Resveratrol improves neurological outcome and neuroinflammation following spinal cord injury through enhancing autophagy involving the AMPK/mTOR pathway

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Abstract. Resveratrol, a natural phenolic compound, provides neuroprotective effects, however, the specific mechanisms of action remain to be elucidated. The purpose of the present study was to examine the neuroprotective effect of resveratrol on spinal cord injury (SCI) and the potential molecular mechanisms of action. A rat model of SCI was induced using Allen's method, and resveratrol (100 mg/kg) was intraperitoneally injected 1 day following surgery. The recovery of neurological function was assessed using the Basso, Beattie, Bresnahan scoring system and an inclined plane test. The concentrations of pro- and anti-inflammatory factors were measured using ELISA. The expression and location of autophagy markers were measured using western blot and immunofluorescence analyses. The results suggested that resveratrol administration resulted in functional improvement of locomotor activity and reduced neuroinflammation following the induction of SCI. In addition, autophagy was activated following SCI, as demonstrated by the significantly increased ratio of microtubule-associated protein light chain 3 (LC3)-II/LC3-I and expression of Beclin-1 in the injured spinal cord. Of note, the enhancement of phosphorylated (p)-AMP-activated protein kinase (AMPK) and the reduction of p-mammalian target of rapamycin (mTOR) following SCI indicated that the SCI-induced activation of autophagy was associated with the AMPK/mTOR signaling pathway. Resveratrol treatment further enhanced the activation of autophagy via the AMPK/mTOR pathway following SCI. By contrast, the autophagic inhibitor, 3-methyladenine, partially inhibited the neuroprotective effects of resveratrol treatment. Together, these findings suggested that resveratrol promoted functional recovery and inhibited neuroinflammation through the activation of autophagy mediated by the AMPK/mTOR pathway following SCI.

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Abbreviations: SCI, spinal cord injury; Res, resveratrol; BBB, Basso, Beattie, Bresnahan; LC3, microtubule-associated protein light chain 3; 3-MA, 3-methyladenine; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-10, interleukin-10; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin

Key words: spinal cord injury, resveratrol, autophagy, neurological outcome, neuroinflammation, AMP-activated protein kinase/mammalian target of rapamycin

Introduction

Spinal cord injury (SCI) is one of the most important causes of mortality and long-term disability among young adults worldwide. In the United States alone, ~273,000 affected individuals and >12,000 new cases are reported annually (1). The pathophysiology of SCI comprises primary injury and the secondary injury; the primary injury is attributed to mechanical forces applied to the spinal cord, whereas the secondary injury is a complex cascade of cellular, molecular and biochemical events, including inflammation, axonal disruption, electrolyte disorders, oxidative stress, apoptosis and autophagy, which are important in the physical and functional deficits following SCI (2,3). These secondary effects can result in the loss of motor neurons and permanent neurological deficits, leading to a wide range of disabilities, which significantly impact on quality of life (4). Although the defined molecular mechanism of this secondary injury remains controversial, the reduction of secondary injury is generally recognized as an effective treatment for SCI.

Autophagy is a highly conserved catabolic mechanism for the degradation of cytoplasmic constituents in the autophagosomal-lysosomal pathway, which can promote cell survival and induce cell death depending on the specific pathological events (5). It is reported that autophagy may rescue neuronal cell death in certain neurodegenerative diseases (6). Evidence

has demonstrated that autophagy is essential in maintaining cellular homoeostasis and protecting against a variety of diseases in the central nervous system (CNS) (7,8). As demonstrated in previous studies, activated autophagy has been implicated in animal models of traumatic SCI (7,9,10); however, its neuroprotective or neurodegenerative effect remains controversial. Whether the function of autophagy induced by SCI is protective or detrimental remains to be elucidated.

Resveratrol (3,4',5-trihydroxystilbene), a plant-derived polyphenolic compound found in red grapes, peanuts and mulberries (11), has a wide variety of beneficial effects on health, including antitumor, anti-inflammatory, anti-oxidation and neuroprotective activities (12). Previous studies have also indicated that resveratrol has neuroprotective properties in various types of acute CNS injuries, including stroke, subarachnoid hemorrhage, traumatic brain injury and SCI (13). It has been demonstrated that resveratrol promotes neurobehavioral and histopathological recovery following experimental SCI in rats (14). In addition, resveratrol treatment alleviates the SCI-induced systemic inflammatory response via suppressing the activity of nuclear factor (NF)-κB and upregulating the expression of sirtuin 1 (SIRT1) (15). However, whether autophagy is associated with the neuroprotective effect of resveratrol treatment following SCI has not been reported. The present study investigated the neuroprotective effects of resveratrol on SCI and the potential molecular mechanisms.

Materials and methods

Animals. A total of 120 adult male Sprague-Dawley rats (250-300 g, 8-12 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were housed in standard temperature conditions (23±25˚C) with a 12-h light/dark cycle, and free access to food and water. All experimental procedures and protocols were approved by the Animal Care and Use Committee of Hebei Medical University (Shijiazhuang, China). All efforts were made to minimize the number of animals used in the experiments and their suffering; there were five rats were in every group at each observation time point in each experiment.

Acute SCI model. The SCI model was established based on Allen's method (16). In brief, the experimental rats were anesthetized via an intraperitoneal injection of sodium pentobarbital (65 mg/kg). Following sterilization with 75% alcohol, a cut was made in the skin and muscles were separated to expose the lamina. The T9/10 spinous process was exposed and clamped with rongeur forceps. The vertebral plates were removed to expose the spinal cord. An impactor (2.0 mm in diameter; 10 g) was vertically dropped from a 25.0 mm height, which directly impacted the spinal cord at T9/10. Hematoma occurred at the injury site, with rapid contraction and tremor of hind limbs, which confirmed successful establishment of the model. The rats in the sham group received the same surgical procedures, but without impaction applied to the spinal cord.

Drug administration. Resveratrol and 3-methyladenine (3-MA) from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) were dissolved in dimethyl sulfoxide and further diluted in PBS to a final dose prior to intraperitoneal injection. The rats in the SCI + Res group were administered with a single dose of resveratrol (100 mg/kg, i.p), Animals in the SCI + Res + 3-MA group were injected with resveratrol (100 mg/kg, i.p) and 3-MA (2.5 mg/kg, i.p) immediately following injury.

Assessment of neurological function recovery. The locomotion recovery of the rats was assessed using the Basso, Beattie, Bresnahan (BBB) open-field locomotor rating scale (17) on days 1, 3, 7, 14 and 21 following SCI. Briefly, the BBB scores range between 0 and 21 points. A score of 0 indicates complete paralysis, and a score of 21 indicates normal locomotion. Each evaluation was completed by two researchers who were blinded to the experimental groups. The inclined plane test was also performed to evaluate locomotion recovery, as described previously (18), at the same time.

ELISA analysis. The spinal cords were collected, homogenized and centrifuged for 20 min at 3,000 x g, 4˚C. The contents of tumor necrosis factor (TNF)-α (cat. no. SMTA00B), IL-1β (cat. no. SMLB00C) and interleukin (IL)-10 (cat. no. SM1000B) in the contused spinal cord were determined using respective ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol, and analyzed using a microplate reader (Dynex Technology, Chantilly, VA, USA). The absorbance was quantified at 450 nm. The levels of TNF-α, IL-1β and IL-10 in each sample were calculated based on the standard curve.

Immunofluorescence staining. The spinal cords were fixed in Tissue OCT-Freeze Medium (NobleRyder Technology, Inc., Beijing, China; http://www.nobleryder.cn) at room temperature for 12 h and cut into 10-μm sections. The sections were blocked with 10% bovine serum albumin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at room temperature. To locate the autophagy marker microtubule-associated protein light chain 3 (LC3) in neurons, LC3/neuronal nuclei (NeuN)/DAPI double fluorescence staining were performed. The sections were incubated overnight at 4˚C with the following mixed primary antibodies: Mouse anti-NeuN antibody (1:500; Abcam, Cambridge, UK; cat. no. GR51694-1) and rabbit anti-LC3 antibody (1:500; Santa Cruz Biotechnology, Inc., CA, USA; cat. no. 12741). The following day, mixed secondary antibodies (1:2,000) corresponding to the primary antibodies were added to the sections at 37˚C for 2 h, which included green donkey anti-mouse IgG (1:2,000) and red donkey anti-rabbit IgG (1:2,000; cat. no. 142401B; both Abbkine, Redlands, CA, USA; cat. no. 133702A). The nuclei were incubated with counterstain solution for 15 min. All images were captured on a Leica DMI4000B microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Western blot analysis. Total proteins in the spinal cord were extracted in RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), suspended and centrifuged at 12,000 x g, 4˚C for 10 min. The protein concentrations were determined using the Pierce BCA method (Thermo Fisher Scientific, Inc. Waltham, MA, USA). The protein samples

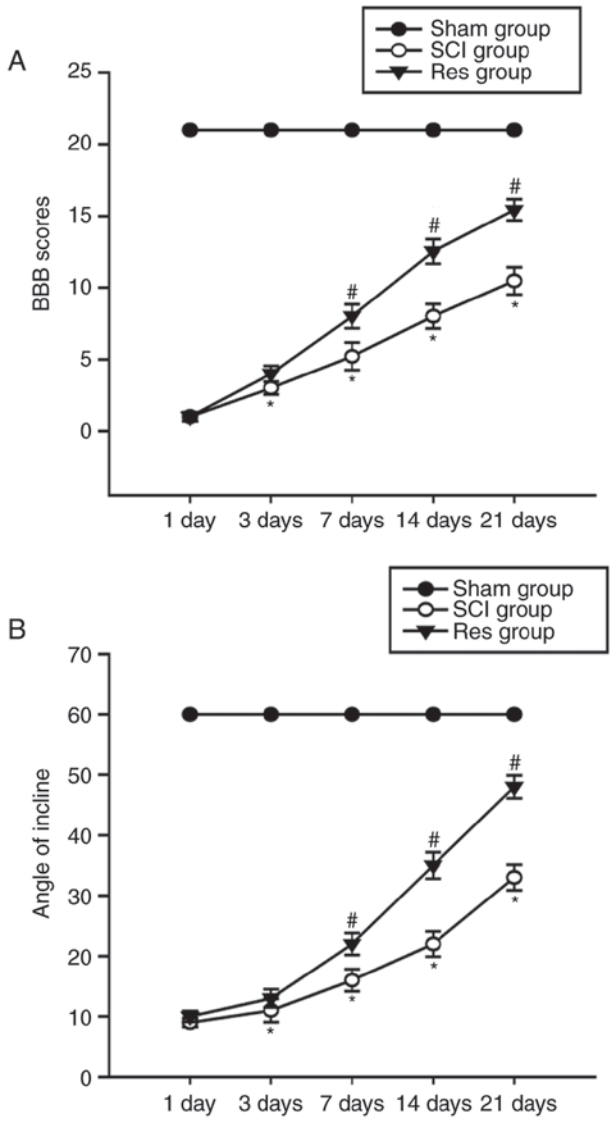

Figure 1. Effects of resveratrol on motor function in rats following SCI. (A) BBB scores of rats in each group at 1, 3, 7, 14 and 21 days post-injury. (B) Inclined plane test scores in each group at 1, 3, 7, 14 and 21 days post-injury. Data are presented as the mean ± standard deviation. *P<0.05 vs. sham group; #P<0.05, vs. SCI group. SCI, spinal cord injury; Res, resveratrol; BBB, Basso, Beattie, Bresnahan.

(20 μg) were separated by electrophoresis on 10% separation gels and then transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA). Following blocking with 5% non-fat milk for 2 h at 37˚C, the membranes were incubated overnight at 4˚C with the following primary antibodies: Rabbit anti-LC3 antibody, and rabbit anti-Beclin-1 antibody (cat. no. 3738), rabbit anti-AMPK antibody (cat. no. 5831), rabbit anti-p-AMPK antibody (cat. no. 2535), rabbit anti-mTOR antibody (cat. no. 2983) rabbit anti-p-mTOR antibody (cat. no. 2971) and rabbit anti-β-actin (cat. no. 8457) from Cell Signaling Technology, Inc. (Danvers, MA, USA) at 1:1,000 dilutions. Following washing in TBST, HRP-conjugated secondary antibodies (1:5,000 cat. no. 7074; Santa Cruz Biotechnology, Inc.) were added for signal development for 2 h at 37˚C. Immunoreactivity was visualized using an ECL kit (GE Healthcare Life Sciences, Amersham, UK) and quantitatively analyzed using ImageJ software v 1.8.0 (NIH, Bethesda, MD, USA). Independent experiments were performed in triplicate, and quantities of band densities were normalized using β-actin.

Statistical analysis. All data are expressed as the mean ± standard deviation of three independent experiments. Statistical analyses were performed with the Prism software package (GraphPad v5; GraphPad Software, Inc., La Jolla, CA, USA). Unpaired Student's t-test was used for comparison of simple effects between two groups. One-way analysis of variance followed by Dunnett's post hoc test was used for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Resveratrol promotes the recovery of neurological dysfunction following SCI in vivo. To investigate the effects of resveratrol on locomotor functional recovery following SCI, BBB scoring and an inclined plane test were performed on days 1, 3, 7, 14 and 21 post-injury. No significant difference in motor function was detected in any rats prior to model establishment. As shown in Fig. 1A, there was no locomotor dysfunction in the rats in the sham group between days 1 and 21. The BBB locomotor scores were lower in the SCI group in comparison to those in the sham group at 3, 7, 14 and 21 days post-SCI. Treatment with resveratrol markedly increased the BBB scores following SCI at 7, 14 and 21 days post-SCI. Similarly, the inclined plane test scores were consistently lower in the SCI group following injury, which were significantly elevated by resveratrol treatment (Fig. 1B). These results suggested that resveratrol improved the behavioral dysfunction caused by SCI.

Resveratrol suppresses SCI-induced neuroinflammation in rats. To determine the effect of resveratrol administration on neuroinflammatory responses, the present study analyzed the levels of pro-inflammatory cytokines TNF-α and IL-1β, and anti-inflammatory cytokine IL-10 in the contused spinal cord using ELISA. As shown in Fig. 2A and B, the levels of TNF-α and IL-1β were significantly increased post-SCI, whereas the level of IL-10 was significantly decreased in the spinal cord of the SCI model (Fig. 2C), compared with that in the sham animals. The intraperitoneal administration of resveratrol following SCI resulted in a significant reduction in the levels of TNF-α and IL-1β, and an upregulation in the level of IL-10 between days 3 and 21 post-injury. These data indicated that resveratrol significantly inhibited the neuroinflammatory response at the lesion site following SCI.

Resveratrol enhances SCI-mediated neuronal autophagy. The present study also evaluated the induction of autophagy following SCI by detecting the expression of autophagy-associated proteins LC3 and Beclin-1, which are considered two primary markers of autophagy. The conversion of LC3-I to LC3-II is essential for the formation of autophagosomes. The results of the immunofluorescence analysis suggested that LC3 was predominantly expressed in NeuN-positive neurons 3 days following SCI (Fig. 3). Western blot analysis demonstrated that the expression levels of Beclin-1 and the ratio of LC3-II/LC3-I

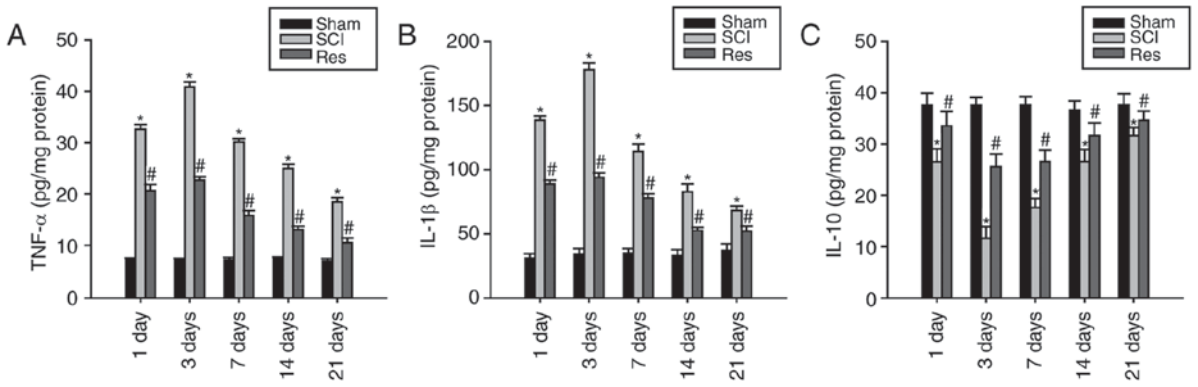

Figure 2. Effects of resveratrol on the expression of inflammatory factors following SCI. The levels of pro-inflammatory factors (A) TNF-α and (B) IL-1β, and anti-inflammatory factor (C) IL-10 at 1, 3, 7, 14 and 21 days following SCI were examined using ELISA analysis. Data are presented as the mean ± standard deviation. *P<0.05, vs. sham group; #P<0.05, vs. SCI group. SCI, spinal cord injury; Res, resveratrol; TNF-α, tumor necrosis factor-α; IL, interleukin.

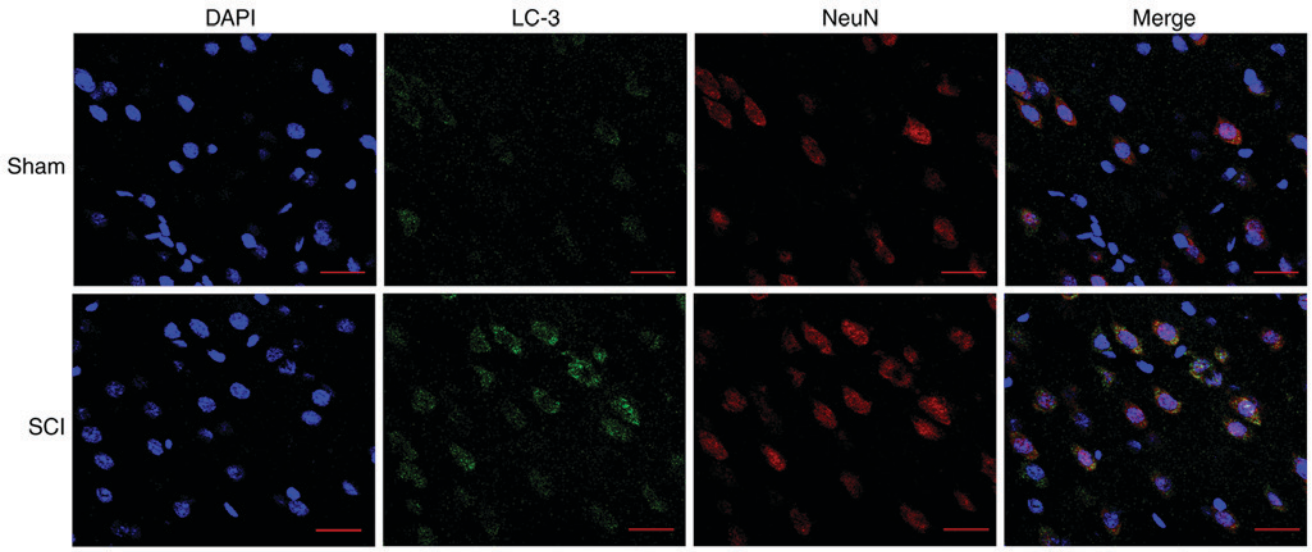

Figure 3. Confocal images of autophagic maker LC3 and NeuN. Immunofluorescence staining of LC3 and NeuN in the damaged spinal cord at 3 days post-SCI. The microphotographs were visualized by confocal laser scanning microscopy. Scale bar, 50 μm. SCI, spinal cord injury; LC3, microtubule-associated protein light chain 3; NeuN, neuronal nuclei.

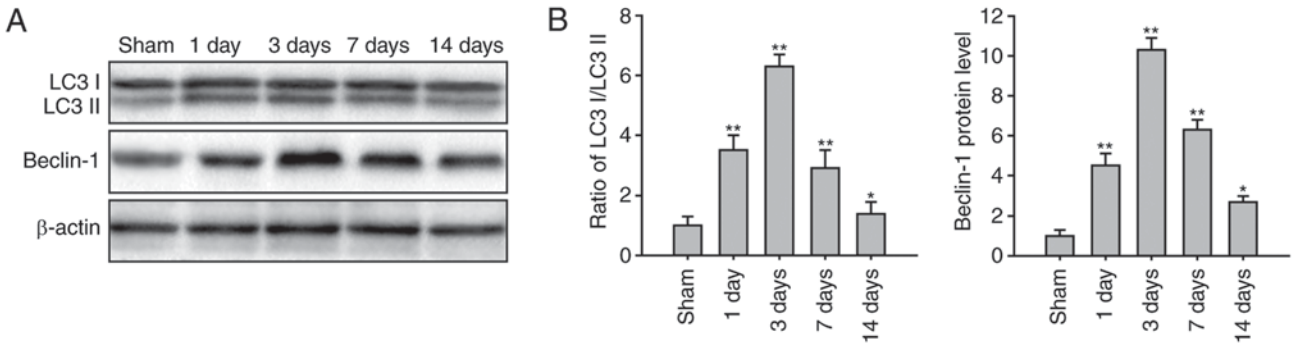

Figure 4. Autophagy is activated in the injured spinal cord of rats following SCI. (A) Immunoblots of autophagic maker proteins LC3 and Beclin-1 in the damaged spinal cord between 1 and 14 days post-SCI. Representative blots are shown from three independent experiments with similar results. (B) Quantitative analysis of levels of the indicated proteins relative to β-actin. β-actin was used as the loading control and for band density normalization. Values are presented as the mean ± standard deviation. *P<0.05 and **P<0.01, vs. sham group. SCI, spinal cord injury; LC3, microtubule-associated protein light chain 3.

were significantly increased 1 day following injury and peaked 3 days following injury, compared with those in the sham group (Fig. 4A and B), which was further enhanced following resveratrol treatment (Fig. 5A and B). Therefore, these results suggested that autophagy was activated in the injured spinal cord following SCI and further enhanced by resveratrol treatment.

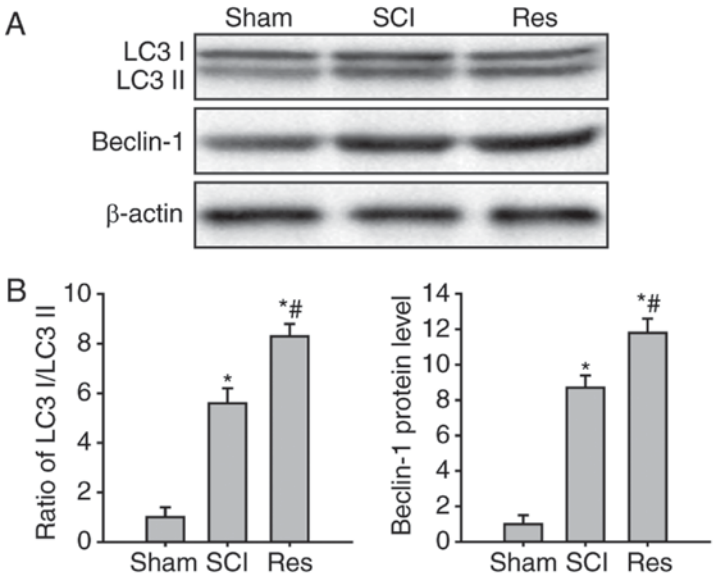

Figure 5. Resveratrol enhances SCI-induced activation of autophagy. (A) Western blot bands; (B) quantitative analysis of the protein expression levels of LC3 and Beclin-1 at 3 days post-SCI +/-resveratrol treatment. β-actin was used as the loading control and for band density normalization. Values are presented as the mean ± standard deviation. *P<0.05, vs. sham group; #P<0.05, vs. SCI group. SCI, spinal cord injury; LC3, microtubule-associated protein light chain 3; Res, resveratrol.

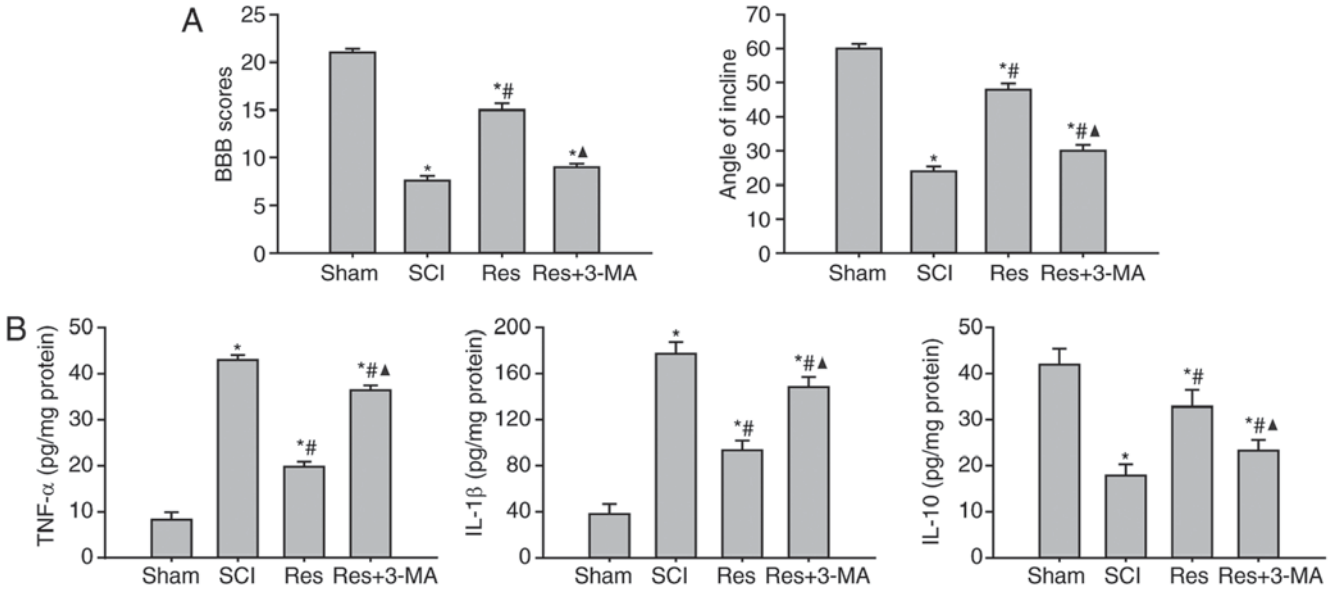

Figure 6. 3-MA treatment partially inhibits the neuroprotective effects of resveratrol in SCI. (A) BBB scores and inclined plane test scores for recovery of locomotor performance at 14 days post-injury. (B) Levels of TNF-α, IL-1β and IL-10 at 3 days post-SCI, as determined using ELISA analysis. Data are presented the mean ± standard deviation. *P<0.05, vs. sham group; #P<0.05, vs. SCI group; ▲P<0.05, vs. Res group. SCI, spinal cord injury; 3-MA, 3-methyladenine; Res, resveratrol; TNF-α, tumor necrosis factor-α; IL, interleukin; BBB, BBB, Basso, Beattie, Bresnahan.

Resveratrol improves functional recovery and inflammation by enhancing autophagy. To investigate the potential mechanism underlying the neuroprotective effects of resveratrol, intraperitoneal injections of resveratrol and 3-MA were performed following SCI surgery. 3-MA, an inhibitor of autophagy, inhibits class III phosphoinositide 3-kinase to suppress the formation of autophagosomes. As shown in Fig. 6A and B, the improvement of functional recovery and neuroinflammatory response induced by resveratrol were partially inhibited by 3-MA treatment. This finding demonstrated that resveratrol treatment protected against SCI in the rats through enhancing the activation of autophagy.

Resveratrol enhances autophagy via the AMPK/mTOR pathway following SCI. To evaluate whether resveratrol induced autophagy through the AMPK/mTOR signaling pathway, the present study examined the effects of resveratrol on levels of AMPK, p-AMPK, mTOR and p-mTOR. The expression of autophagy makers LC-3 and Beclin-1 peaked at 3 days post-SCI, therefore the levels of AMPK, p-AMPK, mTOR and p-mTOR were assessed at 3 days post-SCI. In addition, the recovery of neurological function and axonal regeneration occurred at 21 days post-SCI, therefore, protein expression levels were determined at 21 days post-SCI. An increased expression of

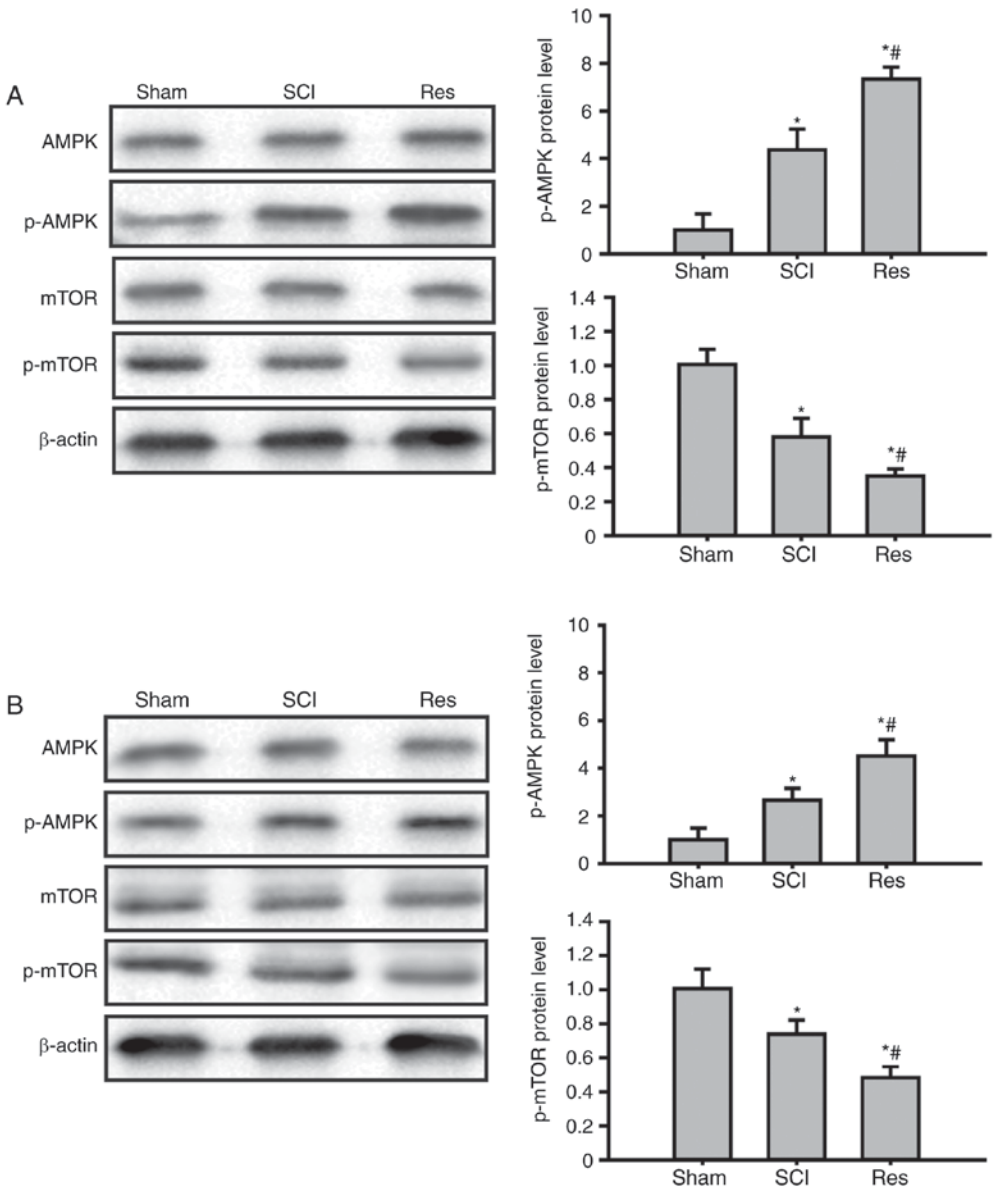

Figure 7. Resveratrol activates autophagy via the AMPK/mTOR signaling pathway. (A) Western blot bands and quantitative analysis of protein levels of AMPK, p-AMPK, mTOR and p-mTOR at 3 days post-SCI. (B) Western blot bands and quantitative analysis of proteins levels of AMPK, p-AMPK, mTOR, and p-mTOR at 21 days post-SCI. β-actin was used as the loading control and for band density normalization. Values are presented as the mean ± standard deviation. *P<0.05, vs. sham group; #P<0.05, vs. SCI group. SCI, spinal cord injury; Res, resveratrol; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; p-, phosphorylated.

p-AMPK and reduced expression of p-mTOR were observed in the local injured spinal cord, compared with levels in the sham group, which indicated that the AMPK/mTOR pathway was involved in the activation of autophagy. Resveratrol treatment significantly enhanced the level of p-AMPK and decreased the level of p-mTOR following SCI at 3 and 21 days (Fig. 7A and B, respectively). Additionally, 3-MA treatment reversed the SCI-induced upregulation of p-AMPK and downregulation of p-mTOR (Fig. 8). These results suggested that resveratrol enhanced the SCI-induced activation of autophagy through activating the AMPK/mTOR signaling pathway.

Discussion

SCI frequently leads to a permanent neurological impairment as a result of primary injury followed by secondary injury without effective treatment. Neuroinflammation and autophagy are important in the progression of SCI. There is currently a focus on identifying drugs that may improve neurological outcome, reduce neuroinflammatory responses and promote neuronal survival following SCI. Resveratrol, a natural phenolic compound, provides neuroprotective effects in acute SCI, however, the specific mechanisms of action have not been elucidated (13). In the present study, on verifying the neuroprotective effects of resveratrol, it was found that resveratrol significantly promoted the recovery of neurobehavioral dysfunction and alleviated neuroinflammatory responses, finally improving outcome post-SCI. Liu *et al* (14) demonstrated that resveratrol improved neuron protection and neurological deficits following SCI *in vivo*, consistent with the findings of the present study, however, the exact mechanism of resveratrol-induced neuroprotection remained

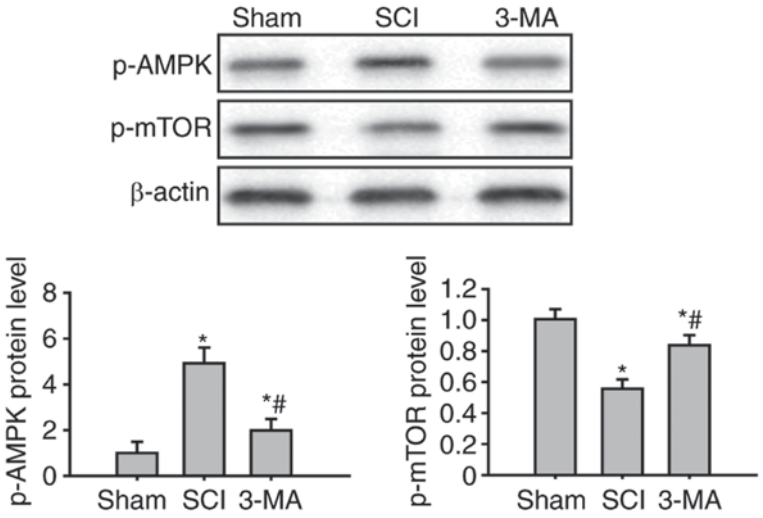

Figure 8. 3-MA treatment regulates the AMPK/mTOR signaling pathway. Western blot bands and quantitative analysis of p-AMPK and p-mTOR at 3 days post-SCI. β-actin was used as the loading control and for band density normalization. Values are presented as the mean ± standard deviation. *P<0.05 vs. sham group; #P<0.05 vs. SCI group. SCI, spinal cord injury; Res, resveratrol; 3-MA, 3-methyladenine; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; p-, phosphorylated

unclear. Liu *et al* (15) also indicated that resveratrol attenuated SCI-induced inflammatory damage in rat lungs. In the present study, it was found that resveratrol suppressed SCI-induced neuroinflammation in the injured spinal cord. The present study was designed to investigate the effects of resveratrol treatment on neurological outcome and neuroinflammation, but also to examine the potential molecular mechanism underlying the neuroprotective effects of resveratrol treatment following SCI in rats.

The activation of autophagy following SCI has been widely observed in a series of studies (9,19). Using a rat model of contusive SCI, Liu *et al* (20) observed the accumulation of LC3-II-positive autophagosomes, appearing at 1 day post-injury. Consistent with previous findings, the results of the present study also showed that autophagy was activated in rats following SCI and was sustained over a period of time, however, the role of autophagy in the pathogenesis of SCI remains controversial. Several studies have reported that the induction of autophagy protects neurons from degradation and that the inhibition of autophagy contributes to neurodegeneration (21). The induction of autophagy may stabilize microtubules and promote axon growth, and finally improve motor behavior recovery following SCI (22). Evidence has also indicated that transient spinal cord ischemia-induced autophagy in motor neurons contributes to neuronal death and inhibits motor functional recovery (18).

In determining whether autophagy is induced following SCI as a neuroprotective mechanism, the present study demonstrated that treatment with 3-MA, an autophagy inhibitor, aggravated neurological impairments and neuroinflammatory responses following SCI. However, whether the neuroprotective effects of resveratrol are associated with the activation of autophagy has not been reported. In the present study, the data demonstrated that the SCI-induced activation of autophagy was enhanced by treatment with resveratrol following surgery. In particular, in the SCI + Res + 3-MA group, it was found that 3-MA administration appeared to partially inhibit the neuroprotective effects of resveratrol in the SCI rats. These findings indicated that resveratrol treatment promoted functional recovery and inhibited neuroinflammation following SCI through enhancing the activation of autophagy.

The specific molecular mechanisms underlying the enhancement of autophagy by resveratrol treatment was investigated in more detail in the present study. A previous study indicated that resveratrol exerts neuroprotective effects on SCI by regulating autophagy and apoptosis mediated by the SIRT1/AMPK signaling pathway (23). The present study investigated whether the AMPK/mTOR pathway is involved in resveratrol-mediated autophagy enhancement. The AMPK/mTOR signaling pathway is primarily involved in regulating autophagy. AMPK, a serine/threonine protein kinase, is not only a major metabolic energy sensor, which regulates energy homeostasis and metabolic stress, but is also important in autophagy and protein degradation (24). mTOR, a downstream target of AMPK and a negative regulator of autophagy, is also important in the modulation of autophagy (25). It is reported that AMPK and mTOR mediate autophagy by directly activating the phosphorylation of Unc-51-like autophagy activating kinase 1 (26). In the present study, the levels of AMPK, p-AMPK, mTOR and p-mTOR were detected 3 and 21 days post-SCI. Rather than a focus on the dynamic alteration post-SCI, the effects of resveratrol treatment on the AMPK/mTOR pathway were examined. It was found that the expression of p-AMPK was significantly increased and that of p-mTOR was markedly decreased in the injured spinal cord tissues following SCI, which suggested that the SCI-induced activation of autophagy was associated with the AMPK/mTOR signaling pathway. Additionally, it was found that the administration of resveratrol further enhanced the level of p-AMPK and reduced the level of p-mTOR, indicating that resveratrol treatment enhanced autophagy via activating the AMPK/mTOR pathway.

The findings of the present study showed that resveratrol promoted functional recovery and attenuated the neuroinflammatory response in rats following SCI. Therefore, resveratrol treatment may be a potential therapeutic strategy for SCI. The present study also confirmed that resveratrol may exert these

neuroprotective effects through the enhancement of autophagy mediated by the AMPK/mTOR signaling pathway following SCI, revealing a novel molecular basis for the neuroprotective role of resveratrol.

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Availability of data and materials

The datasets and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HN and HM designed the experiments; DS, HL and XH performed the experiments; GY and BX analyzed the data; HM wrote and revised the paper; All authors read and approved the final manuscript.

Ethics approval and consent to participate

All protocols in the present study were approved by the Ethics Committee of Hebei Medical University (Shijiazhuang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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