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HIGH-MOBILITY GROUP BOX 1 INDUCES NEURON AUTOPHAGY IN A RAT SPINAL ROOT AVULSION MODEL

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Abstract—Autophagy, a tightly regulated lysosome-dependent catabolic pathway, is implicated in various pathological states in the nervous system. High-mobility group box 1 (HMGB1) is an inflammatory mediator known to be released into the local microenvironment from damaged cells. However, whether autophagy is induced and exogenous HMGB1 is involved in the process of spinal root avulsion remain unclear. Here, we investigated the induction effect of autophagy and the possible role of HMGB1 during spinal root avulsion. It was found that autophagy was activated in the anterior horn of the spinal cord as represented by the increased expression of the autophagic marker microtubule-associated protein light chain 3-II (LC3-II), degradation of sequestosome 1 (p62), and formation of autophagosomes, and that autophagy was inhibited after intraperitoneal injection of anti-HMGB1-neutralizing antibodies in the rat spinal root avulsion model. In addition, HMGB1-induced autophagy and activated mitogen-activated protein kinases (MAPKs) in primary spinal neurons, including c-Jun N-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK), and p38MAPK. Inhibition of JNK or ERK activity significantly blocked the effect of HMGB1-induced autophagy in primary spinal neurons. Finally, HMGB1-induced autophagy increased cell viability in primary spinal neurons under oxygen-glucose deprivation conditions. The above results suggest that HMGB1 is a critical regulator of autophagy and HMGB1-induced autophagy plays an important role in protecting spinal neurons against injury, which may provide new insights into the pathophysiological process of spinal root avulsion.

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Abbreviations: BSA, bovine serum albumin; CQ, Chloroquine; DAPI, diamidino-phenyl-indole; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-signal-regulated kinase; HMGB1, high-mobility group box 1; HRP, Horseradish Peroxidase; JNK, c-Jun N-terminal kinase; LC3, light chain 3; MAPKs, mitogen-activated protein kinases; OGD, oxygen-glucose deprivation; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PLL, poly-L-lysine.

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INTRODUCTION

Autophagy, an intracellular degradative process that recycles cytoplasm for the sake of maintaining cellular survival and stability by way of the autophagosomal-lysosomal pathway, is believed to be involved in numerous physiological and pathological circumstances (Klionsky and Emr, 2000; Shintani and Klionsky, 2004; Kondo et al., 2005; Czaja et al., 2013). The principal role of autophagy is to protect organisms against various pathologies including cancer, neurodegenerative diseases, infections and cardiomyopathy (Levine and Kroemer, 2008). Cytoplasmic contents are segregated into double-membraned autophagosomes during autophagy. After fusing with lysosomes and forming single-membraned autolysosomes, these contents are digested (Kroemer and Jaattela, 2005; Rabinowitz and White, 2010). Therefore, formation of double-layered autophagosomes as detected by electron microscopy is the morphological evidence of autophagy (Gurusamy and Das, 2009). Microtubule-associated protein light chain 3 (LC3) is another widely accepted marker of autophagy (Tanida et al., 2004). To form LC3-II, LC3-I is lipidated and conjugated with phosphatidylethanolamine. LC3-II is present on isolation membranes and autophagosomes. The detection of LC3 by immunofluorescence or immunoblotting is a reliable approach to monitor autophagy-relevant processes (Mizushima and Yoshimori, 2007).

High-mobility group box 1 (HMGB1), a highly conserved nuclear protein, can bind DNA and promote assembly of transcriptional proteins on DNA targets (Muller et al., 2001). Besides its role in the nucleus, numerous studies have reported that HMGB1 is a pro-inflammatory cytokine that mediates response to infection, injury and inflammation (Abraham et al., 2000; Lotze and Tracey, 2005; Tsung et al., 2005; Mantell et al., 2006). HMGB1 is discharged passively from damaged or necrotic cells (Scaffidi et al., 2002) and is secreted actively by inflammatory cells such as macrophages and monocytes after pro-inflammatory stimuli or neurons after ethanol exposure (Gardella et al., 2002; Tang et al., 2007; Zou and Crews, 2014). Released HMGB1 mediates physiopathological responses through several membrane receptors including the receptor for advanced glycation end products (RAGEs) and Toll-like receptors. (Scaffidi et al., 2002; Tang et al., 2005). Previ-

ous studies have demonstrated the autophagy plays a protective role in the nervous system against neurodegenerative diseases such as Alzheimer's (Nixon et al., 2000) and Huntington (Qin and Gu, 2004) diseases. Recent research also suggested that the activity of autophagy was increased after cerebral ischemia-hypoxia, (Adhami et al., 2006) intracerebral hemorrhage, (He et al., 2008) traumatic brain injury (Liu et al., 2008) and spinal cord injury (Kanno et al., 1976). However, there are few studies reporting the induction effect of autophagy in spinal root avulsion.

Spinal root avulsion may lead to a progressive loss of axotomized neurons, resulting in motor dysfunction, neuropathic pain and anesthesia in the lower extremities (Penas et al., 2011; Torres-Espin et al., 2013). Our previous study demonstrated that apoptosis and necrosis occurred in neurons after spinal root avulsion (Jiang et al., 2014). However, the role of autophagy in this process remains unknown. In the present study, we demonstrated that autophagy was activated in a rat spinal root avulsion model and this activation was inhibited after intraperitoneal injection of anti-HMGB1-neutralizing antibodies. In addition, HMGB1 was also able to induce autophagy in primary spinal neurons through the activation of c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK). Finally, we demonstrated that HMGB1-induced autophagy played a role in protecting spinal neurons against injury. These findings may provide new insights into the pathophysiological process of spinal root avulsion.

EXPERIMENTAL PROCEDURES

Chemicals and reagents

HMGB1 was purchased from R&D systems (USA). Chloroquine (CQ) and pharmacologic inhibitors of JNK, ERK and P38 were purchased from Sigma–Aldrich (St. Louis, MO, USA). The choice and concentration of HMGB1, CQ and these inhibitors were according to previous studies on autophagy (Geng et al., 2010; Liu et al., 2011).

Animals and establishment of the spinal root avulsion model

All experiments were performed in accordance with the guidelines of the Animal Ethics Committee of the Second Military Medical University (Shanghai, China). Adult male Sprague–Dawley (SD) rats (200–220 g) were housed under a 12-h light/dark cycle in a specific pathogen-free environment with standard temperature and free access to food and water. All surgical procedures were performed under pentobarbital anesthesia (1%, 40 mg/kg, intraperitoneal injection). After anesthesia and routine disinfection, the right L4–L6 nerve roots were exposed at the intervertebral foramina under an operation microscope of 10× magnification. Then, the right L4–L6 nerve roots were avulsed with a tiny self-made hook. In the sham group, the right L4–L6 nerve roots of rats were only exposed but not avulsed. The rats were sacrificed at 0 h, 4 h, 1 d, 3 d or 7 d after surgery.

Determination of HMGB1 in serum

Rats were anesthetized and blood was collected from the carotid artery at 1 d after model establishment. Serum was prepared by allowing the blood to clot for 30 min at 4 °C followed by centrifugation for 5 min. The serum concentration of HMGB1 was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kit (CLOUD-CLONE, Houston, TX, USA) according to the manufacturer's instructions.

Neuron culture

Highly-enriched spinal neurons were isolated as described previously (Jiang et al., 2006). Embryonic day 14–15 rats were obtained from pregnant female SD rats. The spinal cord was removed from the vertebral canal and cut into 1 mm^3 pieces using a scalpel. The pieces were then digested in 0.05% trypsin (Invitrogen, Carlsbad, CA, USA) for 20 min at 37 °C. After digestion, the supernatant was removed and the remaining trypsin was inactivated with 2 ml DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5% heat-inactivated horse serum (Invitrogen, Carlsbad, CA, USA) at room temperature. After centrifugation at 1500 rpm for 5 min, the supernatant was removed and the tissue was resuspended and re-digested according to the procedures described above. The supernatant containing single cells was collected and diluted to a density of 1×10^6 cells/ml. The cells were plated into a poly-L-lysine-coated (PLL, Sigma, St. Louis, MO, USA) culture plate at the indicated density. Four hours later, the medium was replaced with serum-free neurobasal medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2% B27 supplement (Invitrogen, Carlsbad, CA, USA) and 2 mM glutamine (Invitrogen, Carlsbad, CA, USA). On the following day, 5 μM cytosine-β-D-arabinofuranoside (Sigma, St. Louis, MO, USA) was added into the medium for 24 h to inhibit non-neuronal cell division. Twice a week, half the volume of the medium was replaced with fresh medium.

Identification of cultured neurons

After a 7-day culture on PLL-coated coverslips, cells were fixed with 4% paraformaldehyde (PFA) for 30 min, washed three times in 0.01 M phosphate-buffered saline (PBS), blocked with 10% bovine serum albumin (BSA) for 20 min at room temperature and incubated with monoclonal rabbit anti-rat antibody against β-III tubulin (Sigma, St. Louis, MO, USA) overnight at 4 °C. After three washes in PBS, cells were incubated with the Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit antibody (Sigma, St. Louis, MO, USA) for 2 h at 37 °C. Then diamidino-phenyl-indole (DAPI) (Sigma, St. Louis, MO, USA) was used to stain cell nuclei.