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TREATMENT WITH EDARAVONE ATTENUATES ISCHEMIC BRAIN INJURY AND INHIBITS NEUROGENESIS IN THE SUBVENTRICULAR ZONE OF ADULT RATS AFTER FOCAL CEREBRAL ISCHEMIA AND REPERFUSION INJURY

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Abstract—Edaravone is a novel free radical scavenger that is clinically employed in patients with acute cerebral infarction. However, its effect on stroke-induced subventricular zone (SVZ) neurogenesis is largely unknown. In this study, we investigated the effect and underlying mechanism of edaravone administration on SVZ neurogenesis using a rat model of cerebral ischemia-reperfusion injury. Male Sprague–Dawley rats (200–250 g) were divided into sham operated (n=15), control (n=50), and edaravone-treated (n=50) groups. Rats in the control and edaravone-treated groups underwent 90 min of middle cerebral artery occlusion (MCAO) following reperfusion. Immediately and 12 h after MCAO, the rats received either normal saline (control group) or edaravone (edaravone-treated group) intraperitoneally. 5-bromo-2-deoxyuridine (BrdU) was used to label proliferating cells. Six, 12, and 24 hours after ischemia, reactive oxygen species (ROS) generation, hypoxia-inducible factor 1α (HIF-1α), and vascular endothelial growth factor (VEGF) protein levels in ischemic ipsilateral SVZ were determined. Immunohistochemistry staining for BrdU and doublecortin (DCX) was performed at 1, 4, and 7 days after ischemia. Treatment with edaravone not only mitigated cerebral infarct size (P<0.05) and neurological defects (P<0.05), but also decreased cell proliferation and neural progenitor cells in the ischemic ipsilateral SVZ (P<0.05). Additionally, edaravone reduced effectively ROS generation and HIF-1α as well as VEGF protein levels in the ischemic ipsilateral SVZ (P<0.05). These findings indicate that administration with edaravone, via repressing HIF-1α signaling pathway, inhibits SVZ neurogenesis in rats after cerebral ischemia-reperfusion injury.

Key words: neurogenesis, reactive oxygen species, hypoxia-inducible factor 1α, cerebral ischemia, reperfusion injury.

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Abbreviations: ANOVA, analysis of variance; BrdU, 5-bromo-2-deoxyuridine; DCX, doublecortin; EPO, erythropoietin; HIF-1α, hypoxia-inducible factor 1α; MCAO, middle cerebral artery occlusion; mNSS, modified neurological severity score; NSC, neural stem cell; NSCs, neural stem cells; NSPCs, neural stem/progenitor cells; RMS, rostral migratory stream; ROS, reactive oxygen species; SVZ, subventricular zone; TRITC, tetramethyl rhodamine isothiocyanate; VEGF, vascular endothelial growth factor.

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Stroke is the second leading cause of death in China, with ischemic stroke being the most common type (Shi et al., 2009). Minimization of the infarct area and generation of new neuronal cells in the injured brain are considered important strategic approaches for stroke treatment. The subventricular zone (SVZ), which is located in the lateral wall lining the lateral ventricle, harbors the largest population of neural stem cells (NSCs) that are capable of generating new neurons, astrocytes, and oligodendrocytes in rodents (Emsley et al., 2005), monkeys (Kornack and Rakic, 2001), and humans (Bernier et al., 2000). Proliferating SVZ cells normally migrate through the SVZ and along the rostral migratory stream (RMS) into the olfactory bulb and are finally incorporated as olfactory interneurons (Emsley et al., 2005). Increasing experimental evidence indicates that several pathological conditions such as cerebral ischemia/hypoxia, traumatic and primary degenerative diseases are known to increase neurogenesis in the SVZ of adult brains (Schmidt and Reymann, 2002; Im et al., 2010; Ohira, 2011).

Hypoxia-inducible factor 1α (HIF-1α) is an important transcriptional factor implicated in many cerebrovascular pathological disorders. Several studies indicate that the hypoxia-inducible factor 1 (HIF-1) signaling pathway appears important for stroke-induced SVZ neurogenesis (Androutsellis-Theotokis et al., 2006; Zhang et al., 2006–2007; Panchision, 2009). HIF-1α regulates neurogenesis through increase of vascular endothelial growth factor (VEGF) and erythropoietin (EPO) (Schölzke and Schwaninger, 2007; Tang et al., 2010). VEGF stimulates neural stem cell proliferation and is an attractive guidance cue for the migration of SVZ neural progenitors *in vitro* (Schänzer et al., 2004; Zhang et al., 2003). Moreover, evidence has been accumulating for the involvement of reactive oxygen species (ROS) in the activation of signaling components upstream of HIF-1α such as hydroxylases and kinases (Hwang et al., 2008; Koshikawa et al., 2009).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a novel free radical scavenger. It attenuates ischemic brain injury in patients and animal models (Watanabe et al., 2008; Yamamoto et al., 2009). Edaravone scavenges ROS, suppresses the accumulation of HIF-1α in the nuclei, and reduces HIF-1α binding to VEGF promoter in human astrocytes exposed to hypoxia (Ishikawa et al., 2007; Watanabe et al., 2008). However, it is still unclear whether edaravone plays a role in the neurogenesis following cerebral ischemia. In this study, we used a focal cerebral

ischemia-reperfusion model to explore the effects of edaravone administration on neurogenesis in the ipsilateral SVZ.

EXPERIMENTAL PROCEDURES

Animal modeling

All animals were provided by Experimental Animal Center of Xi'an Jiaotong University School of Medicine (Certificate No. 22-9601018). All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Experimental protocols were approved by the Animal Care and Use Regulation of Xi'an Jiaotong University School of Medicine. Male adult Sprague–Dawley rats, weighing 200–250 g, were maintained on a 12-h light/dark cycle with free access to food and water. Rats in the edaravone group ($n=50$) were treated with intraperitoneal injections (IP) of edaravone (3 mg/kg, immediately and 12 h after middle cerebral artery occlusion (MCAO), Boda, Jilin, China). Rats in the control group ($n=50$) received an intraperitoneal injection of normal saline at a volume similar to that used in the edaravone group. Ischemia was induced by the intraluminal vascular occlusion method as described previously. Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg) intraperitoneally and a 3-0 surgical monofilament nylon suture with rounded tip was introduced into the left internal carotid through the arteriotomy and advanced 16.5–17.5 mm past the carotid bifurcation. Ninety minutes later, the monofilament nylon suture was withdrawn for reperfusion. Rats in the sham-operated group ($n=15$) received the same surgical procedures except that the artery was not incised and occluded. To label the population of proliferating cells during 7 days of stroke, cumulative BrdU labeling was employed; briefly, BrdU (50 mg/kg, Sigma-Aldrich, USA) was injected intraperitoneally at the onset of ischemia and then daily for 1, 4 or 7 consecutive days. The rats were killed 2 h after the final injection (Zhang et al., 2001; Lee et al., 2007). Neurological function was assessed by modified neurological severity score (mNSS) (Zhang et al., 2009). At 24 h ($n=5$ each) after ischemia, 2,3,5-triphenyl tetrazolium chloride (TTC) staining was performed on 2-mm-thick coronal brain sections throughout the brain to evaluate the infarct size as described previously (Hsieh et al., 2006).

Immunohistochemistry

One, 4 and 7 days after ischemia, rats ($n=5$ per time point) were anesthetized with pentobarbital and perfused transcardially with normal saline followed by 4 % paraformaldehyde in PBS. The brains were removed, and tissue from bregma +0.2 mm to bregma −4.0 mm was taken and fixed in 4% paraformaldehyde overnight, then cut into 20-μm-thick coronal sections on a cryostat microtome (HM505 E, Microm, Walldorf, Germany). BrdU staining was performed according to a previous report (Zhang et al., 2011). The sections were incubated in 1% H_2O_2 in PBS for 20 min and in blocking solution (2% goat serum, 0.3% Triton X-100, and 0.1% bovine serum albumin in PBS) for 30 min at room temperature before being treated overnight at 4 °C with the primary antibodies: rabbit-anti-BrdU (1:1,000, Abcam, Cambridge, UK), rat-anti-BrdU (1:1,000, Abcam, UK), and rabbit polyclonal anti-rat doublecortin (DCX) (1:300, Sigma-Aldrich, USA), then washed with PBS/0.3% Triton X-100. For enzyme immunohistochemistry, the sections were incubated with biotinylated goat anti-rabbit immunoglobulin IgG secondary antibody for 2 h at room temperature, rinsed, and placed in avidin-peroxidase conjugate solution for 1 h. The horseradish peroxidase was detected with 0.05% DAB (Sigma) and 0.03% H_2O_2. For double immunofluorescence, the sections were incubated with fluorescein isothiocyanate (FITC)- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated IgG for 2 h at room temperature. Sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA), and fluorescence signals were detected with a laser confocal microscope (TSC SP2, Leica) at excitation/emission wavelengths of 535 nm/565 nm (TRITC, red) and at 470 nm/505 nm (FITC, green). The sections incubated with PBS instead of the primary antibodies were taken as negative controls.

Western blotting

To detect the protein expressions of HIF-1α and VEGF in ischemic ipsilateral SVZ, five rats per time point after 6, 12, and 24 h of ischemia were decapitated, and the brains were removed quickly. The SVZ (bregma +0.2 mm to bregma −4.0 mm) of ischemic hemispheres was cut into small pieces and homogenized in cold protein extraction buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.6, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 20 mM $Na_4P_2O_7$, 10% glycerol, Pierce, Rockford, IL, USA), centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected and measured by BCA assay. Equal amounts of protein (50 μg) were electrophoresed on a 12% SDS-PAGE and transferred to nitrocellulose membrane (0.45 μm, Millipore, USA) for 2 h at 1 mA/cm^2. After incubation with 10% non-fat milk for 2 h at room temperature to mask the non-specific binding site of IgG, the membranes were incubated overnight at 4 °C with rabbit polyclonal anti-HIF-1α antibody (reacts with human, mouse, rat, chicken, monkey; 1:1,000, Abcam, Cambridge, UK), rabbit polyclonal anti-VEGF antibody (reacts with human, mouse, rat; 1:1,000, Abcam, Cambridge, UK), and mouse monoclonal anti-β-actin antibody (1:10,000, Abcam, UK), followed by incubation with anti-rabbit horseradish peroxidase-conjugated IgG (1:5,000, Santa Cruz, CA, USA) and anti-mouse horseradish peroxidase-conjugated IgG (1:1,000, Pierce Biotechnology, USA) for 2 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescent substrate (Thermo Scientific Pierce, USA) using horseradish peroxidase-labeled secondary antibodies (1:5,000, Santa Cruz, CA, USA). The housekeeping protein β-actin was used as a control and tested simultaneously by mouse monoclonal anti-β-actin antibody (1:10,000, Sigma-Aldrich, USA). The luminescent signal is detected by the CCD camera and transmitted to the controller unit, and the data are sent to the computer for analysis and documentation.

In situ superoxide detection

Rats were anesthetized by pentobarbital (40 mg/kg, IP) and sacrificed 6, 12, and 24 h after the onset of ischemia ($n=5$ per time point). The brains were removed and frozen immediately by liquid nitrogen. Twenty-five-μm-thick coronal sections, spaced 200 μm apart, from bregma +0.2 mm to bregma −4.0 mm were prepared for analysis *in situ*. The sections were incubated with dihydroethidium (DHE; 5 μmol/L, Sigma-Aldrich, USA) in PBS for 30 min at 37 °C in a humidified chamber protected from light. DHE is oxidized on reaction with SO^{2-} to ethidium bromide which, in turn, binds to DNA in the nucleus and emits red fluorescence (Shichinohe et al., 2004; Yamamoto et al., 2009). The red fluorescence was detected through a 580-nm long-pass filter using fluorescence microscopy (BX51, Olympus, Japan) and was digitally photographed using a cooled CCD (DP71) camera mounted to the microscope (BX51, Olympus, Japan). On each coronal section ($n=4$ in each rat), the intensity of red fluorescence in ischemic ipsilateral SVZ was semi-quantitatively analyzed using Image-Pro Plus 5.0 for Windows (Media Cybernetics, MD, USA). The fluorescence signal was also measured in the same regions of the