Experimental Neurology 247 (2013) 680–688

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

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

Anti-oxidative, anti-apoptotic, and pro-angiogenic effects mediate functional improvement by sonic hedgehog against focal cerebral ischemia in rats

Shiang-Suo Huang [a,b], Henrich Cheng [c], Ching-Min Tang [d], Mao-Wei Nien [a,b], Yuahn-Sieh Huang [e], I-Huei Lee [d], Jiu-Haw Yin [f], Terry B.J. Kuo [d], Cheryl C.H. Yang [d], Shen-Kou Tsai [g,*], Ding-I Yang [d,**]

[a] Department of Pharmacology and Institute of Medicine, College of Medicine, Chung Shan Medical University, Taiwan
[b] Department of Pharmacy, Chung Shan Medical University Hospital, Taichung, Taiwan
[c] Neural Regeneration Laboratory, Department of Neurosurgery, Neurological Institute, Taipei Veterans General Hospital, Taipei, Taiwan
[d] Institute of Brain Science and Brain Research Center, National Yang-Ming University, Taipei, Taiwan
[e] Department of Biology and Anatomy, National Defense Medical Center, Taipei, Taiwan
[f] Department of Neurology, Cheng Hsin General Hospital, Taipei, Taiwan
[g] Cheng Hsin General Hospital, Taipei, Taiwan

ARTICLE INFO

Article history:
Received 29 November 2012
Revised 22 February 2013
Accepted 4 March 2013
Available online 13 March 2013

Keywords:
Angiogenesis
Anti-apoptosis
Anti-oxidation
Focal ischemia
Middle cerebral artery occlusion

ABSTRACT

Sonic hedgehog (SHH) is a morphogen important for neural development during embryogenesis. Recently, beneficial actions of SHH in ischemic injury have been noted. To test whether epidural application of the biolgically active N-terminal fragment of SHH (SHH-N) may reduce the extent of ischemic brain injury, male Long–Evans rats were exposed to a 60-min episode of middle cerebral artery occlusion (MCAO) with topical application of SHH-N and/or its specific inhibitor, cyclopamine, in fibrin glue over the peri-infarct cortex. We found that epidural application of SHH-N substaintially reduced infarct volumes after 7 days of reperfusion, which was reversed by cyclopamine; SHH-N also improved behavioral outcomes as assessed by global neurological functions, rotarod test, and grasping power test. Furthermore, SHH-N attenuated the extents of protein oxidation, lipid peroxidation, and apoptosis induced by focal ischemia/reperfusion. Immunohistochemical staining coupled with bromodeoxyuridine (BrdU) incorporation revealed that SHH-N enhanced post-ischemic angiogenesis, stimulated the proliferation of nestin-positive (nestin$^+$) neural progenitor cells (NPCs), and suppressed astrocytosis. Our results thus revealed multifaceted protective mechanisms of SHH-N against focal cerebral ischemia/reperfusion.

Introduction

Sonic hedgehog (SHH) is a morphogen involved in the regulation of central nervous system (CNS) polarity (Echelard et al., 1993). In the cells, SHH is first synthesized as a large precursor protein followed by autoproteolysis and lipid modifications of its biologically active N-terminal domain (SHH-N). Binding of SHH-N to the 12-pass transmembrane receptor Patched relieves its suppression of Smoothened, another 7-pass transmembrane receptor, thereby activating downstream transcription factors of the Gli family resulting in the expression of its target genes (Traiffort et al., 2010), which include cyclin D1 (Kenney and Rowitch, 2000), Bcl-2 (Regl et al., 2004), mammalian achaete-scute homolog-1 (Mash1) (Wang et al., 2007), N-Myc (Kenney et al., 2003), and Bmi1 (Leung et al., 2004) that are respectively involved in cell cycle progression, anti-apoptosis, adult neurogenesis, cell proliferation, and self-renewal.

In addition to its well-established roles in tissue patterning during embryonic development, recently a novel protective action exerted by SHH has been revealed in several animal models of ischemia/reperfusion, including cerebral infarction, hind-limb ischemia, and myocardial infarction. For example, SHH may augment blood flow recovery and hence salvage hind limbs from ischemia in aged mice (Pola et al., 2001). Skeletal muscle ischemia induces activation of SHH and the Patched receptor in interstitial mesenchymal cells suggesting responsiveness of these cells to SHH signaling (Pola et al., 2003). SHH gene therapy carries considerable therapeutic potential in individuals with acute and chronic myocardial ischemia by triggering expression of multiple trophic factors and engendering tissue repair in the adult heart (Kusano et al., 2005). SHH is also a critical factor in the pathophysiology of ischemic injury in testis (Dokucu et al., 2009), liver (Tuncer et al., 2007), and kidney (Ozturk et al., 2007). More importantly, expression of endogenous SHH is upregulated in mature hippocampal neurons after focal ischemia and SHH inhibition by cyclopamine suppressed post-ischemic proliferation of subgranular neural progenitor cells (NPCs) (Sims et al., 2009). Intrathecal administration of SHH protein

* Correspondence to: S.-K. Tsai, Cheng Hsin General Hospital, No. 45, Cheng Hsin Street, Beitou District, Taipei City 11212, Taiwan. Fax: +11 886 2 28267411.
** Correspondence to: D.-I. Yang, Institute of Brain Science and Brain Research Center, National Yang-Ming University, No. 155, Section 2, Linong Street, Beitou District, Taipei City 11221, Taiwan. Fax: +11 886 2 28273123.
E-mail addresses: ch9198@chgh.org.tw (S.-K. Tsai), diyang@ym.edu.tw (D.-I. Yang).

0014-4886/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.expneurol.2013.03.004

also increases NPC proliferation in subventricular zone (SVZ) and promotes post-stroke functional recovery (Bambakidis et al., 2012). Similarly, intravenous injection of small-molecule SHH agonist after spinal cord injury in adult rodents reduces cell death, increases neural/oligodendrocyte precursor cells at the rim of the cavity, and improves motor function (Bambakidis et al., 2009, 2010). These findings suggest a beneficial effect of SHH in ischemic tissue injury. Despite these aforementioned reports, whether epidural application of SHH may be beneficial in cerebral ischemia/reperfusion remains to be determined. We therefore tested the potential protective effects of SHH-N in focal ischemia and explored the underlying mechanisms.

Materials and methods

Middle cerebral artery occlusion (MCAO)

All efforts were exercised to minimize animal suffering and to reduce the numbers of animals that were sacrificed. The surgical procedures for MCAO and post-operative animal care have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of National Yang-Ming University. MCAO was performed according to the protocols previously described (Huang et al., 2001; Tsai et al., 2007). Male Long–Evans rats (250–300 g) were anesthetized with a single intraperitoneal injection of chlorohydrate (450 mg/kg, Sigma, St. Louis, MO, USA). The body temperature was maintained during surgery at 37 ± 0.5 °C with a heating pad servo-controlled by a rectal probe. Focal ischemic infarction was produced in the right lateral cerebral cortex in the territory of the middle cerebral artery (MCA). A 1.5-cm scalp incision was cut at the midpoint between the right eye and the right ear. The temporal muscle was separated in the plane of its fiber bundles and retracted in order to expose the zygoma and squamous bone. A 2-mm diameter burr hole was made at the anterior junction of the zygoma and squamous bone using a drill (Dremel Multipro + 5395, Dremel Company, Racine, WI, USA) cooled with saline solution. The dura mater was carefully pierced with fine forceps using a dissecting microscope (OPMI-1, Carl Zeiss Co., Oberkochen, Germany) to expose the right MCA for ligation with 10–0 ophthalmic suture. Both common carotid arteries (CCAs) were then clamped with microaneurysm clips. At the end of 60-min ischemia, the CCAs were unclamped and return of blood flow was visualized; however, the right MCA ligature was left in place permanently. After 7 days of reperfusion, animals were decapitated under anesthesia by intraperitoneal injection of urethane (1.2 g/kg, Sigma). Brains were removed, inspected visually for the anatomy of the MCA and for the signs of hemorrhage or infection, immersed in cold saline solution for 10 min, and sectioned into standard coronal slices at 2-mm thickness using a brain matrix slicer (Jacobowitz Systems, Zivic-Miller Laboratories Inc., Allison Park, PA, USA). Slices were placed in the vital dye 2,3,5-triphenyltetrazolium chloride (TTC, 2%; Sigma) at 37 °C in the dark for 30 min, followed by 10% formalin at room temperature overnight. The extent of cerebral infarction was measured in each section (in mm^2) and the infarct volume (in mm^3) was acquired using the AIS imaging research software (Imaging Research Inc., St. Catharines, Ontario, Canada). Infarct volume was calculated as the sum of infarct area per slice multiplied by slice thickness. Both the surgeon and image analyzer were blinded to the treatment of each animal.

Epidural application of SHH-N and cyclopamine

Fibrin glue (BERIPLAST P COMBI-SET; CSL Behring, Marburg, Germany), an adhesive biopolymer was prepared immediately before use by mixing the fibrinogen (100 mg/mL) with aprotinin solution (200 KIU/ml). This solution was then mixed with calcium chloride (8 mmol/L) and SHH-N (6–200 ng/kg), cyclopamine (2 μg per rat), or both. Immediately at the onset of reperfusion, fibrin glue (20 μl) containing the reagents to be delivered was applied to the surgical cortex to form a glue cast. The same volume of vehicle in the fibrin glue was included as negative controls. Each group contained 9 animals.

Determination of neurological deficits, rotarod test, and grasping power test

The modified Bederson score (Bederson et al., 1986) was used to determine global neurological functions according to the following scoring system: 0, no deficit; 1, forelimb flection; 2, decreased resistance to a lateral push; 3, unidirectional circling; 4, longitudinal spinning; 5, no movement. Neurological scores were always assessed by an independent investigator in a blinded fashion. The rotarod test was applied to assess motor deficit following ischemic insult according to our previous publication (Chen et al., 2010). The rats were placed on rungs of the accelerating rotarod (4–40 rev/min over the time course of 5 min). The latency of the animals staying on the rotarod was recorded. Each animal received three consecutive trials. To quantitatively determine grasping strength, a bar of wires was connected to an ordinary electronic balance. The untested forepaw was temporarily wrapped with adhesive tape to prevent from grasping, whereas the tested one was allowed to grasp the bar while being lifted by the tail with increasing firmness until the grip was lost.

Detection of protein oxidation, lipid peroxidation, and TUNEL assay

The extent of protein oxidation was detected by using an OxyBlot kit (Chemicon, Temecula, CA, USA). The level of oxidized proteins can be measured by detection of protein carbonyl content, which is an index for the oxidative status of proteins. Total proteins from cortices after ischemia/reperfusion were subjected to reaction with 2,4-dinitrophenylhydrazine and derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone). The DNP-derivatized protein samples were then separated on a 15% polyacrylamide gel containing SDS for subsequent Western blotting. The blot was incubated with a rabbit anti-DNP antibody provided in the kit; this was followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody. The extent of lipid peroxidation was determined by using BIOXYTECH® MDA-586 (Oxis International Inc., Foster City, CA, USA) to measure the total malondialdehyde (MDA) levels. Cortices were homogenized in ice-cold PBS and then centrifuged at 3000 ×*g* for 10 min at 4 °C to remove large particles. Samples were hydrolyzed at 60 °C for 80 min in the presence of butylated hydroxytoluene (pH 2.0). Optical density was measured at wavelength 586 nm. The protein concentration was quantitatively determined by using Bradford's reagent. Results are presented as nmol/L/mg proteins. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed by using the DeadEnd™ Fluorometric TUNEL System Kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. The immunostaining of $TUNEL^+$ cells was examined under a fluorescence microscope (Olympus BX61, Tokyo, Japan) equipped with filter sets (excitation/emission: 494-nm/518-nm) to detect the fluorescence signal.

Quantification of blood vessel length, BrdU labeling, immunohistochemistry, and confocal microscopy

Quantification of the total blood vessel length on the surface of the right brain hemisphere was conducted by using AIS imaging research software (Imaging Research Inc.). Cumulative labeling for the population of proliferating cells after cerebral ischemia was achieved by intraperitoneal injection of BrdU (50 mg/kg; Sigma) daily for 7 consecutive days into ischemic rats starting at the day after MCAO. Non-surgical rats receiving the same BrdU injections were sacrificed at corresponding time points to serve as control groups. Rats were