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Regulation of autophagy by AMP-activated protein kinase/ sirtuin 1 pathway reduces spinal cord neurons damage

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ABSTRACT

Objective(s): AMP-activated protein kinase/sirtuin 1 (AMPK/SIRT1) signaling pathway has been proved to be involved in the regulation of autophagy in various models. The aim of this study was to evaluate the effect of AMPK/SIRT1 pathway on autophagy after spinal cord injury (SCI).

Materials and Methods: The SCI model was established in rats *in vivo* and the primary spinal cord neurons were subjected to mechanical injury (MI) *in vitro*. The apoptosis in spinal cord tissue and neurons was assessed by TUNEL staining and Hoechst 33342 staining, respectively. The autophagy-related proteins levels were detected by Western blot. The activation **of** AMPK/SIRT1 pathway was determined by Western blot and immunohistochemical staining.

Results: We found that the apoptosis of spinal cord tissue and cell damage of spinal cord neurons was obvious after the trauma. The ratio of LC3II/LC3I and level of p62 were first increased significantly and then decreased after the trauma *in vivo* and *in vitro*, indicating the defect in autophagy. The levels of p-AMPK and SIRT1 were increased obviously after the trauma *in vivo* and *in vitro*. Further activation of the AMPK/SIRT1 pathway by pretreatment with resveratrol, a confirmed activator of the AMPK/SIRT1 pathway, alleviated the cell damage and promoted the autophagy flux via downregulation of p62 in spinal cord neurons at 24 hr after MI.

Conclusion: Our results demonstrate that regulation of autophagy by AMPK/SIRT1 pathway can restrain spinal cord neurons damage, which may be a potential intervention of SCI.

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Introduction

Spinal cord injury (SCI), a serious traumatic disease of central nervous system, has always been a global medical problem, including primary injury and secondary injury (1). The secondary injury is believed to have a more significant impact on neurofunctional recovery after SCI. Researches have demonstrated that the apoptosis of neural cells occurred in the secondary spinal cord injury and was closely associated with the development of SCI (2, 3). Autophagy, as a rudimental life phenomenon of the eukaryotic cell, is reported to play important regulatory roles in secondary injury (4, 5). Therefore, further studing the regulatory mechanisms of autophagy and apoptosis after SCI will provide a new concept and method for improving the prognosis of SCI.

Silent information regulator 2 (Sir2) family is a kind of NAD+ dependent deacetylases. In mammals, there are seven Sir2 homologues, namely SIRT1-SIRT7. SIRT1, the most studied one, plays key roles in the regulation of autophagy. Recent research suggested that SIRT1 could promote autophagy through regulating autophagy-related genes expressions (6). AMP-activated protein kinase (AMPK) is an important energy metabolism receptor of the body, which can alter energy metabolism via regulating the expressions or activities of downstream molecules. AMPK can transcriptionally activate nicotinamide phosphoribosyl transferase, upregulate NAD+/NADH, then induce the activation of SIRT1 (7). Growing evidence has demonstrated that AMPK-SIRT1 pathway plays pivotal roles in diabetes (8), fatty liver (9), tumor (10), and cardiovascular disease (11) via regulating autophagy. Moreover, the role of AMPK-SIRT1 pathway in the neuroprotection in Parkinson's disease and Alzheimer's disease models has also been confirmed (12, 13). Previous research found that the expressions of SIRT1 (14) and AMPK (15) were changed significantly after SCI. However, the role of AMPK-SIRT1 pathway in autophagy after SCI has not been determined, which remains to be investigated.

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In this study, we first evaluated the regulatory effect of AMPK/SIRT1 pathway on autophagy after SCI and provided theoretical basis for the clinical treatment of SCI.

Materials and Methods

The establishment of SCI model of rats in vivo

Adult Sprague-Dawley rats were obtained from Beijing Vital River Laboratory Animal Co, Ltd (Beijing, China). The rats were randomly divided into two groups: sham group (n=6), SCI model group (n=24). The SCI model was established following a previous method with some alterations (16). Briefly, the rats were anesthetized by intraperitoneally injecting 10% chloral hydrate (3.5 ml/kg), shaved to show the dorsal skin in prone position. Then a 2 cm incision was made along the mid line of back after disinfection and the vertebral column was exposed. Centring on T10 vertebrae, a laminectomy was done. The rats in sham group were suffered the same operation but without injury. After surgery, the subcutaneous fascia tissue and skin are sutured. 50,000U/kg penicillin was intraperitoneally injected to the rats to avoid infections. The spinal cord tissues were collected at 1 d, 7 d, 14 d, and 5 w after the operation for further tests. All experiments for animal were performed strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of China Medical University.

MI model of primary spinal cord neurons in vitro

The primary culture of spinal cord neurons was performed according to a previous method (4). Briefly, adult female rats were mated with male rats(male and female 1:1) and pregnancy test was carried out. The embryos of pregnant rats was removed on the 16th day of the pregnancy. Under the anatomical microscope, the embryonic spinal cord was separated in aseptic conditions, washed, sheared into scraps and digested in 0.25% trypsin for 15 min at 37 °C. The dissociated single cell suspension maintained in DMEM culture medium containing 20% FBS and 1% penicillin and streptomycin was seeded onto polylysine-coated cell culture dishes with medium exchanged every 2-3 days. After 7 days' culture, the neurons were identified by immunofluoresence staining for neuron specific enolase (NSE), as described below. Then the mechanical injury model was carried out as follow: Firstly, a piece of cardboard printed with several transverse and longitudinal lines (5 mm line spacing) was placed under the culture dish. Then the uniform incisions were made along the lines using a sterile razor blade.

Immunofluoresence staining for NSE

The primary spinal cord neurons were seeded on coverslip, fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% TritonX-100 and blocked with 5 % bovine serum albumin (BSA) for 30 min at room temperature. Then the cells were incubated with anti-NSE (1:200, Bioss, China) primary antibody at 4 °C overnight. After washing with PBS, the cells were incubated with Cy3 labeled goat anti rabbit secondary antibody for 1 hr at room temperature. The nuclei were visualized with DAPI. Under a fluorescence microscope, images were obtained at a magnification of 400x.

Treatment with resveratrol in primary spinal cord neurons of rats

The primary neurons were pre-treated with different concentrations of resveratrol (5,10, and 20 μM) before the mechanical injury. Then the primary neurons were collected at 24 hr after the mechanical injury.

Western blot analysis

The protein levels in spinal cord tissues or primary spinal cord neurons were assessed by Western blot analysis. Briefly, total proteins were extracted from spinal cord tissues and primary spinal cord neurons by homogenizing in RIPA buffer and the concentration was determined by BCA Protein Assay Kit (Beyotime, China). Equal amount of protein samples were separated on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) by electrophoresis and transferred onto PVDF membranes. Then the membranes were blocked with 5% skimmed milk and incubated with the following primary antibodies LC3 (1:1000, Abcam, UK), p62 (1:400, Boster, China), SIRT1 (1:1000, Proteintech, China), p-AMPK (1:500, Sangon, China), AMPK (1:500, Sangon, China), and β-actin (1:1000, Santa Cruz, USA) at 4°C overnight, respectively. Membranes were incubated with corresponding secondary antibodies for 1 hr at room temperature. Finally, the results were visualized by chemiluminescent method and the densitometry of the protein band was analyzed by the Image J software (Bethesda, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining

TUNEL staining was adopted to detect the apoptosis rate in spinal cord tissues using *In situ* cell death detection kit (Roche, Switzerland) following the manufacturer's instructions. Briefly, the spinal cord sections were washed with 1 × PBS for 5 min at room temperature for three times and fixed by immersing in 4% paraformaldehyde. After the sections were permeabilized with 0.1% Triton X-100, TUNEL reaction mixture was added to the sections for 60 min at 37 ℃ away from light. Then the sections were stained with DAB and observed under a light microscope at a magnification of 400x.

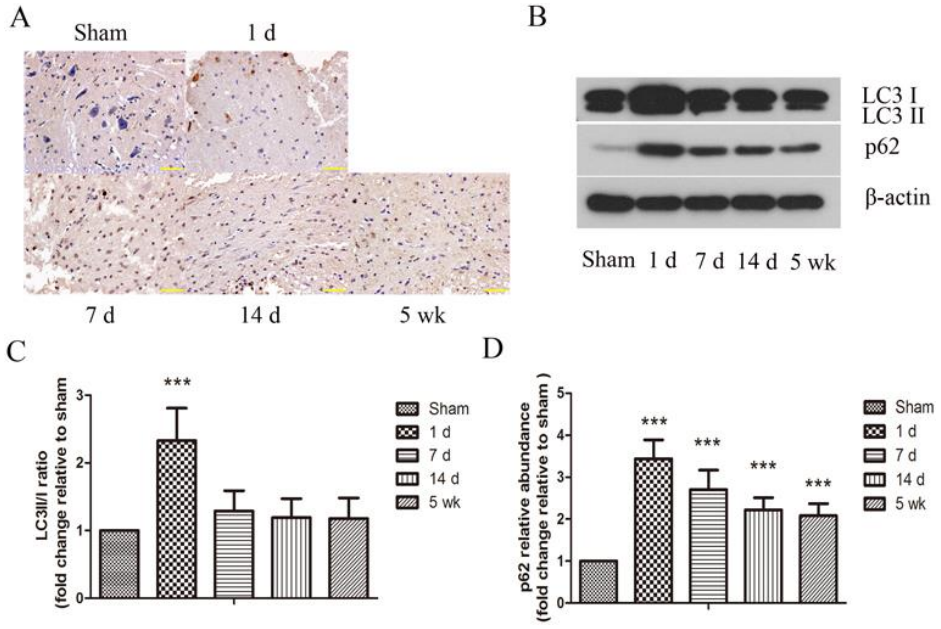

Figure 1. The changes in apoptosis and autophagy flux in spinal cord tissue after spinal cord injury. (A) The apoptosis in spinal cord tissue was detected by TUNEL staining (magnification 400x). (B) The protein levels of LC3I, LC3II and p62 in spinal cord tissue were assessed by Western blot assay. β-actin was used as a loading control. (C)&(D) The gray intensities of the bands were statistically analyzed and shown scale bar in A is 50 μm. The results shown represent at least three independent experiments. Each value represents the mean±SD (n=6) ***P<0.001, versus the sham group

Immunohistochemical staining

The spinal cord tissues were embedded in paraffin and cut into sections of 4 μm-thick. The sections were deparaffinized with xylene. After dehydration in graded concentrations of alcohol, the samples were processed to antigenic recuperation treatment. Then the sections were blocked with goat serum for 15 min at room temperature to avoid non-specific binding. After washing, the samples were incubated with primary antibodies SIRT1 (1:100, Proteintech, China), p-AMPK (1:100, Sangon, China) at 4 °C overnight, respectively. Then a secondary antibody was added to the sections after the washing with PBS for three times. The stains were visualized using DAB horseradish peroxidase color development Kit (Beyotime, China). The sections were counterstained with hematoxylin and observed under a light microscope at a magnification of 400x.

Hoechst 33342 staining

Hoechst 33342 staining was used to observe the morphology changes in nuclei of the apoptosis cells. Briefly, the cells from different treatment groups were washed with PBS, stained with Hoechst 33342 (10 μg/ml) for 5 min. The nuclei were observed under a fluorescence microscope. The condensed or fragmented nuclei were regarded as apoptotic cells.

Statistical analysis

All experimental results were presented as mean ± standard deviation (SD). Statistical differences among multi-groups were performed by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test (GraphPad Prism 5 software). A P<0.05 was considered to be statistically significant.

Results

Effect of SCI on apoptosis and autophagy in spinal cord tissue

The apoptosis induced by SCI in spinal cord tissues was evaluated by TUNEL staining. As shown in Figure 1(A), 1 d after SCI there were obvious apoptotic cells in spinal cord tissue. From the 7th day after SCI, the number of apoptotic cells remained high in spinal cord tissue compared with sham group. Moreover, the levels of autophagy related proteins were assessed by Western blot assay. As shown in Figure 1(B-D), the ratio of LC3II/LC3I (compared with sham group, P<0.001) and level of p62 (compared with sham group, P<0.001 for all) were first increased and then decreased in spinal cord tissue with prolongation of SCI.

Effect of SCI on AMPK/SIRT1 signaling pathway in spinal cord tissue

As shown in Figure 2(A-C), the protein expressions in AMPK/SIRT1 pathway were determined by Western blot assay. The results showed that the levels of p-AMPK (compared with sham group, P<0.05, P<0.001, P<0.05, and P<0.05, respectively) and SIRT1 (compared with sham group, P<0.001 for all) in spinal cord tissue were increased significantly compared with sham group and peaked on day 7 after SCI. To further confirm the increased expressions of p-AMPK and SIRT1 in spinal cord tissue, the immunohistochemical staining was performed. As shown in Figure 2(D), on day 7 after SCI

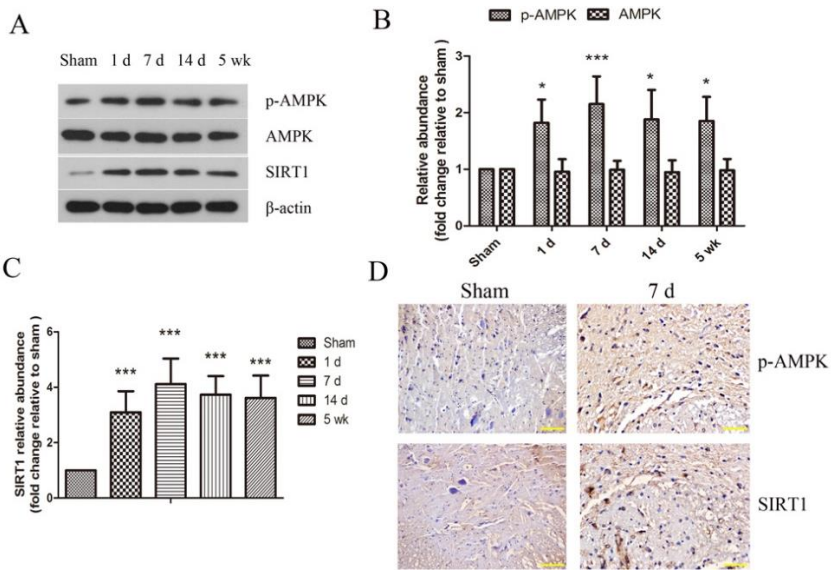

Figure 2. Effect of spinal cord injury on protein levels of phosphorylated-AMP-activated protein kinase and sirtuin 1 in spinal cord tissue. (A) The protein levels of p-AMPK and SIRT1 in spinal cord tissues were detected by Western blot analysis. β-actin was used as a loading control. (B)&(C) The gray intensities of the bands were statistically analyzed and shown. Scale bar in A is 50 μm. The results shown represent at least three independent experiments. Each value represents the mean±SD (n = 6). **P*<0.05, ****P*<0.001, versus the sham group

the expressions of p-AMPK and SIRT1 were obviously enhanced compared with sham group.

Effect of MI on apoptosis and autophagy in primary spinal cord neurons

To simulate SCI *in vitro*, the primary neurons of spinal cord were induced by MI. As shown in Figure 3(A), the primary neurons were identified by immunofluoresence staining of NSE. Moreover, the nucleus damage in primary neurons was assessed by Hoechst 33342 staining. As shown in Figure 3(B), MI resulted in more condensed or fragmented apoptotic nuclei in primary neurons over time compare with control group. As shown in Figure 3 (C-E), the effect of

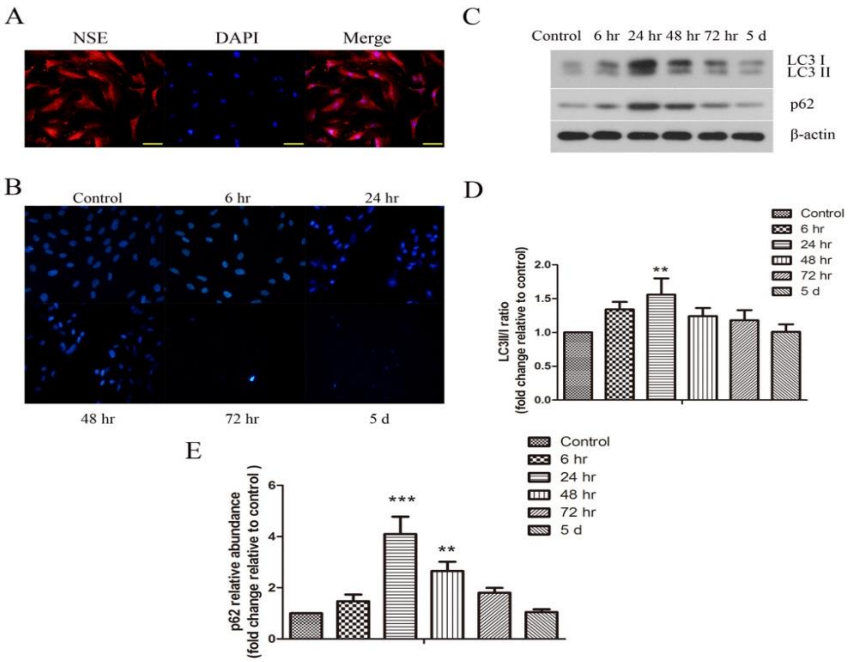

Figure 3. The cell damage and impaired autophagy flux induced by mechanical injury in primary spinal cord neurons. (A) The primary spinal cord neurons were identified by immunofluoresence staining of NSE (magnification 400x). (B) The nucleus damage induced by MI in primary neurons of spinal cord was observed by Hoechst 33342 staining. (C-E) The protein expressions of LC3I, LC3II and p62 in primary spinal cord neurons were determined by Western blot analysis. β-actin was used as a loading control. The gray intensities of the bands were statistically analyzed and shown. Scale bar in A is 50 μm. The results shown represent at least three independent experiments. Each value represents the mean±SD (n=3). ***P*<0.01, ****P*<0.001, versus the control group

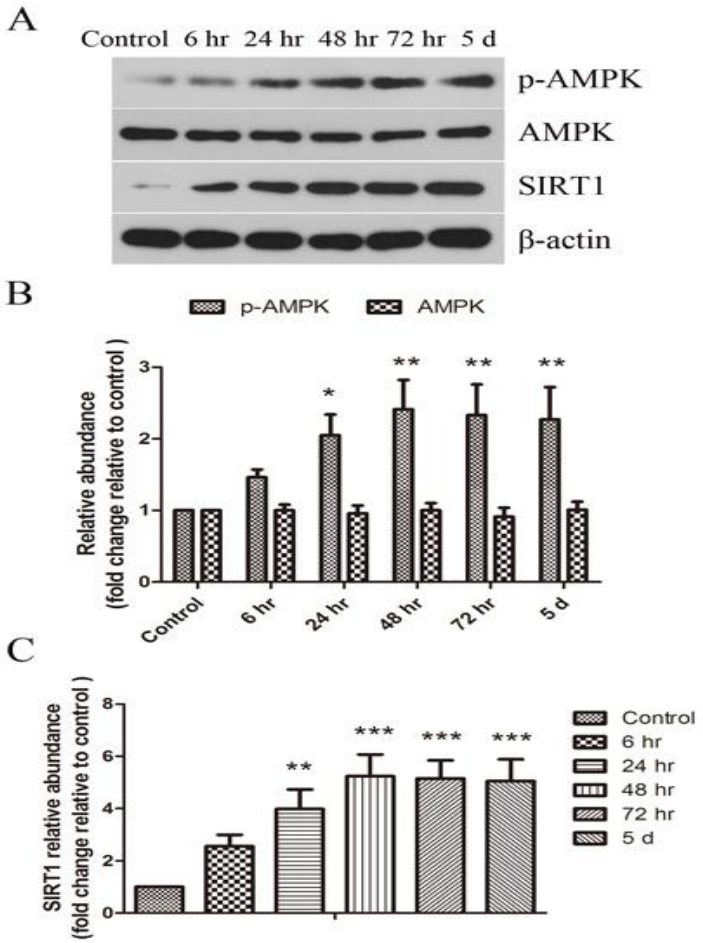

Figure 4. Effect of mechanical injury on protein levels of phosphorylated-AMP-activated protein kinase and sirtuin 1 in primary spinal cord neurons. (A) The levels of p-AMPK and SIRT1 in primary spinal cord neurons were evaluated by Western blot assay. β-actin was used as a loading control. (B)&(C) The gray intensities of the bands were statistically analyzed and shown. The results shown represent at least three independent experiments. Each value represents the mean±SD (n=3). **P*<0.05, ***P*<0.01, ****P* < 0.001, versus the control group

MI on autophagy related proteins in primary neurons was assessed by Western blot. The results indicated that MI resulted in first increase and then decrease in LC3II/LC3I ratio (compared with control group, *P*<0.01) and p62 (compared with control group, *P*<0.001 and *P*<0.01, respectively) level in primary neurons over time, which was similar to that in spinal cord tissue after SCI.

Effect of MI on AMPK/SIRT1 signaling pathway in primary spinal cord neurons

As shown in Figure 4(A-C), the levels of p-AMPK (compared with control group, *P*<0.05, *P*<0.01, *P*<0.01, and *P*<0.01, respectively) and SIRT1 (compared with control group, *P*<0.01, *P*<0.001, *P*<0.001, and *P*<0.001, respectively) were enhanced significantly in MI-treated primary spinal cord neurons as time went on, which was consistent with the *in vivo* results and suggested the activation of AMPK/SIRT1 signaling pathway.

Activation of AMPK/SIRT1 pathway by resveratrol promotes autophagy and reduces cell damage in MI-induced primary spinal cord neurons

To further evaluate the role of AMPK/SIRT1 pathway in the regulation of autophagy, the primary neurons were pretreated with resveratrol, which is confirmed to be an effective activator of the AMPK/SIRT1 pathway. As shown in Figure 5(A-C), pretreatment with resveratrol further upregulated the levels of p-AMPK (compared with MI group, *P*<0.05, and *P*<0.01, respectively) and SIRT1 (compared with MI group, *P*<0.05 for all) induced by MI in primary neurons of spinal cord. Additionally, the nucleus damage was observed by Hoechst 33342 staining and showed in Figure 5(D). The condensed or fragmented apoptotic nuclei induced by MI were restrained by pretreatment with resveratrol obviously. Moreover, the ratio of LC3II/LC3I appeared to be on the rise in resveratrol pretreatment groups compared with MI group, although there was no statistical significance.

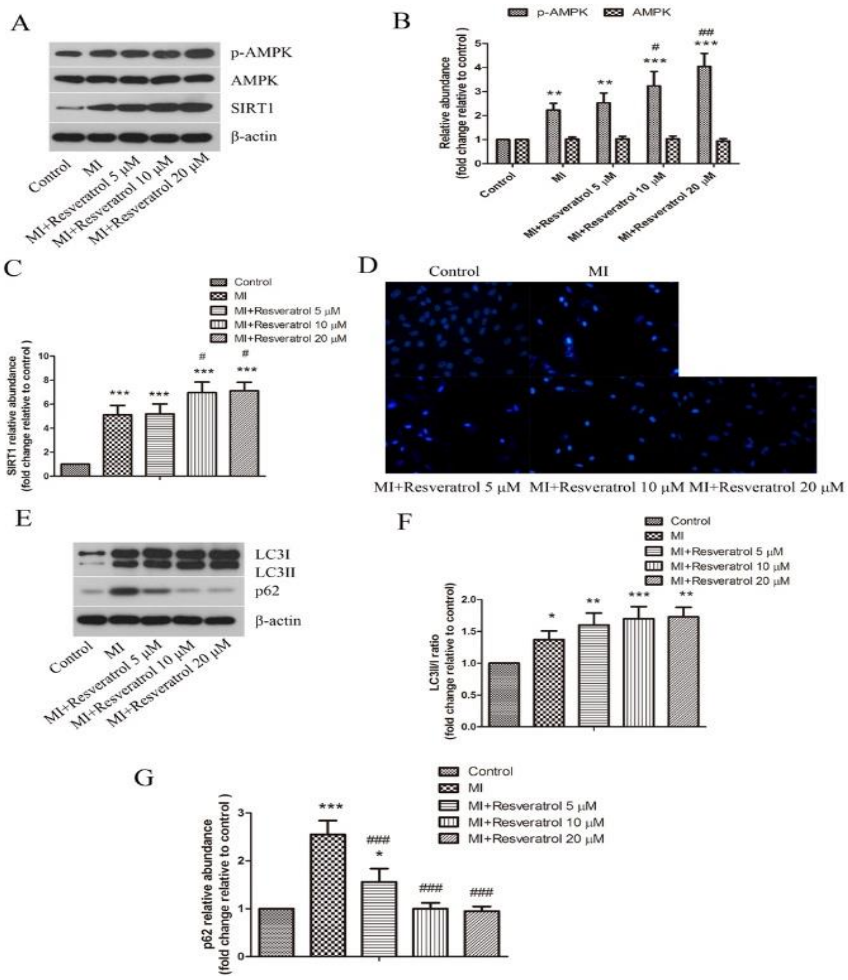

Figure 5. Activation of AMP-activated protein kinase/sirtuin 1 pathway by resveratrol alleviated mechanical injury-induced cell damage and impaired autophagy flux in primary spinal cord neurons. (A-C) The protein levels of p-AMPK and SIRT1 in primary spinal cord neurons were assessed by Western blot assay and the statistical analysis of the gray intensities of the bands were shown. β-actin was used as a loading control. (D) The nucleus damage induced by MI in primary neurons of spinal cord was observed by Hoechst 33342 staining. (E-G) The protein levels of LC3I, LC3II and p62 in primary spinal cord neurons were assessed by Western blot assay. β-actin was used as a loading control. The gray intensities of the bands were statistically analyzed and shown. The results shown represent at least three independent experiments. Each value represents the mean±SD (n= 3). **P*<0.05, ***P*<0.01, ****P*<0.001, versus the control group. #*P*<0.05, ##*P*<0.01, ###*P*<0.001, versus the MI group

Reveratrol pretreatment could significantly downregulate the increased p62 level induced by MI(Figure 5(E-G)) (compared with MI group, *P*<0.001 for all).

Discussion

In our research, the role of AMPK-SIRT1 pathway in the regulation of autophagy flux after SCI was explored for the first time. The MI model of primary spinal cord neurons in rats was adopted for *in vitro* study, which has been reported by previous researches and is able to simulate the damage situation of SCI with steady repetition (4, 17).

Growing evidence has demonstrated the role of autophagy in central nervous system injuries, including SCI. However, there are contradictory results about the role of autophagy during SCI, and autophagy is deemed to be a double-edged sword.

Research showed that G-CSF could inhibit neuronal apoptosis after SCI through inducing autophagy (18). A research by Wang *et al.* suggested that VEGF attenuated SCI by activation of autophagy (19). On the other hand, researches also found that the activation of impaired autophagy after SCI, which played detrimental roles in SCI (15, 20, 21). These contradictory results may be explained by autophagy flux. The autophagy flux is a process from autophagosomes formation to delivery and degradation in the lysosomes (22). The autophagic-lysosomal pathway dysfunction may cause the accumulations of vast immature autophagic vacuoles and block the autophagy flux, which aggravates the damage after SCI (23). LC3 is confirmed to be a specific marker for autophagy and participates in the autophagosomes formation. The conversion of LC3-I to LC3-II subtypes of LC3 suggests the initiation of autophagy (24). While p62 protein is an autophagy substrate that can be degraded by lysosome. The

increased level of p62 suggests the autophagic-lysosomal pathway dysfunction, which results in the inhibition of autophagy flux (23). According to our study, the ratio of LC3II/LC3I was increased at day 1 after the trauma and accompanied by an increased level of p62 *in vivo* and *in vitro,* which indicated the inhibition of autophagy flux was involved in the mechanisms of the damage induced by SCI.

SIRT1 is reported to have protective effects on neurons in nervous system diseases, such as Alzheimer's disease (25) and Prion Disease (26). Recent studies have shown that SIRT1 could regulate autophagy in neurons to delay the progression of neurodegenerative diseases (27). Cunha-Santos *et al.* demonstrated that overexpression of SIRT1 provided important benefits to treat Machado-Joseph disease by regulating autophagy (28). AMPK, the upstream regulator of SIRT1, is demonstrated to regulate autophagy in various physiological and pathological conditions. However, the role of AMPK-SIRT1 pathway in the regulation of autophagy during SCI has not been evaluated. We further investigated the expressions of AMPK and SIRT1 after SCI *in vivo* and *in vitro* and the results showed that the levels of p-AMPK and SIRT1 were increased significantly after the trauma, which might associate with the decreased p62 level in the later period of the trauma.

Resveratrol is a recognized activator of AMPK/SIRT1 pathway. Previous study suggested that the AMPK/SIRT1 pathway activated by resveratrol was involved in autophagy regulation, which protected HUVEC from oxidative damage (29). Wang *et al.* showed that resveratrol induced the activation of the AMPK/SIRT1 signaling pathway, which mediated autophagy activation (30). In our study, we used resveratrol to further activate the AMPK/SIRT1 pathway in MI-treated primary neurons of spinal cord. We found that pretreatment with resveratrol further activated the AMPK/SIRT1 pathway, which promoted autophagy flux by downregulation of p62 protein in spinal cord primary neurons at 24 hr after MI.

Growing evidence has suggested that the molecular pathways that regulate autophagy and apoptosis are inter-connected. Research has showed that autophagy may have protective effect on cells by eliminating damaged organelles and misfolded proteins and the autophagy level negatively correlated with the degree of apoptosis (31). The autophagy related proteins may also degrade pro-apoptotic proteins, such as caspases/Bcl-2 family proteins, so that autophagy plays important role in suppressing apoptosis (32, 33). As evidenced by recent research, the activation of AMPK/SIRT1 pathway may induce autophagy to clear the injured mitochondria and decrease the release of pro-apoptotic substances, thus inhibit apoptosis(12, 34). According to our results, further activation of AMPK/SIRT1 pathway induced by resveratrol could significantly mitigate the cell damage in primary spinal cord neurons at 24 hr after MI, which, at least in part, was mediated by autophagy flux activation.

Conclusion

Our present study demonstrated that SCI resulted in increased damage, impaired autophagy flux and elevated levels of p-AMPK and SIRT1 *in vivo* and *in vitro*. Further activation of AMPK/SIRT1 pathway by pretreatment with resveratrol could mitigate the cell damage, promote the autophagy flux in MI-induced primary spinal cord neurons. Our findings indicate that regulation of autophagy flux by AMPK/SIRT1 pathway may be a potential intervention of SCI.

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