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A Rapamycin-Enhanced Autophagy Reduces Neural Apoptosis by Blocking Bax Mitochondral Translation and Cytochrome C Release in Acute Spinal Cord Injury in Rats

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Abstract

Spinal cord injury (SCI) is a severe central nerve system damage. The actual roles of autophagy in the context of SCI remain controversial. This study was undertaken to determine the effects of enhanced autophagy on neural apoptosis in rat SCI model. Rapamycin or vehicle was given via an intraperitoneal injection after SCI. Spinal cord samples were extracted 24 hr after SCI. The level of phosphorylation of p70S6K was significantly reduced whereas expression of LC3-II/LC3-I was significantly increased after rapamycin treatment. Rapamycin treatment markedly reduced caspase-3 activity and staining density of TUNEL, but increased NeuN expression within the injured spinal cord. Moreover, rapamycin treatment reduced Bax translocation to the mitochondria and the release of cytochrome c into the cytosol. Rapamycin-treated rats showed significantly higher locomotor function in BBB scores compared with vehicle-treated rats. Our results demonstrated rapamycin-enhanced autophagy reduced neuronal apoptosis via inhibiting mTOR signal pathway, and improved locomotory recovery in rat SCI model. The antiapoptotic mechanism was related to the enhancement of autophagy, which ameliorated neural apoptosis through a mitochondrial pathway.

Keywords: Rapamycin; Autophagy; Spinal cord injury; Apoptosis; Mitochondria; mTOR

Introduction

Autophagy is an intracellular degradation mechanism for cytosolic long-lived proteins and organelles [1-3]. This mechanism plays an important role in maintaining cellular homeostasis during stress and normal physiological process. The roles of autophagy in various models of neuronal disorders have been concerned [4]. Previous studies demonstrated that increased autophagy had neuroprotective effects in several neurodegenerative diseases [5,6]. And autophagy induced by rapamycin also could reduce neural tissue damage after traumatic brain injury, cerebral ischemia and neonatal hypoxia-ischemia [7-9]. Kanno et al. firstly reported that autophagic activity was significantly increased in damaged neural tissue after spinal cord injury (SCI) [10]. Some previous studies also confirmed increased autophagic activity at lesion sites after traumatic SCI [11-16]. However, the real role of enhanced autophagy in the condition of SCI remained controversial [10-16].

Traumatic SCI is a severe central nerve system damage, resulting in catastrophic neurological deficits and motor dysfunction. Apoptosis has been recognized as an important process that affects the development of neuronal tissue damage following SCI [17,18]. Apoptotic cell death along the longitudinal axis of the spinal cord may leads to deterioration of spinal cord sensorimotor function. Therefore, a variety of strategies are being investigated to modulate the processes of apoptosis after SCI. The purpose of the present study is to examine whether rapamycin-enhanced autophagy can reduce neuronal apoptosis after SCI. We also investigate the change of the apoptotic signaling in the mitochondrial signal pathway and its relationship with autophagy.

Experimental Procedure

Animals

Adult male Sprague Dawley rats weighing 300–330 g were obtained from Shanghai Super-B&K laboratory animal Corp. Ltd (Shanghai, China). The animals were maintained on a 12-hr light/dark cycle under controlled temperature and humidity conditions. All procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

Study design

Forty-eight rats were randomly assigned to three groups (n=16): sham group, SCI + vehicle group, and SCI + rapamycin group. The SCI + rapamycin group was subjected to SCI and treated with rapamycin. The SCI + vehicle group was subjected to SCI and treated with vehicle. The sham group was subjected

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to a procedure similar to that of the SCI + vehicle group, but without perforation.

Drug administration

Rapamycin (Selleck) was dissolved in dimethyl sulfoxide (DMSO) (25 mg/mL) and further diluted in 0.5 mL aqueous solution containing 5% polyethylene glycol 400 and 5% Tween 80 immediately before injection. Rats in SCI+rapamycin group were injected intraperitoneally with rapamycin at a dose of 1 mg/kg body weight on the onset of SCI based on a previous study [16]. Rats in SCI+vehicle group were injected with an equivalent volume of saline immediately after SCI.

Rat model for SCI

Rats were intra-peritoneally anesthetized using chloral hydrate (400 mg/kg). A 15 mM midline skin incision was made, and the laminae of the T6–T10 vertebrae were exposed. A laminectomy was performed at T8, exposing the dorsal cord surface with the dura intact. Compressive SCI was produced by the application of an aneurysm clip (standardized closing force 30 g) at the T8 level. The clip was removed after a flaccid paralysis in rats' hind legs. The muscles and skin were closed in layers. Bladders were expressed twice a day until spontaneous voiding began. The sham-operated animals received the same surgical procedures, but no impact injury was sustained. Ten rats in each group were sacrificed at 24 h after SCI for terminal dexynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining assay, western blot assays and immunofluorescence staining.

Tissue preparation

At 24 h after SCI and after the sham operation, the rats were overdosed by an intra-peritoneal injection of 100 mg/kg sodium pentobarbital. The rats were transcardially perfused with normal saline, followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Spinal cord samples from rats were divided into two parts, one part was frozen in liquid nitrogen immediately and then stored at 80°C for western blot analysis, and the other was fixed with 4% Paraformaldehyde (PFA) solutions for immunofluorescence staining and TUNEL assay.

Immunofluorescence Staining

For immunofluorescence staining, the spinal cord segments containing the injured site were collected, post-fixed in the same fixative overnight at 4°C, and embedded in paraffin. Serial 7 µm transverse sections around the injured site were mounted on slides. A total of 13 sequential sections at 250 µm intervals, that spanned a 3000 µm length in the spinal cord centered at the epicenter, were collected. The sections were deparaffinized and rehydrated, and then washed in PBS for 10 min, followed by washing with PBS containing 0.3% Tween for 10 min, and blocked with 3% milk and 5% FBS in 0.01 M PBS for 2 h. The sections were incubated with rabbit anti-LC3 antibodies (1:2000; proteintech) diluted in PBS overnight at 4°C. After rinsing with PBS, the sections were incubated with goat anti-rabbit IgG Alexa Fluor 555 secondary antibody (1:500; Beyotime) for 1 h at room temperature.

TUNEL staining assay

The injured spinal cord tissue collected and made into sections as above mentioned. The sections were used for TUNEL staining. Apoptotic cell death was detected using a TUNEL assay, according to the manufacturer's protocol (Roche), and examined under a fluorescent microscope.

Cellular fractionation

Proteins from the enriched fractions of the mitochondria and cytosol were extracted using the Mitochondria Isolation Kit for Tissue protocol (Pierce Biotechnology). Briefly, fresh tissue obtained at 24 h after SCI was disrupted with a Polytron grinder in ice-cold homogenizing buffer and centrifuged at 700 g for 10 min at 4°C to isolate the nuclear fraction. The supernatants were re-centrifuged at 12000 g for 15 min at 4°C. The pellets represented the mitochondrial fraction, while the supernatants represented the cytosolic fraction. The protein content of each sample was determined using a protein assay kit.

Western blot analysis

The spinal cords were homogenized in lysis buffer containing 50 mM Tris HCl (pH 7.6), 20 mM $MgCl_2$, 150 mM NaCl, 0.5% Triton-X, 5 units/mL aprotinin, 5 g/mL leupeptin, 5 g/mL pepstatin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The debris was removed by centrifugation, and the protein levels in the lysates were determined with the aid of the Bio-Rad protein assay (Bio-Rad). The proteins in the lysates were separated by SDS–polyacrylamide gel electrophoresis (PAGE) in 15% gels, and then electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h in TBST buffer (0.01 M Tris HCl, pH 7.5, 0.15 M NaCl and 0.05% Tween 20) containing 3% milk, and incubated with rabbit anti-LC3 antibody (1:2000; proteintech), rabbit anti-phospho-p70S6K antibody (1:2000; Affinity), rabbit anti-NeuN antibody (1:1000; Affinity), rabbit anti-Bax antibody (1:2000; proteintech), rabbit anti-Cyto c antibody (1:1000; proteintech), and rabbit anti-caspase 3 antibody (1:1000; proteintech) diluted in TBST buffer overnight at 4. The membranes were washed with TBS three times and incubated with secondary antibodies for 2h at room temperature. Signals were visualized using the ChemiDicTM XRS + Imaging System (Bio-Rad), and band densities were quantified with Multi Gauge Software for Science Lab 2006 (Fuji Film Corporation, Japan).

Motor function assessment

Motor function recovery after SCI was evaluated with the Basso-Beattie-Bresnahan (BBB) scoring system [19]. This scale is based on the precise observation of hind limb movements, stepping and coordination in an open field. Uninjured animals with normal locomotion exhibit a locomotor score of 21, whereas animals that exhibit complete hindlimb paralysis are

scored as 0. The movements were scored for 4 min by two evaluators blinded to the study groups. Scores are represented as the mean ± SD of all animals (n=6) in a group at a certain time point.

Statistical analysis

Data are expressed as the mean ± SD and were analyzed with SPSS 13.0 software (SPSS, Chicago, IL, USA). Multiple group comparisons of differences in quantitative measurement were made using one-way analysis of variance followed by Dunnett's t-test. A value of *P*<0.05 was considered statistically significant.

Results

Rapamycin treatment improved BBB motor function

BBB scores were assessed for all three groups at different time points after injury, with aim of evaluating the effects of rapamycin on motor function recovery. All rats in the SCI +vehicle and SCI+ rapamycin groups immediately had flaccid paralysis in both hind legs after SCI. However, we found the scores in SCI+ rapamycin group were consistently higher than those in the vehicle-treated animals from 1 day (1.22 ± 0.26 versus 0.43 ± 0.15) to 21 days (8.1 ± 0.3 versus 3.94 ± 0.19, P<0.05, **Figure 1**).

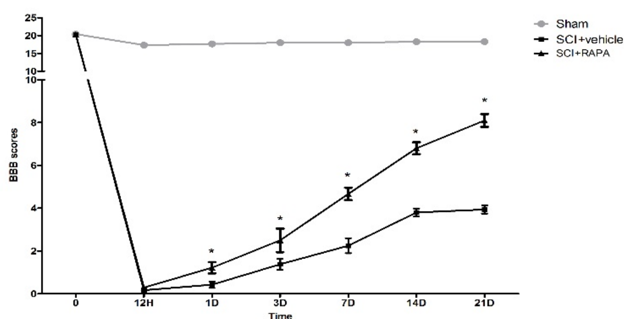

Figure 1 Locomotor recovery after SCI in all three groups from 12 h to 21 days after SCI. The BBB scores were determined from 12 h to 21 days after SCI. The values are the means ± SD, n=6 *P<0.05 versus SCI+vehicle.

Rapamycin treatment reduced loss of neuronal cells after SCI

To investigate loss of neuronal cells after SCI, the expression of NeuN protein within the injured spinal cord tissue was examined. Compared with sham group, the level of NeuN was significantly decreased in rats subjected to SCI.

However, rapamycin treatment blocked the SCI-induced decrease of NeuN expression (P<0.05, **Figures 2A and 2B**).

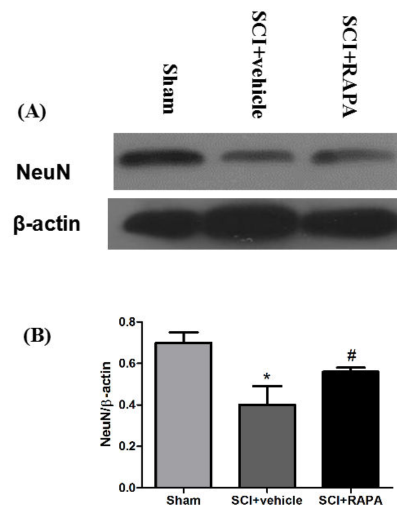

Figure 2 The expression of NeuN in all three groups at 24 h after SCI. (A) Representative Western blots showing levels of NeuN. (B) The relative band densities of NeuN. The densities of the protein bands were analyzed and normalized to β-actin. The bars represent the mean ± SD, n=10, *P<0.05 versus sham, #P<0.05 versus SCI+vehicle.

Rapamycin treatment increased activity of autophagy by down-regulated phosphorylation of p70S6K

LC3 is one biomarker for autophagy activation in mammalian cells. The immunofluorescence analysis showed that the staining density of LC3 was weak in the sham group, but greater in SCI + vehicle and SCI + rapamycin group (**Figure 3**).

Western blot analysis showed that rapamycin treatment significantly up-regulated the ratio of LC3-II to LC3-I compared with the SCI + vehicle and sham groups (P<0.05, **Figures 4A and 4B**). To examine the effect of rapamycin treatment on the mTOR signaling pathway, the level of phosphorylation of p70S6K was evaluated.

The phosphorylated p70S6K protein was significantly decreased in SCI + rapamycin group than that in SCI + vehicle group and sham group at 24 h after SCI (P<0.05, **Figures 5A and 5B**).

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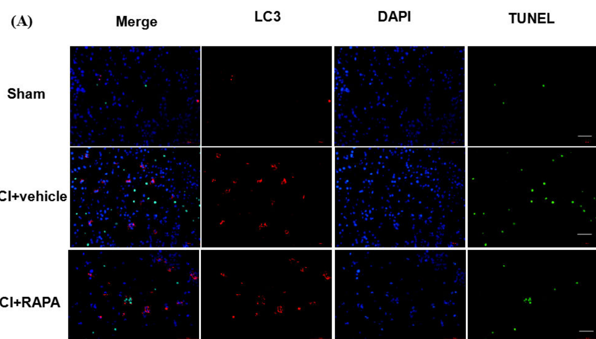

Figure 3 Cortical cellular apoptosis and autophagy in all three groups at 24 h after SCI. Representative TUNEL/LC3/DAPI photomicrographs of the pericontusive cortex in different groups (scale bar =200 um). Fluorescence colors: TUNEL: green, LC3: red and DAPI: blue.

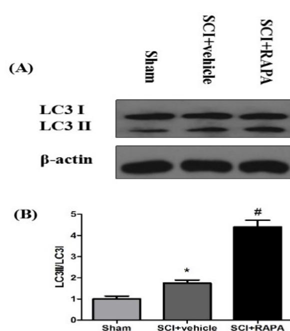

Figure 4 The expression of LC3 in all three groups at 24 h after SCI. (A) Representative Western blots showing levels of LC3. (B) The relative band densities of LC3. The densities of the protein bands were analyzed with LC3II/LC3I. The bars represent the mean ± SD, n=10, *P<0.05 versus sham, #P<0.05 versus SCI+vehicle.

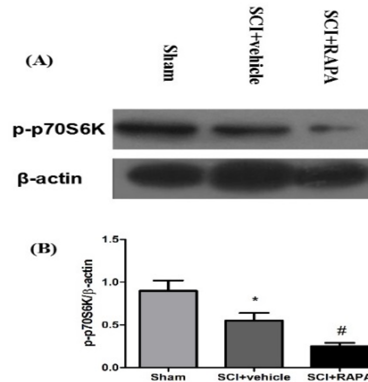

Figure 5 The expression of phosphorylated p70S6K in all three groups at 24 h after SCI.(A)Western blot analysis of phosphorylated p70S6K. (B) The relative band densities of phosphorylated p70S6K. The densities of the protein bands were analyzed and normalized to β-actin. The bars represent the mean ± SD, n=10, *P<0.05 versus sham, #P<0.05 versus SCI+vehicle.

Rapamycin treatment reduced neuronal apoptosis induced by SCI

To investigate expression of neuronal apoptosis after SCI, TUNEL staining was performed. The staining density of TUNEL was weak in sham group, but greater in SCI + vehicle group.

The staining density of TUNEL in the injured spinal cord tissue was obviously decreased after rapamycin treatment (**Figure 3**).

Caspase-3 activation triggers the cleavage of a number of proteins and ultimately leads to DNA fragmentation and apoptosis. Western blot analysis showed the expression of caspase-3 protein was significantly increased after SCI.

Rapamycin treatment reduced expression of caspase-3 induced by SCI compared with vehicle treatment after SCI (P<0.05, **Figures 6A and 6B**).

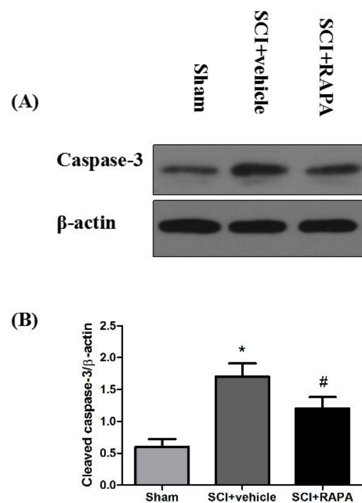

Figure 6 The expression of Caspase 3 in all three groups at 24 h after SCI. (A) Representative Western blots showing levels of Caspase 3. (B) The relative band densities of Caspase 3. The densities of the protein bands were analyzed and normalized to β-actin. The bars represent the mean ± SD, n=10, *P<0.05 versus sham, #P<0.05 versus SCI+vehicle.

Rapamycin treatment blocked the mitochondrial apoptotic pathway

To further investigate expression of apoptotic signaling in the mitochondrial pathway, the levels of proapoptotic factors Bax and Cyto C expression in the mitochondria and cytosol were examined, respectively.

Western blot analysis showed that the levels of Bax protein in the mitochondria and cytosol were significantly increased and decreased, respectively, after SCI compared with the sham group (P<0.05, **Figures 7A, 7D and 7E**).

On the contrary, levels of Cyto C in the mitochondria and cytosol were significantly decreased and increased, respectively, after SCI compared with the sham group (P<0.05, **Figures 7A to 7C**).

However, rapamycin treatment inhibited the translocation of Bax to the mitochondria and the subsequent release of Cyto c into the cytosol compared with vehicle treatment after SCI (P<0.05, **Figures 7A to 7E**).

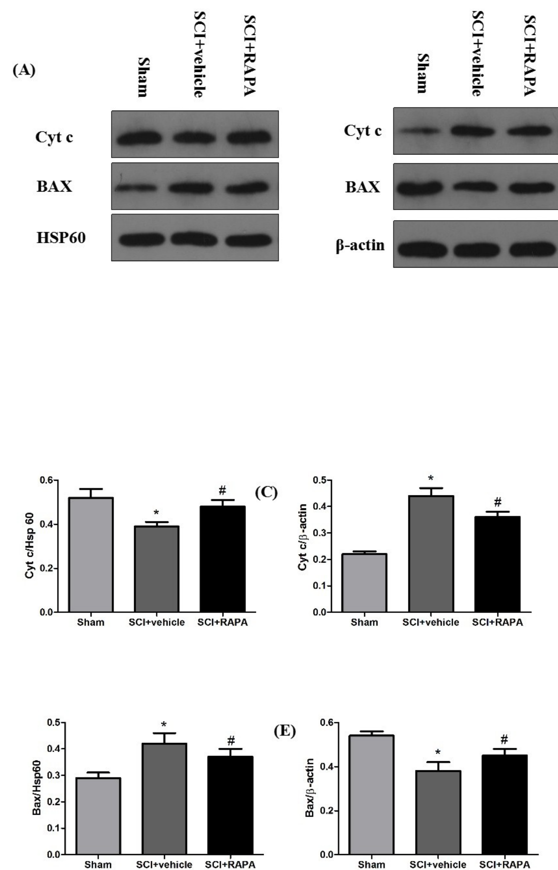

Figure 7 The expression of Bax and cytochrome c within the mitochondria and cytosol in all three groups at 24 h after SCI. (A) Representative Western blots showing levels of Bax and cytochrome c. (B, D) The relative band densities of mitochondrial Bax and cytochrome c. (C, E) The relative band densities of cytosolic Bax and cytochrome c. The densities of the protein bands were analyzed and normalized to β-actin or Hsp 60. The bars represent the mean ± SD, n=10, *P<0.05 versus sham, #P<0.05 versus SCI +vehicle.

Discussion

Autophagy is a physiological phenomenon that plays an important role in maintaining cellular homeostasis [1-6]. Previous studies demonstrated that autophagy participated in the pathophysiological process of central nerve system damage, including brain trauma and cerebral ischemia, and traumatic SCI [7-16]. Several signal pathways have been reported to participate in the regulation of autophagic activity. The mTOR appears to be one key gene/protein that regulates mammalian autophagy [20]. p70S6K is conceived as a direct downstream protein and the most well characterized target of mTOR [21]. In the present study, the reduction of phosphorylated p70S6K indicated that rapamycin inhibited mTOR signaling after SCI. Expression of LC3 expression was

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significantly increased in the damaged neural tissue following rapamycin treatment. Our results demonstrated that rapamycin treatment could enhance activity of autophagy by inhibiting mTOR after SCI.

Previous studies showed rapamycin-enhanced autophagy could reduce neural tissue damage after traumatic brain injury and neonatal hypoxia-ischemia [7-9]. However, the actual function of autophagy in the damaged neural tissue after SCI remains controversial. Kanno et al. firstly reported autophagy was activated in the damaged neural tissue after SCI [10]. They found the neurons expressing Beclin 1 displayed the characteristics of autophagic cell death, which indicated the role of autophagy may be detrimental for neural tissue after SCI. Wei et al. also demonstrated intrathecal injection of autophagy inhibitor 3-Methyladenine reduced neuronal damage and promoted functional recovery via autophagy attenuation after SCI [15]. These results indicated deleterious role of autophagy in SCI. In contrast, other authors found the neuroprotective effects of autophagy in reducing spinal cord damage after SCI [12-14,16]. In the present study, we confirmed rapamycin-enhanced autophagy had benefits in reducing loss of neurons and improving motor function in rats of SCI.

Apoptosis is one important mechanism in the secondary damage of SCI. Previous study has showed apoptotic cell death following SCI is highly mitochondrial dependent [17,18]. In response to apoptotic stimuli, The Bcl-2 family member Bax proteins act downstream in the mitochondrial apoptotic pathway [22]. The outer mitochondrial membrane becomes permeabilized, resulting in the insertion of Bax into the outer mitochondrial membrane and cytochrome c release. That finally promotes the caspase-3 activation and cleavage of specific cellular substrates leading to DNA fragmentation and cell death [23,24]. In the present study, we found that rapamycin treatment markedly reduced the expression of Bax, cytochrome c and caspase-3 induced by SCI. The number of TUNEL-positive cells was also significantly decreased after rapamycin treatment. Our results showed autophagy activiated by rapamycin reduced neuronal apoptosis by inhibited the mitochondrial apoptotic pathway, in agreement with findings of previous studies [12-14,16].

The mechanism of rapamycin-enhanced autophagy in mediating mitochondrial apoptosis following SCI is still concerned. Chen et al. demonstrated enhanced autophagy by melatonin reduced neural apoptosis in a model of subarachnoid hemorrhage, by virtue of inhibiting the translocation of Bax to the mitochondria and the subsequent release of Cytochrome c [25]. In the present study, we also found rapamycin treatment resulted in striking change of Bax and Cytochrome c expression in the mitochondria and cytosol compared with vehicle treatment. Therefore, Bax mitochondrial translocation and cytochrome c release induced by SCI could be blocked by rapamycin treatment. Jorgensen et al. have found clearing damaged mitochondria by autophagosome can reduce mitochondria-dependent apoptosis, which is also called as mitophagy [26]. Recently, the induction of neuronal mitophagy was also found in acute SCI [27]. We speculated the inhibitive effects of rapamycin on neural apoptosis may be achieved by autophagy-mediated mitochondrial elimination.

Conclusion

In the present study, we demonstrated that rapamycin increased autophagic activity by inhibited the mTOR signaling pathway, and reduced mitochondria-dependant neuronal apoptosis in a rat model of SCI.

Acknowledgments

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