Original Article

Autophagy plays a protective role in motor neuron degeneration following spinal cord ischemia/reperfusion-induced spastic paralysis

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Abstract: Spinal cord ischemia and reperfusion (SCIR) injury can lead to neurologic dysfunction and paraplegia, which are serious complications after shock or thoracoabdominal aortic surgery. Autophagy is a fundamental cellular process in eukaryotes, and homeostasis of autophagic activities in the cytoplasm is critical for the maintenance of neuronal function. To date, no studies have addressed the involvement of autophagy in the regulation of motor neurons in the ventral horn of the spinal cord area following SCIR-induced spastic paralysis or the underlying mechanisms of this process. In this study, we investigated spastic paralysis in rats following SCIR injury. The number of autophagosomes increased 3 h following the injury, and subsequently decreased slowly to near-normal levels in the sham group as indicated by the autophagy markers microtubule-associated protein 1 light chain 3 (LC3), beclin-1, and p62. Furthermore, after treatment with the autophagy inhibitor 3-Methyladenine (3-MA) and autophagy activator rapamycin following SCIR, autophagy in the SCIR-3-MA group decreased significantly, while that in the SCIR-Rap group increased, compared with SCIR-DMSO controls group. Moreover, the assessment of motor neurons function, using Reuter’s score and motor evoked potentials (MEP) and somatosensory evoked potentials (SEP), indicated that promoting autophagy reduced scores compared with SCIR controls, while inhibiting autophagy increased the scores, and hence motor neurons function. Autophagy in the SCIR model protected motor neurons function and morphology. These results would provide more evidences for better understanding function of autophagy in motor neurons degeneration and mechanisms underlying spastic paralysis. Autophagy would be a novel target for prevention and therapy in SCIR damage.

Keywords: Spinal cord ischemia reperfusion injury, spastic paralysis, motor neurons, autophagy, rapamycin and 3-MA

Introduction

Spinal cord ischemia and reperfusion (SCIR) injury causes paraplegia, which is a serious complication after thoracic or thoracoabdominal aortic surgical interventions [1]. This complication has been attributed to temporary or permanent ischemia of the spinal cord by interruption of the blood supply. Prolonged clamp times, rupture and dissection are related to incidence of paraplegia [2]. The complications incidence rate of spinal cord caused by ischemia/reperfusion are reported higher than 10% [3], and the patients with SCIR have a poor prognosis, usually resulting in paralysis and mortality, which places burdens on family and society [4]. The progressive nature of the injury and the delayed occurrence of the complication suggest that the pathological mechanisms are associated with not only the initial ischemic injury but also ongoing insults after the reperfusion phase.

Motor neurons are nerve cells that connect the ventral horn of the spinal cord to effector organs, mainly muscles and glands, either directly or indirectly. Motor neurons transfer signals from the spinal cord to the effectors to produce an action. The progressive degeneration and death of motor neurons in the brain and spinal cord lead to prototypical neurodegenerative diseases, such as amyotrophic lateral sclerosis, secondary denervation, and muscle wasting (amyotrophic) [5]. After spinal cord injury,
motor neurons degenerate in a time-dependent fashion according to the neurotransmitters’ deregulation [6].

Autophagy is a fundamental cellular process in eukaryotes that enhances the ability of cells to respond and adapt to environmental changes [7]. During the autophagy process, an autophagosome is formed to isolate targeted or non-specific materials within a double-membrane-bound vesicle. The subsequent fusion of the autophagosome with endolysosomal vesicles leads to enzymatic degradation and recycling of the sequestered substrates [8]. A crucial function of autophagy is to breakdown macromolecules such as proteins to provide amino acids and other factors necessary to generate energy and to synthesize new proteins [9]. The ability to capture large materials distinguishes this pathway from proteasomal degradation, making autophagy necessary for maintaining cellular homeostasis. Many of the substrates found within autophagosomes are those that threaten cell viability, and include damaged organelles, protein aggregates, and intracellular pathogens [7, 10]. Autophagosome formation requires the localization of phosphatidylethanolamine conjugated microtubule-associated protein 1 light chain 3 (LC3) to the autophagosomal membrane [11]. Both excessive activation of autophagy and its inhibition by inhibitory agents may lead to cell death [12, 13].

Ischemia and reperfusion injury in other organ systems have been studied more extensively than those in the spinal cord. Therefore, only few studies have addressed the function of autophagy in SCIR-induced spastic paralysis. In the present study, we aimed to illustrate the function of autophagy in protecting motor neurons degeneration.

Materials and methods

Spinal cord ischemia-reperfusion injury model

The Animal Use and Care Committee of School of Medicine, Third Military Medical University approved all experiments, and all surgical interventions and postoperative animal care conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. A total of 72 adult Sprague-Dawley SD rats (210±20 g) were supplied by the Animal Breeding Center of Institute of Surgery Research/Daping Hospital. The rats had no any neurological intact before operation. The spinal cord ischemia-reperfusion injury model was established according to the previous study [4]. To established spinal cord ischemia-reperfusion injury model, rats were divided normal group and ischemia-reperfusion group (SCIR). In the drug treatment group, 3-MA (50 mg/kg) and rapamycin (1 mg/kg), DMSO (an equivalent volume of vehicle) were intraperitoneal injected 4 hours, 1 day and 2 days after surgery, respectively. In SCIR group, rats were anesthetized with anintraperitoneal injection of 4% sodium pentobarbital with an dose of 50 mg/kg. Ischemia of the spinal cord was produced by occlusion of aortic arch for 14 min modified from previous study [4]. In the sham group, the aortic arch was exposed with no occlusion.

Immunofluorescence

The immunofluorescence staining was used to observe autophagy after SCIR through the expression of beclin-1 and P62. In brief, spinal cord segment containing the entire injury site 3-6 mm rostral and 3-6 mm caudal was fixed and sectioned into 20 μm slice with a Leica CM1900 cryostat. After blocked with 10% bovine serum albumin for 1 h at room temperature, sections were incubated with the primary antibody, anti-beclin-1 (Abcam, ab62557, 1:400, USA) and anti-p62 (Sigma-Aldrich, P0067, 1:500) at overnight 4°C, respectively. After incubation with fluorescent-conjugated secondary antibodies (FITC, 1:200; ZSGB-Bio, China) for 1 hour at 37°C, sections were washed 3 times in PBS followed by 4’,6-diamidino-2-phenylindole (DAPI) incubation for 5 min. All images were captured at the ventral horn of spinal cord using a laser scanning confocal microscope (Leica SP-2, Germany).

Western blotting

Spinal cord tissues were homogenized in ice-cold lysis buffer containing 1 mM EDTA, 20 mM Tris-HCl (pH7.5), 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl₂, and a protease inhibitor cocktail (1:100) (Pierce, Rockford, IL, USA), then centrifuged at 12, 000 rpm for 20 min at 4°C. Protein concentration of the supernatant from extract was measured with BCA assay kit (Beyotime, Beijing, China). Equivalent amounts of proteins were loaded on SDS-PAGE and transferred to PVDF mem-
branes (Millipore, Bedford, MA). The membranes were blocked in TBS Tris-Tween 20 and probed with anti-LC3 II (1:1000; L7543, Sigma-Aldrich, USA), anti LC3 I (1:1000; Sigma-Aldrich, USA), anti-beclin-1 (Abcam, ab62557, 1:400, USA) or anti-p62 (1:1000; N05114, Cell signaling, USA). After washing, the membranes were incubated with secondary antibodies (1:10000, zombie, Beijing, China) for 60 min at room temperature. All blots were probed with antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:3000. Millipore, Bedford, MA, USA) as loading control. Semi-quantitation of scanned image was analyzed as previous study indicated [14].

Neurologic function assessment

Neurologic function was assessed according to the method of Reuter’s scores [15], observed at 1 day, 3 days, and 7 days after the procedure. Animals were scored with a 11-point scale: 11: hind-limb complete paralysis, 0: normal function; the higher score indicates more severe dysfunction. Blind scoring ensured that two observers without knowledge of the treatment received by individual animals.

To evaluate the sensory and motor function recovery of hind limb, somatosensory evoked potential (SEP) and motor evoked potential (MEP) were recorded at 7 d after reperfusion. Rats were placed in a stereotaxic frame to make the skull horizontal between the bregma and lambda. A 3-4 mm diameter hole drilled in the cranium and somatosensory and motor cortex of right hemisphere were exposed. Stereotaxic coordinates were measured from bregma and calculated using rat atlas [16] (SEP: P.-0.42-2.20 mm; L. 1.50-3.00 mm; MEP: P.-0.48-3.14 mm; L. 1.00-3.00 mm). SEP were recorded from right somatosensory cortex with electrical stimulation of left sciatic nerve. MEP were recorded from left sciatic nerve right with electrical stimulation of right motor cortex. The electrical stimulation was produced by a constant stimulator (AD Instruments, Australia) with parameter (4HZ, 4.5 mA 0.2 ms duration). Signals from metal microelectrode were band-pass filtered (2-2000 HZ) and recorded digitally by computer (PowerLab system/16SP, AD Instruments). Three repeats of SEP and MEP measurement were taken on each rat. The latency from the first prominent peak were evaluated by software (Scope 3.7.1).

Statistical analysis

A chi-square test or Fisher’s exact test was used to compare the differences in categorical variables. One-way analysis of variance (ANOVA) was used to analyze differences in continuous variables and multiple comparisons were used to compare each two groups. Values are expressed as the mean ± standard error of the mean. SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to analyze all data. P<0.05 was regarded as statistically significant.

Results

Autophagy was induced in the rat spinal cord after SCIR

To observe the autophagy phenomenon in the ventral horn of the spinal cord, tissue transections at 3 h, 6 h, 1 d, 3 d, and 7 d post injury (dpi) were stained by immunofluorescence against anti-beclin-1 or anti-p62 primary antibodies and DAPI. Beclin-1 is a marker of autophagosome formation, while p62 binds to the autophagosomal membrane proteins LC3 and autophagy-related protein 8 (Atg8), bringing p62-containing protein aggregates to the autophagosome. We observed a higher signal of beclin-1 in motor neurons in the ventral horn in injured rats compared with sham controls (Figure 1A). Furthermore, the rate of autophagy progressively decreased in the SCIR group after injury. Similar to beclin-1, the higher percentage of p62-positive cells indicated that autophagy increased significantly shortly after SCIR, and subsequently decreased slowly (Figure 1B). Moreover, we detected autophagy markers.
Autophagy protect motor neuron from degeneration

Including LC3, beclin-1, and p62 in the tissue lysates of both sham and SCIR groups by western blot (Figure 1C, Supplemental Figure 1). LC3 is cleaved at its C-terminus by Atg4 to form LC3-I, which is covalently conjugated to phosphatidylethanolamine to form LC3-II. LC3-II is specifically targeted to Atg5-12 associated and expanded phagophores, and remains associated with autophagosomes even after fusion with lysosomes. After normalization to GAPDH, we found that LC3 II/LC3 I ratio (Figure 1D), beclin-1 (Figure 1E), and p62 (Figure 1F) increased significantly after SCIR injury and subsequently decreased to normal levels as in the sham group at 7 dpi. These results suggested that autophagy increased following SCIR injury and subsequently decreased gradually to near-normal levels.

Rapamycin induced autophagy in the rat spinal cord after SCIR

Given that autophagy is a protective process for cell survival, we treated the rats with the

Figure 1. Autophagy was induced in the rat spinal cord after SCIR. (A, B) Immunofluorescence detection of autophagy marker Beclin-1 (A) and p62 (B) in the ventral horn of spinal cord area after SCIR. The nuclear was stained by DAPI (Blue). Ischemia of the spinal cord was produced by occlusion of aortic arch for 14 min (SCIR group) or no occlusion (sham). The tissues from control (sham), and 3 h, 6 h, 1 d, 3 d and 7 d post operation were collected and stained as indicated. Scale bar: 150 μm. (C) Expression of the autophagy marker LC3 II, LC3 I, Beclin-1 and p62 were detected by western blot in sham and indicated time post SCIR as treated in (A, B). GAPDH was loading control. (D-F) LC3 II/LC3 I (D), Beclin-1 (E) and p62 (F) expression levels were expressed in the histograms as fold changes in treated versus sham sample, after GAPDH normalization. All histograms show the mean values of three independent experiments. Bars indicate means ± std. (n=3, **P<0.01, compared with sham group).
Autophagy protect motor neuron from degeneration

Figure 2. Rapamycin induced autophagy in the rat spinal cord after SCIR at 3 days. (A) Immunofluorescence detection of autophagy marker Beclin-1 and p62 (Green) in the ventral horn of spinal cord area. Ischemia of the spinal cord was produced by occlusion of aortic arch for 14 min (SCIR group) or no occlusion (sham). 3-MA (50 mg/kg) and rapamycin (1 mg/kg), DMSO (an equivalent volume of vehicle) were intraperitoneal injected 4 hours, 1 day and 2 days after surgery, respectively. The nuclear was stained by DAPI (Blue). The tissue from control (sham), SCIR group and SCIR group treated by DMSO (solvent), autophagy inhibitor 3-MA or autophagy activator Rapamycin were collected 3 days after surgery and stained as indicated. Scale bar: 150 μm. (B) Expression of the autophagy marker LC3 II, LC3 I, Beclin-1 and p62 were detected by western blot after SCIR as treated in (A). GAPDH was loading control. (C-E) LC3 II/LC3 I (C), Beclin-1 (D) and p62 (E) expression levels were expressed in the histograms as fold changes in treated versus sham sample, after GAPDH normalization. All histograms show the mean values of three independent experiments. Bars indicate means ± std. (n=3, **P<0.01, compared with sham group; ###P<0.01, compared with SCIR-DMSO group).

autophagy promoter rapamycin (SCIR-Rap group) and autophagy inhibitor 3-Methyladenine (3-MA) (SCIR-3-MA group) 4 hours, 1 day and 2 days after SCIR injury. We found that the rate of autophagy in motor neurons in the SCIR-3-MA group was much lower than that in the control
Autophagy protect motor neuron from degeneration

Group treated with DMSO (SCIR-DMSO group), while rapamycin promoted autophagy in the SCIR-Rap group (Figure 2A), which was most obvious at 3 days after injury. Similar to beclin-1, the percentage of p62-positive cells indicated that autophagy increased significantly shortly after SCIR, and either decreased subsequently after treatment with the inhibitor 3-MA or increased after autophagy activation by rapamycin (Figure 2A). Moreover, we detected autophagy markers in the tissue lysates by western blot (Figure 2B, Supplemental Figure 2). Compared with the SCIR-DMSO control group, LC3 II/LC3 I ratio (Figure 2C), beclin-1 (Figure 2D), and p62 (Figure 2E) were downregulated significantly after 3-MA treatment following SCIR injury, while they were upregulated after rapamycin treatment. These results suggested that 3-MA significantly repressed autophagy, while rapamycin promoted autophagy after SCIR injury.

*Induction of autophagy mitigate SCIR-induced spastic paralysis in rats*

Paraplegia was identified according to the Jacobs score, and the percentage of paraplegia in injured groups were higher than that in sham

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Figure 3. Induction of autophagy mitigate SCIR-induced spastic paralysis in rats. (A) Neurologic function was assessed according to Reuter’s score at 1 day, 3 days and 7 days post occlusion of aortic arch for 14 min (SCIR group) or no occlusion (sham), and 3-MA (50 mg/kg), rapamycin (1 mg/kg) or DMSO (an equivalent volume of vehicle) were intraperitoneal injected 4 hours, 1 day and 2 days after surgery, respectively. 0 indicates normal state and 11 indicates most severe dysfunction state in Reuter’s score. (B) Spastic paralysis was identified after treatment as in (A) through stretch reflex: 0, normal; 1, slightly increase/decrease; 2, excite/disappear; and muscular tension: 0, normal; 1, high/low tension; 2, flaccid/spastic. (C-E) Motor evoked potentials (MEP) and somatosensory evoked potentials (SEP) were examined 7 days post-operation (C); the N1 wave latency of MEP (D) and SEP (E) was analyzed. (mean ± std, n=8, **P<0.01, compared with sham group; ##P<0.01, compared with SCIR-DMSO group).
Autophagy protect motor neuron from degeneration

control group, while no significant difference in percentage of paraplegia was observed SCIR groups, including drugs treated groups. As rapamycin activated autophagy in the SCIR rat model, we evaluated neurological function delicately using Reuter’s score (0-11 point-scale). At 1 dpi, the mean Reuter’s score was 10.38±0.79, followed by gradual recovery at 3 and 7 dpi in the SCIR group. In addition to urinary and fecal incontinence, limb rigidity was observed, especially of the flexor and extensor muscles, which were both highly tense. The mean Reuter’s score at 7 d after SCIR was significantly higher in 3-MA treated group (10.61±0.93), while it was significantly lower in rapamycin treated group (8.96±0.76), compared with DMSO treated controls (9.72±0.69; Figure 3A). Further, spastic paralysis was identified through stretch reflex and muscular tension. The percentage of spastic paralysis had no significant difference among drug treated groups, which meant that autophagy had no effect on the incidence of spastic paralysis. What’s more, the spastic paralysis became much more serious over time than normal control. The severity of dysfunction at 7 d after SCIR was significantly higher in 3-MA treated group, while it was significantly lower in rapamycin treated group, compared with DMSO treated controls (Figure 3B). To assess the hind limb function after SCIR injury and the different treatments, both motor evoked potentials (MEP) and somatosensory evoked potentials (SEP) were examined at 7 dpi (Figure 3C). We found that N1 wave peak latency of both MEP (Figure 3D) and SEP (Figure 3E) in all the three SCIR groups (3-MA, Rap, and DMSO) were significantly longer than those in the sham group. More importantly, the MEP and SEP N1 wave peak latency were significantly longer in rats treated with 3-MA compared with the DMSO treated SCIR rats; while the MEP and SEP N1 wave peak latency were significantly shorter in the rapamycin treated group compared with the SCIR-DMSO controls. These results suggested that SCIR injury induced neurons especially motor neurons dysfunction and that the rapamycin-induced increase in autophagy could extenuate SCIR induced spastic paralysis.

Induction of autophagy extenuate SCIR-induced motor cell degradation

To further assess motor neurons in the SCIR injury model and the effects of 3-MA and rapamycin, we performed a Nissl staining pro-
Autophagy protect motor neuron from degeneration

Procedure and evaluated motor neurons morphology. After 7 d of reperfusion, in the sham group, a large number of normal neurons contained Nissl bodies apparently in cytoplasm, with prominent nucleoli and loose chromatin. In contrast, motor neurons of rats placed in SCIR group were morphologically damaged, with shrunken and dark nucleoli and few Nissl bodies contained in cytoplasm. And edema was observed around the motor neurons. Compared with the SCIR-DMSO group, the number of normal motor neurons in SCIR-3-MA group decreased distinctly, while the morphology was comparably damaged. Oppositely the number of damaged motor neurons in the SCIR-Rap group was remarkably decreased (Figure 4A, 4B). These results demonstrated that increased autophagy following SCIR injury protects motor neurons from degeneration.

Discussion

Autophagy is a tightly regulated catabolic pathway for lysosomal degradation of cytoplasmic organelles or cytosolic components and the recycling of the resulting macromolecules [17]. Homeostasis of autphagic activities in the cytoplasm is critical for the maintenance of neuronal function [18]. There is increasing evidence that failure in clearing the aggregated proteins or impaired organelles contributes to programmed cell death or apoptosis [19]. Intriguingly, abnormal autophagic activity has been described in Alzheimer's disease [20], Huntington's disease [21], Parkinson's disease [22], and Creutzfeldt-Jakob disease [23].

In the present study, we established the rat SCIR-induced spastic paralysis model and evaluated the autophagy in motor neurons of injured spinal cord. We found motor neurons in the ventral horn of the spinal cord area were clearly positive for autophagy markers at 3 h post-reperfusion; subsequently, autophagy decreased slowly to near-normal levels at 7 dpi. Investigation of the molecular mechanisms suggested that autophagosomes significantly increased after SCIR injury as indicated by the autophagosome formation marker LC3 II and autophagy substrates marker p62. After intervention with 3-MA and rapamycin, the autophagy level increased and decreased respectively. It indicated that autophagy occurred in motor neuron located in ventral horn of spinal cord, where mainly contains motor neurons. The results would provide some evidences for relationship between autophagy and SCIR-induced spastic paralysis.

We have previously demonstrated temporal loss of motor neurons in the ventral horn of the spinal cord following SCIR. The suggested mechanism indicated decreasing glycine level satan early stage and gamma aminobutyric acid (GABA) levels at a later stage, which was not sufficient to antagonize the excitotoxicity induced by glutamate that caused intracellular calcium overload resulting in neuronal death. Ling et al. [24] have demonstrated the temporal and spatial profiles of cell loss following experimental spinal cord injury and that decrease in motor neurons occurred at 1 h post-surgery and lasted over a week. Moreover, no recovery of motor neurons was observed during the process [24]. In combination with the pathogenesis of SCIR-induced spastic paralysis, there are many animal studies aimed at inhibiting inflammatory cells or chemokines and up-regulating protective proteins or factors. Patients with spinal cord compression were treated with low-doses of dexamethasone (0.025 mg/kg) and amino guanidine (75 mg/kg) before and after spinal cord decompression, regardless of whether they had spinal cord injury. Preoperative and postoperative use can reduce secondary edema after spinal cord injury, release tumor necrosis factor-α (TNF-α) and interleukin 1β (IL-1β), and reduce cell inflammatory necrosis [25, 26]. Experiments show that the expression of microtubule-associated protein-2 (MAP-2) and acetylcholine transferase (ChAT) has an important effect on the central nervous system function. Monosialotetrahexosylganglioside (GM-1), a widely used nutritional neurotrophic drug, can protect the function of spinal cord by stimulating the expression of MAP-2 and ChAT in rats after spinal cord injury [27]. In our study, we used autophagy interference 3-MA and rapamycin to observe the effect of autophagy to motor neurons and neurologic function.

As spastic muscular tension and excited stretch reflex are the main characteristics to identify spasticity in clinical [28-30], they were used to assess the extent of spasticity in paralysis rats. The classification guidelines are according to the widely used Reuter's score [15]. Our results indicated that the degree of motor neuron injury in the SCIR group is obviously increased. Moreover, after rapamycin treatment, rats
Autophagy protect motor neuron from degeneration

scored lower on the Reuter’s score, while it was higher after 3-MA treatment. These findings suggest that rapamycin-induced autophagy had a protective effect on motor neurons function, while the autophagy inhibitor 3-MA had a negative effect. Additionally, higher Reuter’s scores and prolonged latency of MEP/SEP were strongly enhanced following SCIR. Furthermore, rapamycin exerted a protective effect on motor neurons in SCIR rats, while 3-MA damaged further motor neurons. Our results proved that autophagy had a protective effect on motor neurons and provided a novel and effective approach for developing protective therapy for SCIR injury.

In this investigation, we identified and evaluated autophagy in SCIR-induced spastic paralysis in rats. We demonstrated more extensive autophagy following rapamycin treatment, which had a protective effect on motor neurons in the ventral horn of the spinal cord following SCIR. In contrast, the 3-MA induced inhibition of autophagy aggravated the excitotoxicity and subsequent injury. Our results identified that autophagy may play an important role in neuronal degeneration and pathogenic mechanisms of SCIR damage. Furthermore, this strategy would provide a novel and effective target for protecting SCIR in the future.

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Disclosure of conflict of interest
None.

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Autophagy protect motor neuron from degeneration


Supplemental Figure S1. The original photo of LC3I, LC3II, Beclin-1 and p62 that were detected by western blot in sham and indicated time post SCIR after treated.
Supplemental Figure S2. The original photo of LC3I, LC3II, Beclin-1 and p62 that were detected by western blot in sham, SCIR, SCIR-DMSO, SCIR-3-MA and SCIR-Rap.