78, ATF3 and ATF6 in spinal cord tissue was determined by western blot. *P<0.05, compared with Sham group. **P<0.05, compared with the LV-Ctrl group.

Knockdown of RyR2 improves mitochondrial dysfunction and ER stress after SCI

Next, we examine the possible effect of knockdown of RyR2 on mitochondrial and ER function. In Fig. 4A, we showed that contusion injury significantly decreased the oxygen consumption rate, compared with that of Sham group. In contrast, knockdown of RyR2 markedly inhibited the decrease of oxygen consumption rate in LV-Ctrl group. The results implicated that contusion-associated upregulation of RyR2 mediated mitochondrial dysfunction and knockdown of RyR2 prevented mitochondrial dysfunction. In addition, ER function was evaluated by the detection of sensitive markers. In Fig. 4B, we showed that expression of glucose-regulated protein 78 (GRP78), activating transcription factor 3 (ATF3) and ATF6 were significantly increased in rats with SCI, compared with that in Sham group. In LVshRNAi-RyR2 group, the increase of GRP78, ATF3 and ATF6 expression was significantly prohibited (Fig. 4C). The data demonstrated that upregulation of RyR2 may be involved in contusion-induced ER stress.

Knockdown of RyR2 reduces oxidative stress after SCI

As shown in Fig. 5A, TBARS content was significantly increased after SCI, compared with that of Sham group. Knockdown of RyR2 markedly inhibited the increase of TBARS (Fig. 5A).

Fig. 5. Effect of knockdown of RyR2 on oxidative stress in spinal cord. 28 days after the injury, the spinal cord tissues were homogenated and the content of TBARS (A), GSH (B) and GSSG (C) levels were determined by commercial assay kits. mRNA expression of NOX2, NOX4 and P66^{shc} was measured by real-time PCR. *P<0.05, compared with Sham group. **P<0.05, compared with the LV-Ctrl group.

Moreover, GSH level was markedly reduced after SCI, which was inhibited by knockdown of RyR2 (Fig. 5B). The alteration of GSSG was opposite to that of GSH (Fig. 5C). The results indicated that contusion resulted in significant oxidative stress which may contribute to SCI and upregulation of RyR2 was involved in contusion-associated oxidative stress in spinal cord.

To explore the mechanism of RyR2-mediated oxidative stress, several key factors that are responsible for reactive oxygen species (ROS) generation were determined. As shown in Fig. 5, NADPH oxidase 2 (NOX2), NOX4 and p66^{shc} mRNA expression was measured by real-time PCR. mRNA expression of NOX2 in rats with SCI was significantly increased, compared with that of Sham group (Fig. 5A). Knockdown of RyR2 markedly inhibited the increase of NOX2 expression (Fig. 5D). In addition, NOX4 and p66^{shc} mRNA expression were also increased by contusion in spinal cord (Fig. 5E and F). However, no significant effect of knockdown of RyR2 on NOX4 and p66^{shc} mRNA expression was observed (Fig. 5E and F). The results implicated that NOX2 may be involved in RyR2-mediated oxidative stress through ROS generation.

Discussion

Previous studies have shown that RyR2 expression is markedly increased after hypoxic injury of spinal cord dorsal column [14]. However, role of RyR2 in traumatic injury to spinal cord was largely unknown. In the present study, we knocked down the expression of RyR2 using LV construct and examined the effect of downregulation of RyR2 on SCI recovery. We found that knockdown of RyR2 significantly promoted the recovery of structural and functional injury in spinal cord, as evidenced by reduction of lesion volume and increase of BBB and CBS scores. Inflammation plays an important role in the injury of spinal cord after trauma and reduction of inflammation was considered to be an important intervention for SCI therapy [23, 24]. Since downregulation of RyR2 prohibited the increase of proinflammatory

Fig. 6. Schematic mechanism of role of RyR2 in spinal cord injury.

cytokines IL-1 β and TNF α , it was indicated that RyR2 was involved in the development of inflammation, which contributed to traumatic injury of spinal cord.

To further explore the mechanism of RyR2-associated reduction of inflammation and improvement of SCI, mitochondrial and ER function was evaluated. Mitochondrial dysfunction is an important pathophysiological process that is associated with various diseases. Especially, mitochondrial dysfunction is believed to be an important contributor to SCI [25-27]. In addition, ER stress participates in various types of damage after traumatic injury of spinal cord [28-30]. In the current study, we found that downregulation of RyR2 significantly ameliorated mitochondrial dysfunction and ER stress, as evidenced by increased oxygen consumption rate and decreased expression of GRP78, ATF3 and ATF6.

Under a variety of conditions, mitochondrial dysfunction and ER stress are closely associated with excessive ROS generation, also called oxidative stress. Oxidative stress was considered to be a central event in the development of SCI [31-34]. Activation of RyR2 contributes to excessive ROS generation which in turn results in mitochondrial dysfunction and ER stress [35-37]. In the present study, we further examined the effect of downregulation of RyR2 on oxidative stress. We showed that knockdown of RyR2 significantly reduced oxidative stress, as evidenced by decrease of TBARS and GSSG content and increase of GSH content. Furthermore, we also tested the possible source of ROS generation induced by RyR2. NOX2 and NOX4 are important ROS-generating enzymes mainly locating at membranes [38, 39]. P66^{shc} is a novel redox adaptor that could generate ROS in mitochondria [40]. We found that NOX2, NOX4 and p66^{shc} were increased in SCI rats. It was previously found that inhibition of NOX2 reduced locomotor impairment, inflammation, and oxidative stress after SCI [41]. In the study, we found that knockdown of RyR2 significantly decreased NOX2 expression, but had no evident effect on NOX4 and p66^{shc} expression. These results indicated NOX2 may be involved in RyR2-induced ROS generation which mediated contusion-induced SCI.

In conclusion, we show that knockdown of RyR2 decreased NOX2 expression, reduced oxidative stress, ameliorated mitochondrial dysfunction and ER stress, prohibited inflammation, resulting in improved recovery of SCI. Given the special role of RyRs in mediating the release of calcium ions from intercellular storage room and the fact that calcium could stimulate NOX2 [42], we speculate that calcium may participate in RyR2-induced regulation of NOX2 in SCI. Further studies are needed to testify that hypothesis. Overall, we suggest that RyR2 play an important role in SCI through NOX2-mediated regulation of redox state and thus mitochondrial and ER dysfunction and inflammation (Fig. 6). The data provide novel insights into the mechanism of RyR2-mediated injury and the potential therapeutic targets for injury in spinal cord.

Acknowledgments

This work was supported by National Natural Science Foundation of China (No. 81372045).

Disclosure Statement

The authors declare that there are no conflicts of interest.

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