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ZL006 protects spinal cord neurons against ischemia-induced oxidative stress through AMPK-PGC-1α-Sirt3 pathway

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ABSTRACT

Spinal cord ischemia (SCI) induces a range of cellular and molecular cascades, including activation of glutamate receptors and downstream signaling. Post-synaptic density protein 95 (PSD-95) links neuronal nitric oxide synthase (nNOS) with the N-methyl-D-aspartic acid (NMDA) receptors to form a ternary complex in the CNS. This molecular complex-mediated cytotoxicity has been implicated in brain ischemia, but its role in SCI has not been determined. The goal of the study was to investigate the potential protective effects of ZL006, a small-molecule inhibitor of the PSD-95/nNOS interaction, in an in vitro SCI model induced by oxygen and glucose deprivation (OGD) in cultured spinal cord neurons. We found that ZL006 reduced OGD-induced lactate dehydrogenase (LDH) release, neuronal apoptosis and loss of cell viability. This protection was accompanied by the preservation of mitochondrial function, as evidenced by reduced mitochondrial oxidative stress, attenuated mitochondrial membrane potential (MMP) loss, and enhanced ATP generation. In addition, ZL006 stimulated mitochondrial enzyme activities and SOD2 deacetylation in a Sirt3-dependent manner. The results of western blot analysis showed that ZL006 increased the activation of AMPK-PGC-1α-Sirt3 pathway, and the beneficial effects of ZL006 was partially abolished by AMPK inhibitor and PGC-1α knockdown. Therefore, our present data showed that, by the AMPK-PGC-1α-Sirt3 pathway, ZL006 protects spinal cord neurons against ischemia through reducing mitochondrial oxidative stress to prevent apoptosis.

1. Introduction

Spinal cord ischemia (SCI), caused by the decreased or interrupted blood supply in the spinal cord tissues, is a devastating event for patients. It is a consequence of various pathological conditions, including thoracoabdominal aortic aneurysm surgery, systemic hypoperfusion, vasculitis, and cardiogenic embolism, and the incidence of SCI after thoracic and thoracoabdominal aortic repair surgery is reported to be up to 32% (Panthee and Ono, 2015; Yu et al., 2016). SCI induces a range of complex cellular and molecular cascades, such as generation of oxygen free radicals, lipid peroxidation, intracellular calcium overload, inflammatory cytokines release, and mitochondrial dysfunction (Yang et al., 2016). Although many advances in medical technologies have been achieved in the past few decades, the incidence of SCI-induced paraplegia is still 5.1% after open thoracoabdominal aortic aneurysmrepair surgery (LeMaire et al., 2012; Nardone et al., 2016).

The sirtuins (or Sir2-like proteins) are a conserved family of class III histone deacetylases (HDACs), and have been reported to be involved in transcriptional silencing, genetic control of aging and longevity of organisms ranging from yeasts to humans (Michan and Sinclair, 2007). Among the known sirtuin members, Sirt3 is characterized by its localization to the mitochondria, and has been identified as a stress responsive deacetylase recently shown to play a role in protecting cells under stress conditions (Bause and Haigis, 2013; Lombard et al., 2007; Pillai et al., 2010). Sirt3 functions as a downstream target of peroxisome proliferator-activated receptor (PPARγ) coactivator-1α (PGC-1α), which is directly regulated by

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adenosine monophosphate-activated protein kinase (AMPK), a serine/threonine kinase that senses energy status in various cells and tissues (Zhou et al., 2014). Previous studies showed that this AMPK-PGC-1α-Sirt3 pathway was involved in the molecular mechanisms underlying various neurological disorders (Chen et al., 2015; Fu et al., 2012; Huh et al., 2016; Yu et al., 2012).

Glutamate is the major excitatory CNS transmitter, and it is well established that release of glutamate and overactivation of multiple glutamate receptors, especially N-methyl-D-aspartate (NMDA) receptor, is an important molecular mechanism in the setting of ischemic injury in CNS (Arundine and Tymianski, 2004). Although many NMDA receptor antagonists have been shown to exert neuroprotective effects in pre-clinical ischemia studies, the clinical development was halted in the pilot phase because of the limited administration time window and adverse effects on endogenous NMDA receptor mediated neuronal survival mechanisms (Ginsberg, 2008). Activation of the NMDA receptors induces intracellular calcium overload and regulation of several downstream signaling cascades, including neuronal nitric oxide synthase (nNOS). This process requires a scaffold protein named as post-synaptic density protein 95 (PSD-95), which can recruit nNOS to NMDA receptors to forms an NMDAR/PSD-95/nNOS complex (Cao et al., 2005). Blocking the formation of this molecular complex through selective inhibitors or gene knockdown techniques has been demonstrated to be effective in alleviating brain ischemia in both in vitro and in vivo experiments (Bach et al., 2015; Gardoni and Di Luca, 2006; Zhou et al., 2010). However, the effect of this strategy on SCI has not been determined. Thus, the present study was designed to investigate the effect of ZL006, a small-molecule inhibitor at the PSD-95/nNOS interface, on ischemia-induced injury in spinal cord neurons.

2. Materials and methods

2.1. Spinal cord neuron cultures

All animal studies were approved by the Institutional Animals Ethics Committee. Cortical neurons were cultured from Sprague-Dawley rats using a modified method reported by Chen et al. (Chen et al., 2012). Rat spinal cords were removed from embryos at 16–18 days, stripped of meninges and blood vessels and minced. Tissues were cut into small pieces and dissociated by 0.25% trypsin digestion for 15 min at 37 °C. Neurons were resuspended in Neurobasal medium containing 2% B27 supplement and 0.5 mM L-Glutamine and plated at a density of 3×10^5 cells/cm^2. Neurons were maintained at 37 °C in a humidified 5% CO_2 incubator and half of the culture medium was changed every other day.

2.2. OGD

OGD was preformed to mimic ischemia in vitro using previous published methods (Chen et al., 2011). To initiate OGD, culture medium was removed and rinsed with PBS for three times. The cultured neurons were placed into a specialized, humidified chamber containing 5% CO_2, 95% N_2 at 37 °C with glucose-free DMEM, which was pre-gassed with N_2/CO_2 (95%/5%) to remove residual oxygen. After 2 h challenge, neurons were removed from the anaerobic chamber, and the culture medium was replaced with Neurobasal medium containing 2% B27 supplement and 0.5 mM L-Glutamine. The neurons were incubated for further 24 h to generate reperfusion insult.

2.3. Neuronal viability

Neurons were cultured at a concentration of 5×10^4 in microplates in a volume of 100 μl/well culture medium. After various treatments, 10 μl cell proliferation reagent, WST-1, was added into each well and incubated for 4 h at 37 °C. Then, 100 μl/well culture medium and 10 μl WST-1 was added into one well in the absence of neurons, and its absorbance was used as a blank position for the ELISA reader. Cells were shaken thoroughly for 1 min on a shaker and the absorbance of the samples was measured using a microplate reader.

2.4. Lactate dehydrogenase (LDH) assay

Neuronal cytotoxicity was determined by the release of LDH, a cytoplasmic enzyme released from cells, and a marker of membrane integrity. Briefly, 50 μl of supernatant from each well was collected, incubated with reduced form of nicotinamide-adenine dinucleotid (NADH) and pyruvate for 15 min at 37 °C and the reaction was stopped by adding 0.4 M NaOH. The activity of LDH was calculated from the absorbance at 440 nm and background absorbance from culture medium that was not used for any cell cultures was subtracted from all absorbance measurements.

2.5. TUNEL staining

Apoptotic cell death was detected by the TUNEL assay, a method to observe DNA strand breaks in nuclei. Briefly, spinal cord neurons were seeded on 1.5 cm glass slides at a density of 3×10^5 cells/cm^2. Twenty-four hours after OGD, neurons were fixed by immersing slides in 4% methanol-free formaldehyde solution in PBS for 20 min and permeabilized with 0.2% Tri-ton X-100 for 5 min. Cells were labeled with fluorescein TUNEL reagent mixture for 60 min at 37 °C according to the manufacturer's suggested protocol (Promega, Madison, WI, USA), and the slides were examined by fluorescence microscopy and the number of TUNEL-positive (apoptotic) cells was counted.

2.6. Caspase-3 activity

The activity of caspase-3 was measured using the colorimetric assay kit according to the manufacturer's instructions (Cell Signaling Technology, MA, USA). Briefly, after being harvested and lysed 10^6 cells were mixed with 32 μl of assay buffer and 2 μl of 10 mM Ac-DEVD-pNA substrate. Absorbance at 405 nm was measured after incubation at 37 °C for 4 h. Absorbance of each sample was determined by subtraction of the mean absorbance of the blank and corrected by the protein concentration of the cell lysate. The results were described as relative activity to that of control group.

2.7. Mitochondrial oxidative stress

A novel reporter gene Mito-Timer was used to measure the mitochondrial oxidation level. Briefly, we transferred a mixture of plasmid and Lipofectamine 2000 into cells incubated with serum-free DMEM, and the medium was changed to 10% serum DMEM after 8 h. After 24–48 h, a confocal microscope was used to acquire images that contained green (488/518 nm) and red (543/572 nm) channels.

2.8. ATP generation

Neurons were subjected to fission and centrifuged at 12 000 *g* for 5 min. In 24-well plates, 100 μl of each supernatant was mixed with 100 μl ATP working dilution. Luminance was measured using a monochromator microplate reader. The ATP release levels were expressed as a fold of the luminescence levels in the treated control cells.

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