Neuroscience 329 (2016) 193–200

PROBUCOL INHIBITS NEURAL CELL APOPTOSIS VIA INHIBITION OF mTOR SIGNALING PATHWAY AFTER SPINAL CORD INJURY

ZIPENG ZHOU,[a] SHURUI CHEN,[b] HAOSEN ZHAO,[a] CHEN WANG,[a] KAI GAO,[a] YUE GUO,[a] ZHAOLIANG SHEN,[c] YANSONG WANG,[a] HONGYU WANG[a] AND XIFAN MEI[a]*

[a] *Department of Orthopedics, First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China*

[b] *Jinzhou Medical University, Jinzhou, China*

[c] *Department of Orthopedics, Second Hospital of Jinzhou, Jinzhou, China*

Abstract—Autophagy plays an essential role in neurodevelopment, axonal guidance, neuropathic pain remission, and neuronal survival. Inhibiting the mammalian target of rapamycin (mTOR) signaling pathway can induce the occurrence of autophagy. In this study, we initially detected the effect of probucol on autophagy after spinal cord injury (SCI) by intraperitoneally injecting spinal cord-injured rats with probucol for 7 days. The levels of Beclin1 and LC3B were evidently enhanced at 7 days post-operation. However, the increase in the phosphorylated AMP-activated protein kinase (AMPK) protein and the decrease in ribosomal protein S6 kinase p70 subtype (p70S6K) phosphorylation level simultaneously occurred after SCI. Moreover, the expression levels of apoptosis-related proteins of Caspase-3, Caspase-9, and Bax were significantly reduced. Immunofluorescence results indicated that the expression of Caspase-3 protein was evidently decreased and that of Beclin-1 protein was increased by probucol. Nissl staining and Basso, Beattie, and Bresnahan scores showed that the quantity and function of motor neurons were visibly preserved by probucol after SCI. This study showed that probucol inhibited the mTOR signaling pathway to induce autophagy, reduce neural cell apoptosis and promote recovery of neurological function after SCI.

Key words: spinal cord injury, probucol, apoptosis, autophagy, mTOR.

INTRODUCTION

Spinal cord injury (SCI) is widely recognized as a worldwide medical problem (Wyndaele and Wyndaele, 2006). The related secondary injuries including inflammation, oxidative stress, apoptosis, and autophagy significantly affect SCI prognosis (Hayashi et al., 2000; Dery et al., 2009). The difficulties in SCI treatment can be attributed to autophagy and apoptosis, which perform major roles in physical and functional deficits (Blight, 2002; Rabchevsky et al., 2011). Standard treatment options for SCI have not been established; thus, an effective treatment is urgently needed (Courtine et al., 2011). In recent studies, neural cell autophagy performed a significant part in SCI. The increase in autophagy level can reduce nerve cell apoptosis and promote neural functional recovery after SCI (Komatsu et al., 2006; Chen et al., 2008; Ching et al., 2014). In addition, a lot of studies have demonstrated the important functions of mammalian target of rapamycin (mTOR) signaling pathway to regulate the level of autophagy (Kanno et al., 2012). Under lack of energy or stress, the mTOR signaling pathway is suppressed mainly through the mechanism of AMP-activated protein kinase (AMPK) activation (Inoki et al., 2012). In the research of Lin et al. (2014), mTOR activation in Merkel cell carcinoma was found to inhibit autophagy, whereas mTOR inhibition can induce autophagy. Wu et al. (2013) also found that the induction of autophagy simultaneously increases AMPK phosphorylation level, accompanied by phosphorylation of reducing S6 kinase p70 subtype (p70S6K). The findings suggest that ethanol extracts induce autophagy and the AMPK/mTOR pathway, further proving that the signal through mTOR pathway induces autophagy.

Probucol is a lipid-lowering drug with a variety of effects such as anti-inflammatory and anti-oxidation, used to lower cholesterol and prevent coronary atherosclerotic and heart disease in clinical. (Yamashita et al., 2008; Yamashita and Matsuzawa, 2009). Recently, an increasing number of studies have reported that probucol performs a protective function in neurotoxicity and neurodegeneration models, such as Alzheimer's disease and Huntington's disease (Santos et al., 2012; Ribeiro et al., 2013). However, Falk et al. (2011) found that probucol has significantly more effective and sustained beneficial effects in mitochondrial respiratory chain disease, in addition to activating PPAR signaling pathways. And then based on this study, Peng et al. showed probucol also inhibits mTORC1 to induced autophagy (Peng et al., 2015). It can directly inhibit mTORC1-regulated downstream activities. But few reports have elucidated the effect of probucol on autophagy level after SCI through the mTOR signaling pathway.

In this research, we used a rat model of SCI to investigate the possible neuroprotective mechanism of

*Corresponding author.
E-mail address: meixifan1971@163.com (X. Mei).

Abbreviations: AMPK, AMP-activated protein kinase; BBB, Basso, Beattie, and Bresnahan; BSA, bovine serum albumin; CNS, central nervous system; DMSO, dimethyl sulfoxide; mTOR, mammalian target of rapamycin; p70S6K, S6 kinase p70 subtype; PBS, phosphate-buffered saline; SCI, spinal cord injury.

http://dx.doi.org/10.1016/j.neuroscience.2016.05.019

SCI treatment by probucol. The results of our research may supply a new molecular basis by which probucol exerts its therapeutic action in SCI treatment and may have potential clinical application value in the future.

EXPERIMENTAL PROCEDURES

Animals and probucol treatment

Adult male Sprague–Dawley rats (weight range 180–220 g) were bought from the Laboratory Animal Center of Jinzhou Medical University. All experimental procedures were approved by the Animal Care and Use Committee of Jinzhou Medical University. All rats were fed under a specific-pathogen-free laboratory at 22 ± 1 °C with a normal circadian cycle and the right amount of water and food was added. After adaptation to the new environment, the rats were divided into the following three groups with the method of completely random design: sham group, vehicle group, and probucol group. The sham group was only performed laminectomy. The vehicle and probucol groups were established SCI model and respectively intraperitoneally injected with or without probucol. Probucol (Sigma, St. Louis, MO, USA) was dissolved in 10% dimethyl sulfoxide (DMSO) diluted in 0.9% saline. The probucol group received 10 mg/kg of probucol intraperitoneally (2 mL/kg; i.p.) (Santos et al., 2012) once a day for 7 days (first administration at 1 h after SCI). Simultaneously, the vehicle group was treated with the vehicle (0.9% saline with 10% DMSO, i.p.) in a similar manner as the probucol group.

SCI model

A contusive SCI rat model was set up by modified weight-drop method as described previously (Yacoub et al., 2014). Briefly, rats were anesthetized by i.p. injection of chloral hydrate (10%) (0.33 mL/kg) and placed in a solid framework, and aseptic technique was applied during the entire operation, using the highest point of the back as a guide of T10 prominence. A standard T9/10 laminectomy was performed to expose the spinal cords. A 10-g impactor (tip diameter: 2 mm) was dropped rapidly from 25-mm height, producing a moderate-to-severe cord contusion, resulting in lower limb paralysis and neurogenic bladder dysfunction. The spinal cord was rinsed with 0.9% saline and sutured the incisions after surgery. Then, antibiotics were intramuscular injected for three consecutive days and the bladder was massaged three times daily to aid urination until bladder function regained to normal.

Assessment of locomotion behavioral

The behavior of rats was determined by the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale before the operation and at 1, 3, 7, 14, 21, and 28 days after SCI (Basso et al., 1995) to observe the recovery of motor function. In brief, choose three highly trained examiners to assess the scores of rats according to the BBB score standard with a double blind method. The range of BBB scores from 0 indicative of complete paralysis to 21 representative of normal locomotion. Drawing a line chart based on average scores of rats in each group.

Western blot

At 7 days post-surgery, each group, which included three rats, was anesthetized by chloral hydrate (10%) (0.33 mL/kg), and the damaged spinal cord (1 cm from the center of the injury point) was immediately removed, rinsed with 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for three times, save backup in −80 °C. Tissues were cut into small pieces by using a pair of eye scissors and then dissolved in RIPA buffer with PMSF (Beyotime Biotechnology, Shanghai, China) for 20 min on ice and dissociated by an ultrasonic homogenizer. The protein supernatant was separated by centrifugation at 12,000 rpm for 25 min at 4 °C. The final protein concentration (2 μg/μL) was quantified according to BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China), with bovine serum albumin (BSA) as protein standard. The same amount of protein samples (40 μg) were emitted into polyacrylamide gels with different concentrations. The proteins were separated with SDS–PAGE and transferred onto a polyvinylidene fluoride membrane, after subsequently blocking with 10% BSA in TBST (25 mM Tris–HCl, 0.15 M saline, and 1% Tween 20) at room temperature for 2 h. Then, the membranes were incubated with the following primary antibodies at 4 °C overnight, including anti-phospho-AMPK antibody (1:2000; Cell Signaling Technology, Inc., Boston, USA), anti-phospho-p70SK antibody (1:1000; Cell Signaling Technology, Inc., Boston, USA), anti-LC3B antibody (1:1000; Cell Signaling Technology, Inc., Boston, USA), anti-Beclin-1 antibody (1:1000; Novus Biologicals, Littleton, CO, USA), anti-Caspase-3 antibody (1:1000; Cell Signaling Technology, Inc., Boston, USA), anti-caspase-9 antibody (1:1000; Cell Signaling Technology, Inc., Boston, USA), anti-Bcl2 antibody (1:1000; Abcam, Cambridge, UK), anti-Bax antibody (1:1000; Abcam, Cambridge, UK), and anti-β-Tubulin antibody (1:1000; TransGen Biotech, Beijing, China). On the second day, membranes were incubated with the following secondary antibodies at room temperature for 2 h, including HRP AffinPure Goat Anti-Rabbit IgG (1:10,000; EarthOX, Millbrae, CA, USA) and HRP AffinPure Goat Anti-Mouse IgG (1:10,000; EarthOX, Millbrae, CA, USA). The membranes were developed using ChemiDoc-It™TS2 Imager (UVP, LLC, Upland, CA, USA), and relative optical density was analyzed by ImageJ2x software (National Institute of Health, Bethesda, MD, USA).

Tissue preparation

Each group, which included three rats, was anesthetized by chloral hydrate (10%) (0.33 mL/kg) at 7 days after operation and then transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde. A segment of spinal cord about 5 mm that included the site of injury was removed. The spinal cord segments were fixed in 4% paraformaldehyde for 1 day and then moved to 30% sucrose in 4% paraformaldehyde until the segments sink to the bottom of the liquid. The 10-μm transverse frozen sections (3 mm from the center of injury) were cut by the cryostat Microtome for immunofluorescence and Nissl staining.