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Role of Autophagy in Rat Acute Spinal Cord Injury Induced by Rapamycin

Haicheng Yan[1,2#], Changwu Dou[2#], Wei Wang[3], Fuming Tian[1,2], Youle Su[1,2], Songtao Qi[1*]

1. Department of Neurosurgery, Nanfang Hospital, Southern Medical University, Guangzhou, 510515, China.
2. Department of Neurosurgery, The Affiliated Hospital of Inner Mongolia Medical University, Inner Mongolia Medical University, Hohhot,010050, China
3. The First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou 510120, China.

[*]Corresponding author at: Department of Neurosurgery, Nanfang Hospital, Southern Medical University, Guangzhou, 510515, China.
Email address: nmgyhc1981@126.com
[#]These authors contribute equally to this article (co-first author).

Abstract: Several clinical predictors of neurological outcome after traumatic spinal cord injury (SCI) have been identified, including severity of injury, level of injury, and a zone of partial preservation. The process of autophagy plays a role in protecting cells from damage. in this study,we explored the effects of rapamycin-induced autophagy on apoptosis in a rat model of acute spinal cord injury (SCI), and explored the effect of rapamycin on apoptosis in primary spinal cord cell culture. Our study found that The expression of the apoptosis-related protein caspase-3 did not significantly increase until 21 days following injury, while increases in LC3II and LC3I began 10 days after injury, but then declined. TUNEL staining and electron microscopy confirmed that following injury autophagy occurred before apoptosis, but by 14 days after the injury, the level of autophagy had decreased significantly while the level of apoptosis showed a continued increase. Following treatment with rapamycin, apoptosis was significantly higher than in the vehicle control group, but significantly lower than in the sham-operated group, showing a protective effect of rapamycin. Gale scale grades in rats treated with rapamycin were significantly higher compared with the vehicle control group, suggesting a functional effect of rapamycin-induced inhibition of apoptosis. Our results indicate that rapamycin significantly improved the prognosis of acute SCI in rats by inhibiting cell apoptosis.

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【Key words】Autophagy , Rapamycin,spinal cord injury

The process of autophagy plays a role in protecting cells from damage. Autophagy degrades macromolecules and organelles in the cytoplasm, thereby facilitating the renewal of cellular components and maintenance of the active physiological state of cells in extreme environments [1-3]. Autophagy also enables the recycling of cellular products and thus the maintenance of intracellular homeostasis[4].In addition, autophagy is also affected by other factors such as hormonal factors, temperature, oxygen concentration, and cell density [5]. Although the primary role of autophagy is protecting cells, it paradoxically can also have a role in cell death[6]. Recent studies have demonstrated that following acute SCI, numerous nerve cells undergo apoptotic death, which is believed to be an important part of secondary injury to the spinal cord [7–10]. Therefore, how to inhibit nerve cell apoptosis effectively is an intriguing area for current SCI research[11-12]. Studies have revealed that during the process of nerve cell death, autophagy occurs prior to apoptosis and plays an important role in cell death regulation. Conventional treatment to prevent secondary damage in neuronal tissue is to administer methylprednisolone (MP), a synthetic glucocorticoid[13]. While MP can inhibit lipid oxidation and suppress the production of reactive oxygen species and free radicals, it is well known that high doses of glucocorticoids may cause severe complications such as cardiac arrest, respiratory arrest, osteoporosis,and osteonecrosis[14-15]. This necessitates the development

of new treatments against secondary damage. Rapamycin is a new macrocyclic lactone agent with potent immunosuppressive effects, which is mainly used to prevent rejection in organ transplantation[16]. Rapamycin blocks the signals of multiple cytokines and inhibits the calcium-dependent and -independent signaling pathways in T and B lymphocytes, thereby suppressing the immune response and inflammation. Inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), can induce apoptosis in different cell types [17].Rapamycin has been found to increase autophagy by inhibition of the mammalian target of rapamycin (mTOR) [18-19].This suggests that since autophagy has a protective effect on cells, rapamycin might have potential as a therapeutic agent in acute SCI. Therefore, the present study was primarily carried out to investigate the effects of rapamycin-induced autophagy on neural apoptosis in a rat model of acute SCI.

Materials and methods

Animals and Reagents

Female adult Sprague-Dawley rats (250–300g, aged 8 weeks) and pregnant Sprague-Dawley rats for establishment of embryonic primary neuronal cultures were purchased from SLRC Laboratory Animal Co. Ltd. The rats were housed in an animal facility with 12-hour light-dark cycles at 22–25 ° C and had free access to water and food. After surgery, urine was manually squeezed from the bladder periodically from each rat. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Fujian Medical University. rapamycin were purchased from Sigma-Aldrich (St. Louis, Mo., USA), and 3-methyladenine (3-MA; autophagy inhibitor) was purchased from Merck Millipore (Billerica, Mass., USA).

SCI Induction

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate and fixed in the prone position. The spinal cord was exposed at the T10 level and SCI was induced using a modified Allen weight drop technique as has been previously described [20] .Briefly, under aseptic conditions, a dorsal thoracic incision was performed, followed by blunt dissection of the dorsal fat pad. The lamina was then exposed with the surgical opening centered at levels T9–T11. The rat spinal cords were injured at T10 using a force of 8g/2.5 cm, which has been shown previously to result in complete paraplegia [21] . The rats in the sham-operated control group were also surgically exposed at the T10 level, but without induction of SCI.

Drug Administration after SCI Induction

Four hours after SCI induction some rats were administered either DMSO (vehicle control) (10ml/kg/day); MP, a synthetic glucocorticoid drug (30mg/kg/day, Roche, Shanghai, China); rapamycin (5 mg/kg/day, Sigma-Aldrich), or both MP and rapamycin for 30 days. MP was injected intravenously, while rapamycin was administered intraperitoneally. Primary Spinal Cord Cell Culture The rat primary spinal cord cell culture was prepared from embryonic day 15 Sprague-Dawley rats according to the published protocols with modification. After the meninges and blood vessels were removed, rat embryonic spinal cords were cut into small pieces in cold HBSS. The tissues were digested with 0.05% trypsin in HBSS for 15 min at 37 ° C with constant, gentle agitation, and then an equal volume of serum-containing culture medium was added to inactivate trypsin.After trituration,the cells were seeded in noncoated dishes and cultured with DMEM supplemented with 20% heat-inactivated fetal calf serum, penicillin, and streptomycin (all from Gibco, Grand Island, N.Y., USA) at 37 ° C in a 5% CO 2 humidified incubator for 2 h to allow nonneuronal cells to adhere to the dishes. The supernatants were collected and the viable cells were seeded at the density of 1 . 10 6 /ml in polylysinecoated dishes. On the second day, the medium was replaced with neurobasal medium supplemented with 2% B27 supplement and 2mM glutamine (all from Gibco). After 24 h, 3 μg/ml Arabinofuranosyl Cytidine (AraC, Sigma-Aldrich) was added into the medium to suppress glial cell proliferation. The medium was renewed twice a week. At 7–8 days in vitro, the spinal cord cell cultures were subjected to the experiments described below.

Western Blot Analysis

For Western blot analysis, 5-mm spinal cord segments at the T10 level were obtained followed by protein extraction using tissue lysis buffer (Promega, Fitchburg, Wisc., USA). Cell lysates (40 μg/lane) were separated by SDS-PAGE. Blots were incubated with a 1: 400 dilution of rabbit anti-caspase-3, or goat anti-LC3 (all from Santa Cruz Biotechnology, Santa Cruz, Calif., USA), followed by incubation with a 1: 5,000 dilution of HRP-conjugated donkey anti-rabbit IgG or bovine anti-goat IgG (Santa Cruz Biotechnology).Proteins were visualized and quantified using chemiluminescence assay.

Real-Time PCR

Cultured spinal cord cells were treated with DMSO (vehicle control), IL-1β (30 ng/ml), IL-1β (30 ng/ml) plus rapamycin(1mmol/l), IL-1β (30 ng/ml)

plus 3-MA (2 mmol/l), or IL-1β (30 ng/ml) plus rapamycin (1mmol/l) plus 3-MA (2 mmol/l). After 36-hour treatment, cells were subjected to RNA extraction and subsequent real-time PCR for quantification of Bcl-2, which inhibits apoptosis, and Bax, which promotes apoptosis. Data were expressed in comparison to the vehicle control group. Total RNA was extracted from the collected primary cell cultures by Trizol (Invitrogen, Carlsbad, Calif., USA), followed by complementary DNA synthesis. Real-time PCR assays were performed using a real-time PCR kit (Invitrogen) to quantify the mRNA expression level of Bax, Bcl-2, and β-actin. β-Actin was used as the internal control. The PCR primer sequences are listed in table 1 . The PCR amplification protocol consisted of an initial denaturation step of 2 min at 94 ° C followed by 35 cycles of 94 ° C for 30 s, 58 ° C for 30 s, and 72 ° C for 1 min. The relative mRNA expression of Bcl-2, Bax,and β-actin were determined by the 2 -ΔΔCt method.

Table 1. Primer sequences used for quantitative real-time PCR

Gene	Primer sequence (5′-3′)	Product size, bp
Bcl-2	F: CGGGAGATCGTGATGAAGTAC R: AGGCTGGAAGGAGAAGATGC	126
Bax	F: TGGTTGCCCTCTTCTACTTTGC R: GAAGTCCAGTGTCCAGCCCAT	101
β-Actin	F: AGATTACTGCCCTGGCTCCTAG R: TCATCGTACTCCTGCTTGCTG	145

Statistical Analysis

Data were presented as means ± SD. Comparisons of 3 or more groups were performed with analysis of variance with post hoc comparison using a Bonferroni correction for type I error adjustment.Comparisons of 2 groups were performed using an independent t test. Data were analyzed using SPSS 15.0 statistics software, and $p < 0.05$ was considered statistically significant.

Results

1.Temporal Expression Profiles of Inflammatory Cytokines in Injured Spinal Cord Tissue after SCI Induction

To depict the protein expression profiles of IL-1β and TNF-α during the progression of SCI, 42 rats were randomly divided into 7 groups, one sham-operated control group and 6 SCI groups. The rats in the 6 SCI groups were sacrificed at 1, 3, 10, 14, 21, and 30 days after SCI induction. The rats in the sham-operated group were sacrificed 30 days after the sham procedure. After injury, protein levels of TNF-α and IL-1β in the damaged spinal cord tissues significantly increased by day 3 compared to the corresponding levels of the sham controls, peaked at 10 days, and then subsequently declined. At day 21 after injury, the protein levels of both TNF-α and IL-1β were similar to those of the sham controls.

2. The Effect of Rapamycin on Autophagy and Apoptosis

To further investigate the effect of autophagy on cell apoptosis, the autophagy activator rapamycin was intraperitoneally injected into the rats after SCI. The SCI rats were sacrificed at 3, 10, 14, 21, and 30 days after SCI induction. The rats in the vehicle control group were sacrificed at 30 days after SCI induction. The injured spinal cord samples were subjected to Western blot analysis to quantify the protein profiles of caspase-3,LC3I, and LC3II. The representative gel images are shown in figure1. Upon stimulation of rapamycin, the ratio of LC3II/LC3I significantly increased up to more than fourfold that of the vehicle control at day 10 after SCI and further increased thereafter, while without stimulation of rapamycin, the ratio of LC3II/LC3I on day 10 after SCI was about twofold that of the corresponding vehicle control. On the other hand, rapamycin significantly increased caspase-3 protein expression on day 10 after SCI, whereas without stimulation of rapamycin, caspase-3 was not significantly different from that of the vehicle control until day 21 after SCI..

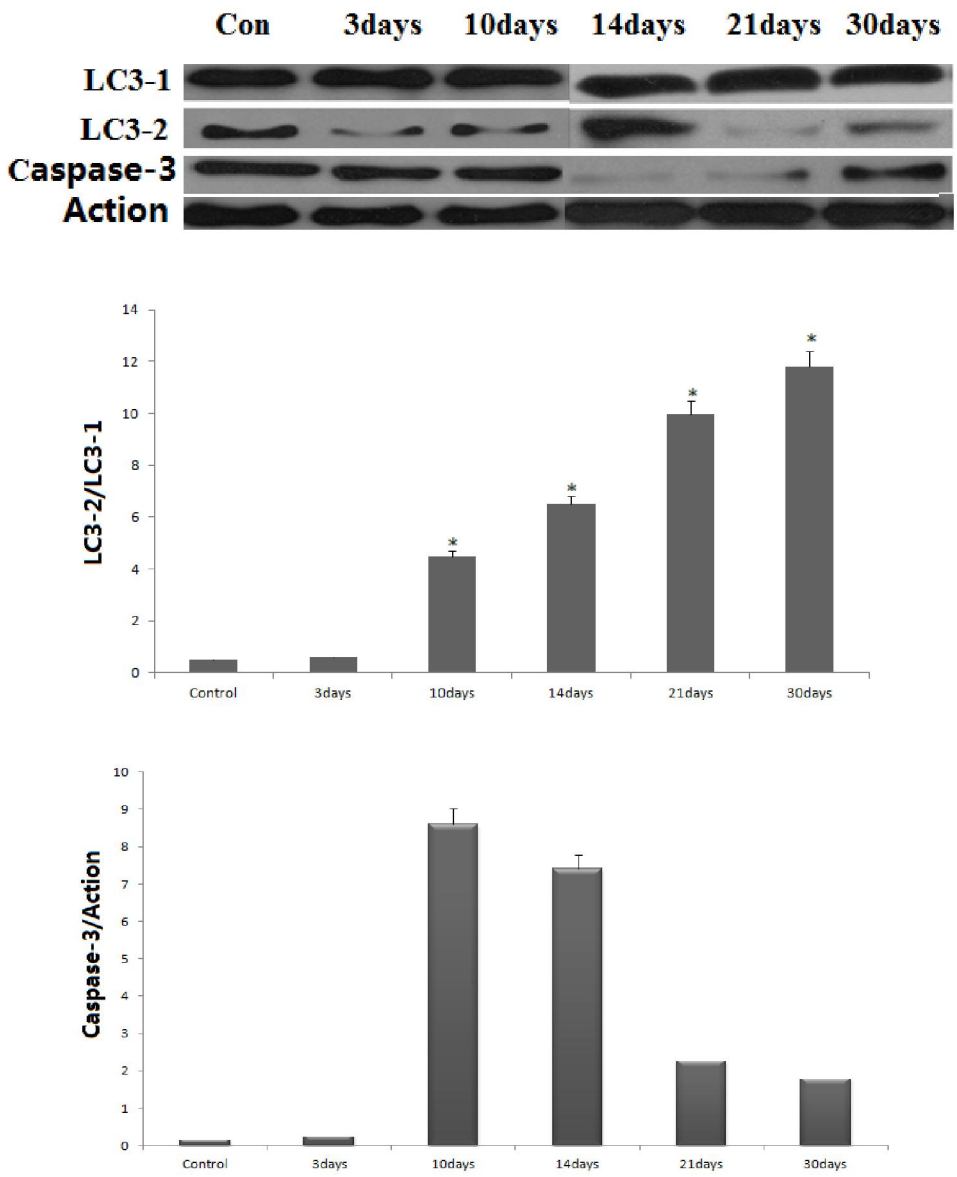

Fig.1. The effect of rapamycin on cell apoptosis and autophagy in the injured spinal cord tissue after SCI.
NOTE: Changes in protein expression levels of LC3 and caspase-3 in injured spinal cord tissue 3, 10, 14, 21, and 30 days after SCI. The animals in the vehicle control group were induced with SCI and treated with DMSO, and were sacrificed 30 days later. The data are presented as the fold of the vehicle control. For each time point n = 4. * $p < 0.05$ vs. the control.

3. Effect of Rapamycin on Primary Spinal Cord Cell Culture

The apoptosis rates in the IL-1β groups were significantly different from the corresponding vehicle control groups at both 24 h and 48 h (fig.2); the significant difference at 48 h was slightly larger than at 24 h. Rapamycin reduced IL-1β-induced apoptosis at both 24 and 48 h,which was reversed by 3-MA.Rapamycin significantly increased the Bcl-2 expression in IL-1β-treated cells (fig.3). Notably, 3-MA suppressed Bcl-2 expression induced by IL-1β plus rapamycin,but without reaching statistical significance.In contrast,rapamycin significantly inhibited IL-1β-induced Bax expression, which was reversed by 3-MA (fig.3).Furthermore, rapamycin significantly

increased the ratio of Bcl-2/Bax compared to that of IL-1β, which was significantly inhibited by 3-MA (fig.3).

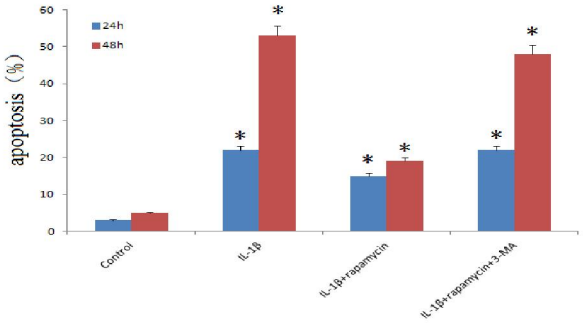

Fig.2. The effect of rapamycin on IL-1β-induced apoptosis in primary spinal cord cell culture. n = 6 for each group. Cells were treated with DMSO (control), IL-1β, rapamycin, or 3-MA for 24 h or 48 h followed by flow cytometry. * $p < 0.05$ compared to the control group.

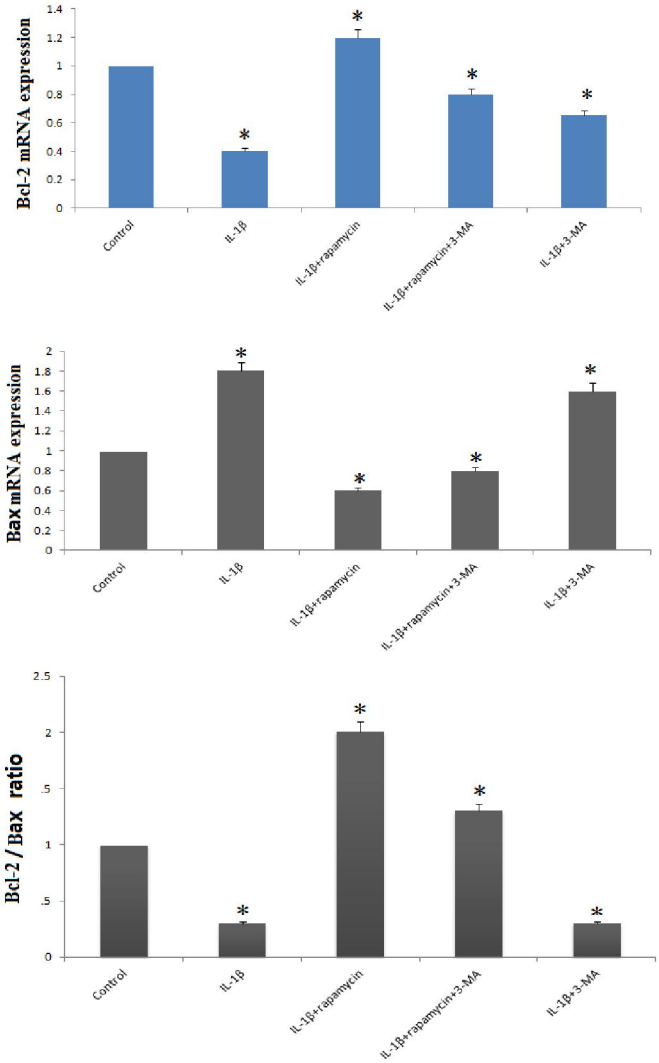

Fig. 3 The effect of rapamycin on the mRNA expression level of Bcl-2 and Bax in primary spinal cord cell culture. NOTE:Cells were treated with DMSO (control), IL-1β, rapamycin, or 3-MA for 36 h. The data are expressed as the fold of the vehicle control group. n = 6 for each group. * $p < 0.05$ compared to the control group.

Discussion

Autophagy is a controllable cellular process of lysosomal degradation. Under extreme conditions, autophagy can produce ATP and reusable materials such as amino acids by degrading long-lived proteins and some organelles, and thus ensure the survival of cells. When the autophagic capacity of cells is insufficient,the mitochondrial pathway of apoptosis will be initiated, resulting in dysfunction of mitochondria, release of apoptosis-inducing factors, and subsequent cell death.Our findings show that in a rat model of acute SCI in which the compression injury was induced at T10, the administration of rapamycin reduced locomotor impairment in the hind limbs of the rats. We also provided evidence that the improvement in hind limb function involved increased autophagy activity in spinal cord cells which reduced apoptosis.Our results strengthen the evidence supporting this conclusion and indicate that rapamycin should be studied further to assess its therapeutic potential as an agent for use in spinal cord trauma.We found that the autophagosomes were detected by transmission electron microscope in the injured spinal cord nerve cells at different time points, and the number of autophagosomes increased over time, suggesting the activation of autophagy after injury.mTOR plays an important role in central nervous system neuroprotection and neuroregeneration. Inhibition of mTOR has been found to reduce neural tissue damage and locomotor impairment in mice with SCI.However,it has not been completely clarified how inhibition of the mTOR signaling pathway by rapamycin leads to neuroprotection. It is believed that phosphatidylinositol-3-kinase (PI3K)/Akt signaling with involvement of downstream mTOR plays a role in regulating rapid cellular necrosis at the center of central nervous system trauma and the delayed onset of apoptosis and macrophagy. Further studies on the mechanism of the mTOR pathway appear warranted to provide more information on how spinal cord injuries might be treated pharmacologically.

It has been demonstrated that IL-1β can remarkably induce apoptosis of cells at the junction of the inner annulus and intervertebral disc in rats [22-27] . TNF-α and IL-1β have also been reported to induce apoptosis of rat intervertebral disc cells cultured in serum-free medium [28-32] . Therefore, it is likely that the elevated expression of TNF-α and IL-1β in the injured spinal cord may induce the occurrence of nerve cell apoptosis. In our tissue culture experiments we found that rapamycin reduced IL-1β-induced apoptosis at 24h and 48 h.Sekiguchi et al.[28]reported that administering rapamycin after SCI reduced p70S6K protein phosphorylation and increased the expression levels of LC3 and Beclin1 and reduced neuronal loss and cell death. Chen et al.[29] found that rapamycin given after SCI increased LC3-II expression and decreased p70S6K phosphorylation.Also, TNF-α production was reduced as was microglial activation. In addition, neuron preservation was noted. In our study, we observed that rapamycin increased the expression level of Bcl-2 in cells treated with IL-1β and inhibited IL-1β-induced Bax expression.The gold standard for detecting autophagy is observation of the ultrastructure of autophagosomes and autophagolysosomes under a transmission electron microscope,which is capable of recording the dynamic morphological chan ges in autophagosomes.

In the current study, we assessed hind limb function after SCI in rats administered MP or rapamycin, or both.We found that the rats given MP had improved hind limb function compared with the vehicle controls beginning on day 10, and the rats given rapamycin had improved hind limb function compared with the vehicle controls beginning on day 14. Rats that received both MP and rapamycin had improved hind limb function beginning on day 1. A protective effect was found as evidenced by decreased levels of serum and tissue myeloperoxidase and serum malondialdehyde,decreased activity of xanthine oxidase and caspase- 3, and increased serum and tissue levels of catalase.

In conclusion, our data demonstrated that intervention with the autophagy activator rapamycin significantly improved the prognosis in rats with acute SCI and suggested that this may involve inhibiting cell apoptosis. Our results suggest that activating autophagy after acute SCI is a promising potential therapeutic strategy for SCI.

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