Int. J. Devl Neuroscience 33 (2014) 49–56

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Gypenosides pre-treatment protects the brain against cerebral ischemia and increases neural stem cells/progenitors in the subventricular zone

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ARTICLE INFO

Article history:
Received 27 July 2013
Received in revised form 5 December 2013
Accepted 5 December 2013

Keywords:
Subventricular zone
Neurogenesis
Stroke
Cell proliferation
Gypenosides

ABSTRACT

Gypenosides (GPs) have been reported to have neuroprotective effects in addition to other bioactivities. The protective activity of GPs during stroke and their effects on neural stem cells (NSCs) in the ischemic brain have not been fully elucidated. Here, we test the effects of GPs during stroke and on the NSCs within the subventricular zone (SVZ) of middle cerebral artery occlusion (MCAO) rats. Our results show that pre-treatment with GPs can reduce infarct volume and improve motor function following MCAO. Pre-treatment with GPs significantly increased the number of BrdU-positive cells in the ipsilateral and contralateral SVZ of MCAO rats. The proliferating cells in both sides of the SVZ were glial fibrillary acidic protein (GFAP)/nestin-positive type B cells and doublecortin (DCX)/nestin-positive type A cells. Our data indicate that GPs have neuroprotective effects during stroke which might be mediated through the enhancement of neurogenesis within the SVZ. These findings provide new evidence for a potential therapy involving GPs for the treatment of stroke.

1. Introduction

MCAO in adult rodents results in an infarct that encompasses the cerebral cortex and striatum on the occluded side of the brain. In the adult brain, pathological processes, including cerebral ischemia, can enhance neurogenesis. In the adult mammalian brain, NSCs are located primarily in two areas: the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus (DG) of the hippocampus (Ming and Song, 2011; Taupin and Gage, 2002). Many studies have reported a significant enhancement in progenitor proliferation following MCAO in the SVZ and DG of adult rats and mice (Jin et al., 2001; Kokaia and Lindvall, 2003; Komitova et al., 2002; Parent et al., 2002; Zhang et al., 2001). Newly generated neural precursors from the SVZ migrate toward the areas of the brain that are injured as a result of ischemia (Kokaia and Lindvall, 2003; Sun et al., 2004; Thored et al., 2006; Yan et al., 2007). Some studies have reported that the increase in SVZ neurogenesis appears to be associated with cell replacement in the injured striatum (Arvidsson et al., 2002; Hou et al., 2008; Kokaia and Lindvall, 2003; Parent et al., 2002; Sun et al., 2004; Thored et al., 2006; Yan et al., 2007). No such evidence exists to support a similar form of cell replacement in the hippocampal dentate gyrus following stroke or other cerebral insults (Lichtenwalner and Parent, 2006).

In the post-ischemic brain, many BrdU+/NeuN+ cells and a subset of BrdU+/DCX+ cells with the morphology of migrating neurons can be seen at the infarct site (Arvidsson et al., 2002). By co-labeling with BrdU and DCX and labeling SVZ cells with the fluorescent tracer DiI, the progenitor cells from the SVZ could be found to have directly migrated to the striatum in the post-ischemic rat brain (Jin et al., 2003). These newly generated cells can survive in the ipsilateral striatum and corpus callosum for more than 3 months. In all, 3–10% of the surviving cells were shown to express the immature neuronal marker Class IIIβ-Tubulin (TuJ1) and mature neuronal markers such as MAP-2 and NeuN 2–3 months after ischemia (Gu et al., 2000; Jiang et al., 2001). Although the SVZ-derived

Abbreviations: SVZ, subventricular zone; MCAO, middle cerebral artery occlusion; NSCs, neural stem cells; RMS, rostral migratory stream; DG, dentate gyrus; OB, olfactory bulb; GFAP, glial fibrillary acidic protein; DCX, doublecortin.

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http://dx.doi.org/10.1016/j.ijdevneu.2013.12.001

progenitors migrated into the damaged areas of the brain, many newly formed cells were also found in the olfactory bulb (OB) following focal ischemia in primates, indicating that some cells still follow their natural route of migration (Koketsu et al., 2006).

Gypenosides (GPs) are dammarane-type saponins that are extracted from *Gynostemma pentaphyllum* (Thunb) Makino (Cucurbitaceae). The active component of gypenoside is the hydroxyl group attached to the twentieth or twenty-first carbon in the dammarane-type ring (Shang et al., 2006). GPs are widely used in Asia in traditional Chinese medicine. Studies have shown that GPs have antioxidative stress effects in both primary cultures of rat cortical cells treated by glutamate (Shang et al., 2006) and in the cortex and hippocampal CA1 region in a rat model of chronic cerebral hypoperfusion (Zhang et al., 2011). Some studies have shown that GPs have neuroprotective effects on MPP^+-induced dopaminergic neurons in primary nigral cultures (Wang et al., 2010b) and in the substantia nigra of mouse and rat models of Parkinson's disease (Choi et al., 2010; Wang et al., 2010a). By using hippocampal slices, the ethanolic extract of *G. pentaphyllum* has been shown to have protective effects from hypoxia/hypoglycemia-induced injury (Schild et al., 2009, 2012). GPs have been shown to decrease the damage to the DNA and RNA of rat neurons in an acute global ischemia model (Qi et al., 2000). However, it remains unknown whether GPs can reduce the infarct volume *in vivo* and improve motor function in an ischemic animal. This study investigated the protective effects of GPs during stroke and the activity of GPs on the NSCs within the SVZ of MCAO rats.

2. Materials and methods

2.1. Experimental materials

Adult male Sprague-Dawley (SD) rats weighing 280–350 g were used for the experiments and were obtained from Vital River Laboratories (Beijing, China). The rats were kept under standard lighting conditions (12 h light/dark cycles). This study was approved by the ethics committee of the School of Medicine of Shandong University. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Shandong University.

2.2. MCA occlusion model

SD rats were anesthetized with 10% chloral hydrate by intraperitoneal injection (3 mL/kg body weight). The left common carotid arteries were identified and isolated through a ventral midline cervical incision. A nylon filament (0.28 mm in diameter) with a rounded tip was introduced from the common carotid artery lumen into the internal carotid artery to block the origin of the left middle cerebral artery (MCA). The left MCA was occluded with the filament for 120 min, and the filament was withdrawn to allow for 24 h of reperfusion. In the sham group, a left neck incision was made to expose the arteries, but the nylon thread was not inserted into internal carotid artery. The animals were returned to their home cages following recovery from the anesthesia.

2.3. Study design in animal models

The rats were randomized into six groups: sham group ($n = 13$), MCAO group ($n = 11$), the GPs 200 group treated with 200 mg/kg GPs per day (sham + GPs 200, $n = 5$; MCAO + GPs 200 group, $n = 9$) and the GPs 400 group treated with 400 mg/kg GPs per day (sham + GPs 400, $n = 5$; MCAO + GPs 400 group, $n = 9$). The rats in the GPs 200 and GPs 400 groups were treated with different doses of GPs according to their body weights by oral gavage. The rats were treated once a day for 10 days before MCA occlusion. The rats in the sham and MCAO groups received same amount of saline for 10 days.

2.4. Examination of neurological deficits

The behavioral assays were scored using a modification of the system used by Bederson et al. (1986). The rats were examined after the 24-h reperfusion while suspended 20–30 cm above the testing table and were scored according to the following criteria: 0, rat extends both forelimbs straight; 1, rat keeps one forelimb to the breast and extends the other forelimb straight; 2, rat shows decreased resistance to a lateral push without circling; 3, rat shows decreased resistance to a lateral push with circling; and 4, rat loses spontaneous walking and consciousness. Rats scoring 0 or 4 were not used in the evaluation of this experiment.

2.5. Evaluation of infarct volume

Twenty-four hours following reperfusion, the rats were killed while under deep anesthesia. The brains were removed carefully and sliced into 2.0 mm thick coronal sections. The fresh brain slices were incubated in a 2% triphenyltetrazolium chloride (TTC, Sigma) in normal saline for 30 min at 37 °C and then transferred to a 4% paraformaldehyde solution for fixation. After TTC staining, digital photographs were taken. Areas of the infarct regions were analyzed in NIH Image J. The infarct areas of each section were the average of the sum of two sides. The volume of infarction for each animal was calculated by taking the product of the average slice thickness (2 mm) and summing the infarct areas in all brain slices. The results were represented as the percentage of the total volume.

2.6. BrdU labeling

After MCAO, the rats were injected with BrdU by intraperitoneal injection (Sigma, 50 mg/kg), four times at 2-h intervals. The rats were killed 2 h after the last injection ($n = 3$ in each group). Non-surgical rats in the sham group received the same injection of BrdU and were killed at corresponding time points and used as control groups. Rats were transcardially perfused with normal saline followed by 4% paraformaldehyde. The brains were removed and fixed in 4% paraformaldehyde.

2.7. Immunohistochemistry

Serial 40-μm sagittal or coronal sections were cut with a cryostat and stored at −80 °C. For BrdU immunostaining, DNA was first denatured by incubating brain sections in 50% formamide/2x standard saline citrate at 65 °C for 2 h and then in 2 N HCl at 37 °C for 30 min. After washing, sections were incubated with blocking solution and then with a sheep polyclonal antibody against BrdU (1:1000; Abcam) at 4 °C overnight followed by an Alexa Fluor 594-conjugated donkey anti-sheep IgG (1:1000; Invitrogen) in blocking solution at room temperature for 2 h. For double immunolabeling of DCX/nestin and GFAP/nestin, the primary antibodies used were mouse monoclonal anti-nestin (1:500; Millipore) and rabbit polyclonal anti-GFAP (1:1000; Millipore) or anti-DCX (1:500; Cell Signaling Technology). The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) (1:1000; Invitrogen), Alexa Fluor 594-conjugated goat anti-rabbit IgG (H + L) (1:1000; Invitrogen). Sections were mounted with Vectashield (Vector) and 4′,6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei.