Sonic Hedgehog Regulates Ischemia/Hypoxia-Induced Neural Progenitor Proliferation

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Background and Purpose—Sonic hedgehog (Shh) protein is required for the maintenance of neural progenitor cells (NPCs) in the embryonic and adult hippocampus. Brain ischemia causes increased proliferation of hippocampal NPCs. We therefore examined whether Shh regulates the increase in proliferation of NPCs after ischemia/hypoxia.

Methods—Male SV129 mice were exposed to a 20-minute middle cerebral artery occlusion; hippocampi were then analyzed for Shh mRNA and protein expression by real-time polymerase chain reaction, immunoblot, and immunohistochemistry. Primary cell cultures of neurons, astrocytes, and NPCs were exposed to 16 hours of hypoxia (1% O₂) and analyzed by real-time polymerase chain reaction and immunoblot for Shh expression. Proliferation of NPCs, in vivo and in vitro, was measured by bromodeoxyuridine incorporation.

Results—Among the cell types examined in vitro, only NPC and neurons increased Shh mRNA under hypoxic conditions. Furthermore, hypoxia increased proliferation of NPCs and this proliferation was enhanced by the addition of recombinant Shh or blocked by the pathway-specific inhibitor, cyclopamine. Middle cerebral artery occlusion was associated with a transient 2-fold increase in the mRNA encoding both Shh and its transcription factor, Gli1, 0.5 days after ischemia. Within the hippocampus, Shh protein was increased approximately 3-fold 3 and 7 days after ischemia and was observed predominantly within cells in the CA3 and hilar regions. Shh was expressed only in mature neurons. In vivo, cyclopamine suppressed ischemia-induced proliferation of subgranular NPCs.

Conclusion—The Shh pathway plays a role in the proliferation of NPCs induced by ischemia/hypoxia and might participate in injury remodeling. (Stroke. 2009;40:3618-3626.)

Key Words: brain ischemia ■ dentate gyrus ■ gene expression ■ Sonic hedgehog ■ stem cell

Materials and Methods

Animals
SV129 male mice 10 to 12 weeks old were obtained from Taconic (Hudson, NY) for middle cerebral artery occlusion (MCAO). SV129 pregnant mice, E15, and P1 pups were obtained for cell culture. Our institutional Subcommittee on Research and Animal Care approved all animal use.

Neural Progenitor Culture
E15 mouse cortices were minced and incubated at 37°C for 20 minutes in a solution of trypsin, 0.25%, and DNase I. The tissue was washed in media containing 10% fetal bovine serum and then passed through a 70-μm nylon filter (BD Falcon). Cells were resuspended in defined media containing 1% fetal bovine serum, Dulbecco’s modified Eagle’s medium/F12, N2 supplement (Gibco Invitrogen), 0.6% glucose, penicillin/streptomycin/amphotericin B (1:100 dilution; Gibco Invitrogen), 50 mmol/L HEPES, FGF-2 (20 ng/mL), and EGF (20 ng/mL; Gibco Invitrogen) and plated at 2×10⁵ cells/well into 24-well plates. After 24 hours, 1% fetal bovine serum was removed.

Astrogial Culture
The cells harvested as described previously except that P1 mice pups were used. Cells were plated on 24-well plates at same density as
described previously in Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum.

**Neuronal Culture**

E15 to 17 cortices were harvested as described previously and plated at a density of 3 x 10⁶ cells/well. Cells were cultured in Neurobasal with glutamine (2 mmol/L), B27 (2% vol/vol), and penicillin/streptomycin antibiotics at all times in 24-well dishes coated with poly-D-lysine and laminin (Biocoat). On Days 1 to 3, glutamate (25 μg/mL) and β-mercaptoethanol (10 μmol/L) were added. On Day 3, cytarabine 10 μmol/L was added for 24 hours.

**Hypoxia and Drug Treatment**

Cultures were treated in a hypoxia chamber with an oxygen control sensor (Biospherix, Ltd. Redfield, NY) to maintain O₂ at 1% ± 0.2% (hypoxia). Controls remained in the incubator in 95% air/5% CO₂. Either cycloamine (Toronto Research Chemicals) and/or recombinant mShh (Stem Cell Technologies) was added to cells at the time of hypoxic exposure.

**mRNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction**

mRNA from hippocampi was isolated with Trizol Reagent (Invitrogen). mRNA from culture experiments was isolated with the RNeasy Mini Kit (Qiagen). cDNA was synthesized according to the SuperScript III First-Strand Synthesis System for Reverse transcription–polymerase chain reaction (Invitrogen) using random hexamer primers. Samples were run on an ABI Prism 7000 (Applied Biosystems, Foster City, Calif) using Taqman FAM labeled probes (Applied Biosystems). Gene-specific amplicons were normalized to an endogenous control of 18S rRNA.

**Proliferation Assay**

Assay was performed according to the manufacturer’s recommendation with some modification (Roche Molecular Biochemicals); additional washing steps and blocking with 1% bovine serum albumin in phosphate-buffered saline, before antibody incubation, were added. Cells were incubated with 5-bromodeoxyuridine for 16 hours. Plates were read with a Wallac VICTOR® V luminometer (Perkin Elmer Life Sciences, Inc, Boston, Mass).

**Western Blotting**

NPC or hippocampal protein was extracted with urea lysis buffer (8 mol/L urea, 200 μL/mL Tris pH 7.4, 2.5 mmol/L EDTA, 2.5 mmol/L EGTA, 1% DTT, 4% CHAPS, 6% NP-40, protease inhibitor; Roche Applied Science). Total protein (20 μg for Shh or 50 μg for hypoxia-inducible factor 1α [HIF1α]) was loaded in 15% or 8%, respectively, Tris-glycine gel. Proteins were transferred onto Hybond ECL nitrocellulose membrane (Amersham Biosciences) or polyvinylidene difluoride, respectively, and blocked (1% bovine serum albumin in phosphate-buffered saline). Membrane was incubated overnight with rabbit anti-Shh 1:2500 for cell culture and 1:500 for tissue (SantaCruz Biotechnology) or mouse anti-HIF1α 1:500 (Novus Biological). Membrane was washed and incubated with anti-rabbit/mouse–horseradish peroxidase 1:5000 (Zymed). Bands were detected with ECL plus (Amersham Biosciences). For internal loading control, mouse anti-β-actin 1:10 000 (Sigma-Aldrich) or β-tubulin 1:5000 (Upstate) and antimonuse–horseradish peroxidase 1:10 000 (Amersham Biosciences) or antimouse-Cy5 1:400 (Jackson ImmunoResearch Laboratories) was used.

**Animal Surgery MCAO**

The procedure was performed as previously described. Briefly, mice were anesthetized with 3% isoflurane and maintained on 1% to 1.5% isoflurane in 70% N₂O/30%O₂ using a Fluotec 3 vaporizer (Colonial Medical Supply, Franconia, NH). Regional blood flow was monitored during ischemia and reperfusion by laser Doppler flowmetry (FF2B; Perimed, Järfälla, Sweden). The middle cerebral artery was occluded with an 8–0 nylon monofilament (Ethicon) coated with silicone for 20 minutes. During surgery, body temperature of mice was maintained at 37°C with a heating pad and rectal probe. A total of 4 to 6 mice per variable underwent surgery. A control group of mice was subjected to surgery, but the nylon filament was not introduced (sham). In 6 separate animals, an oxygen probe (Catalog BF/OT/E; Oxford-Optronix, Oxford, UK) was placed in the CA3 region of the hippocampus (–1.7 mm bregma, 3 mm lateral, 1.8 to 2 mm depth at a 45° angle).

**Intraventricular Catheter**

The intraventricular catheter with an osmotic pump (1007D, Alzet; Durect Corp, Cupertino, Calif) was placed into the right lateral ventricle (ipsilateral to ischemia). The catheter placement was 1.4 mm lateral and 0.6 mm caudal from the bregma and 1.8 mm deep. The pump was loaded with either 5 μmol/L cycloamine or vehicle (0.1% DMSO in phosphate-buffered saline). The delivery rate of the pump was 0.5 μL/h (cycloamine delivery, 60 pmol/d).

**5-Bromodeoxyuridine Injections**

Mice received intraperitoneal injections of 5-bromodeoxyuridine (BrdU; 50 mg/kg), every 4 hours for a total of 4 injections. Mice were euthanized 30 minutes after the last injection.

**Tissue Preparation**

Brains were removed after transcardial perfusion with 4% paraformaldehyde and fixed overnight then placed in 30% sucrose in phosphate-buffered saline for 24 hours. The brains were cut in 20-μm sections on a cryotome. Brains processed for Shh immunostaining were paraffin-embedded and cut in 8-μm sections.

**Immunohistochemistry**

**Proliferation BrdU Studies**

Slides were treated with 2 N HCl for 30 minutes for antigen retrieval and then 3% hydrogen peroxide for 10 minutes. Slides were blocked (10% horse serum). Slides were incubated with primary antibody, mouse anti-BrdU (1:100; Becton-Dickinson), and secondary antibody, biotinylated horse antimouse (1:250; Jackson ImmunoResearch Laboratory), each for 1 hour. Slides were stained with Vectastain elite ABC kit using diaminobenzidine (Vector Laboratories).

**Shh Staining**

Slides were processed as described previously except that slides were treated with 10 mmol/L sodium citrate pH 6 and microwaved instead of 2 N HCl. Antibodies used were anti-Shh (1:100, H-160; Santa Cruz Biotechnology) at room temperature for 3 hours and biotinylated antirabbit (1:250; Jackson ImmunoResearch Laboratory) at room temperature for 1 hour. Slides were counterstained with hematoxylin. Controls were performed by omitting primary antibody and/or substituting the primary antibody with a control mouse IgG antibody. For double labeling, Shh immunofluorescence was performed as described previously but streptavidin-Alexa 546 (1:500 dilution; Invitrogen) was used instead of diaminobenzidine. Other primary antibodies used included mouse anti-NeuN (1:100 dilution; Chemicon), rabbit anti-glial fibrillary acidic protein (GFAP)-Cy3 (1:1000 dilution; Sigma), and goat antidoublecortin (1:250 dilution; Santa Cruz Biotechnology).

**Data Analysis**

**Real-Time Polymerase Chain Reaction**

Analysis of real-time polymerase chain reaction was performed on Q-gene. Target gene expression is expressed as normalization to internal control 18S rRNA. Statistical comparison of mean normalized expression was performed by analysis of variance.

**Immunohistochemical Analysis**

Quantification of BrdU was similar to our previous description. Briefly, the modified stereological counting methods involved every 20th section in a series of 80 sections. For measures of proliferation,
diaminobenzidine-positive cells, viewed under brightfield with a Nikon Eclipse TE2000, 40× objective lens, were counted only at the subgranular zone. The average number of BrdU-positive cells was multiplied by the total number of sections. The number of cells/hippocampus was averaged across the group and then differences were compared by analysis of variance.

**Hypoxia Proliferation**

Comparisons of conditions were performed by analysis of variance with appropriate post hoc analysis for significance. Significance was considered at a value of \( P < 0.05 \).

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**Results**

### Shh Expression and Response to Hypoxia in Primary Cell Cultures

We sought to determine if Shh was upregulated in primary cell cultures exposed to hypoxia. We exposed neurons, astrocytes, and NPCs to hypoxia (1% \( O_2 \)) for 4 hours and 16 hours. There were no changes in Shh in any of these cultures over the 4-hour exposure period (data not shown). However, there were significant increases in Shh mRNA in both NPCs
and neurons, but not astrocytes, after 16 hours of hypoxia (Figure 1). A lactate dehydrogenase assay demonstrated no evidence of cell death in hypoxic cultures compared with controls. Shh protein levels were also increased in NPC in response to hypoxia at this time point (Figure 1G). In addition, neuronal cultures treated with cobalt chloride, a hypoxia-mimicking agent, also increased Shh protein levels (data not shown). When absolute levels of expression were compared, astrocytes expressed levels of Shh at much higher levels than neurons or NPC (Figure 1H). So we tested whether a more intense hypoxic stimulus (0.1% O2 for 3 hours) would also stimulate increased Shh expression in astrocytes. Our results show that despite high levels of Shh expression in glia, they do not increase Shh expression when exposed to hypoxia (Figure 1F). These results suggest that hypoxia upregulates Shh in neurons or neural progenitors but not in astrocytes.

Our results demonstrate that Shh gene expression and protein levels increase in neural progenitors after hypoxia. We then tested whether adding exogenous recombinant Shh increased proliferation of neural progenitors under hypoxia and whether this hypoxia-induced proliferation could be blocked with the Shh pathway-specific inhibitor, cyclopamine. NPCs were exposed to normoxia or hypoxia (1% O2 for 16 hours) and treated with 20 nmol/L Shh and/or 5 μmol/L cyclopamine. Indeed, hypoxia did increase proliferation of neural progenitors. Also, hypoxia and exogenous Shh had an additive effect on proliferation (Figure 1J–L). Cyclopamine reduced the proliferation stimulus of hypoxia or Shh and their combined treatment.

**MCAO Induces a Transient But Functional Hypoxia in the Hippocampus**

Our data suggested that in vitro NPCs are responsive to hypoxia through upregulation of Shh and that Shh might modulate hypoxia-induced proliferation. We developed a mouse model of mild ischemia in which the middle cerebral artery is occluded (MCAO) for 20 minutes and then reperfused. Occlusion of the middle cerebral artery in the mouse causes transient hypoxia as measured with an intrahippocampal oxygen probe placed at the level of the CA3 and hilus (Figure 2A). The mean partial pressure of oxygen in brain tissue at the CA3 before MCAO was 17.22 ± 7.1 mm Hg; during ischemia, the mean partial pressure of oxygen in brain tissue was 3.98 ± 2 mm Hg. Brain temperature before and during MCAO was unchanged, 36.9 ± 0.8 and 37.5 ± 1.2°C, respectively. As a measure of functional hypoxia within the hippocampus, we analyzed whether HIF1α, a protein, which increases in the setting of low oxygen tension, was altered after hypoxia. After 3 hours, after 20-minute MCAO, the HIF1α increased an average of 2.7-fold in the hippocampus (Figure 2B). Vascular endothelial growth factor A transcription was measured by real-time polymerase chain reaction as increased 6 hours after reperfusion.
factor-A increased an average of 360% over the contralateral control hippocampus (Figure 2C). These results support the concept that the transient ischemia induces a hypoxic stress on the hippocampus in our MCAO model.

MCAO Upregulates Shh Gene and Protein Expression in the Hippocampus

To determine whether the hippocampal hypoxia induced a proliferative response similar to our in vitro model, proliferating hippocampal NPCs were pulse-labeled with BrdU over a 12-hour period, after injury at specified times, and then the animals were immediately euthanized. This method allowed us to quantitate NPC in the S-phase in the subgranular zone. In this model, there is a significant increase in proliferation in the subgranular zone seen at 7 and 10 days after injury (Figure 3). These proliferating subgranular cells are almost entirely doublecortin-positive and represent newly generated migrating neurons (Figure 3A–C). With this model, we next examined Shh gene expression and its pathway constituents, smoothened, patched, and Gli1 after ischemia within ipsilateral and contralateral hippocampi. We analyzed 4 time periods, 0.5, 2.5, 7, and 10 days after ischemia, and compared gene expression with sham-operated controls. There was a transient increase in Shh and its transcription factor Gli1 confined to the ipsilateral hippocampus at 12 hours (Figure 3D). There were no significant changes in smoothened or patched mRNA in the ipsilateral or contralateral hippocampus (data not shown). Shh protein levels were increased approximately 3-fold in the ipsilateral hippocampi 3 and 7 days after ischemia (Figure 3E).

Figure 3. Ipsilateral subgranular zone proliferation and Shh gene expression after ischemia. Ischemia induces an increase in subgranular zone neural progenitors 7 and 10 days after ischemia as measured by a 12-hour pulse of BrdU 50 mg/kg before euthanasia (analysis of variance $P<0.02$, mean $\pm$ SD, n=3). These newly born subgranular cells (BrdU, green nuclei) colocalize with immature neuronal marker doublecortin (red) 7 days after 20 minutes of MCAO. A, Dentate ipsilateral to ischemia demonstrates increased colabeling of BrdU (green) and doublecortin (red) compared with the contralateral dentate (B) and the ipsilateral side of a sham animal (C). Scale bar=60 $\mu$m. D, Relative mRNA expression measured by real-time polymerase chain reaction normalized to baseline expression at Time 0 (sham) shows early increases in Shh and Gli1 (*$P<0.008$ and **$P<0.02$ compared with Time 0, respectively; mean $\pm$ SEM, n=6). E, Representative fluorescent immunoblot demonstrates a significant 3-fold increase in Shh protein by 7 days ($P=0.0025$; n=4 for sham and 7 days, n=2 for 3 days; ROD, relative optical density).
We confirmed this upregulation by immunohistochemistry and showed that Shh was predominantly expressed in cells within the CA3 and the subgranular region (Figure 4). We performed double immunohistochemistry for Shh and BrdU to determine if upregulation of Shh occurred in proliferating cells. At 3, 7 (not shown), or 10 days after ischemia, there were no cells that colocalized BrdU and Shh in the subgranular zone or hilus region (Figure 5). Double immunostaining for NeuN or GFAP demonstrated that almost all Shh-positive cells were NeuN-positive (only 5% of Shh cells do not label with NeuN) and there was no colocalization with GFAP for any Shh-positive cell (Figure 6). These data suggest that mature neurons are likely the source of ischemia-induced Shh upregulation.

**Inhibition of Shh Blocks Proliferation After Ischemia**

In the developing cerebellum, Shh is released from mature Purkinje neurons and induces proliferation of immature external granule neurons.2 We sought to test whether a similar system might be present in the dentate gyrus, where mature neurons may stimulate progenitor proliferation. We showed that Shh and hypoxia can increase NPC proliferation and that this proliferation can be blocked by cyclopamine, a specific inhibitor of the Shh pathway. Using the same mouse model of ischemia, we chronically infused cyclopamine or vehicle through an osmotic pump into the lateral ventricle starting 3 days after ischemia. After 7 days of cyclopamine infusion, proliferating hippocampal NPCs were pulse-labeled with
BrdU as described previously. Compared with the sham-operated group, the vehicle-treated ischemic group showed a 95% increase in proliferation 10 days after ischemia. Treatment with cyclopamine significantly reduced the ischemia-induced proliferation by 30% (Figure 6). Our results indicated that cyclopamine could suppress the ischemia-induced proliferation response in the subgranular progenitors.

**Discussion**

We have provided evidence that both NPCs and neurons upregulate Shh in response to hypoxia, whereas astrocytes do not. NPC also respond with increased proliferation to hypoxia and Shh. We show that upregulation in the hippocampus after ischemia is predominantly limited to the ipsilateral side to the ischemia. This upregulation is limited to CA3, the hilus, and subgranular zone. Double immunohistochemistry suggests that neurons within these regions overwhelmingly account for the increased Shh. However, the Shh pathway inhibitor can reduce hypoxia and ischemia-induced NPC proliferation. We believe this study is the first to provide evidence supporting a role for Shh in ischemic brain injury. Similar to previous studies of ischemic muscle and lung epithelial injury, we too have shown that Shh and its transcription factor, Gli1, are upregulated after ischemia.

Unlike our in vitro results, Studer et al. did not find Shh upregulated in NPCs after hypoxia. A few possible explanations for this discrepancy include our more sensitive detection technique of real-time polymerase chain reaction or our more severe hypoxia treatment (1% versus 3% oxygen). Furthermore, they used mesencephalic neural progenitors, which may have phenotypic differences between our cortically derived progenitors. They reported a similar increase in proliferation in cortical progenitors exposed to hypoxia but did not comment on Shh changes in these cells. Interestingly, their results showed that hypoxia increased dopaminergic differentiation of NPCs. Dopaminergic differentiation is a function in which Shh is known to play a significant role. It has been well established that Shh

![Figure 6. Shh is produced by mature neurons and inhibition decreases ischemia-induced proliferation. Shh/NeuN or GFAP double immunohistochemistry 7 days after ischemia. Almost all Shh-positive cells (red) except for a few (noted by arrows) colocalize the mature neuronal marker NeuN (green) and no cells localize with GFAP (green). Cyclopamine blocks ischemia-induced NPC proliferation in the subgranular zone. A, Vehicle group demonstrates ischemia-induced proliferation compared with sham. Cyclopamine (60 pmol/d) blocked the ischemia-induced proliferation (mean±SD, n=4). B, Cyclopamine and C (vehicle) representative photomicrographs of BrdU immunohistochemistry after ischemia of the subgranular zone demarcated by line. Bar=100 μm.](http://stroke.ahajournals.org/issue/31/11/StROKE-IssueArticle-Image19546.html)
induces a dose-dependent proliferation in nestin-positive stem cell/progenitors in vitro cultures as well as in an overexpression model of Shh in vivo. We now build on this observation by demonstrating that both hypoxia and Shh are additive and that the hypoxic induction of proliferation of these nestin-positive progenitors is partially Shh-dependent. The mechanisms of interaction between Shh and hypoxia remain to be elucidated. Recently, Hwang et al. also showed that hypoxia upregulates Shh in cardiomyoblasts and that there is likely an interaction between Shh and HIF1α. Our previous results suggested that this interaction is not through a direct stimulation of vascular endothelial growth factor by Shh and thus if there is a HIF1α interaction, it is not directly through vascular endothelial growth factor. There may be an interaction with angiopoietins.

It is unclear by what mechanism ischemia/hypoxia upregulates Shh. The mechanism is currently a focus of investigation. The increase in expression is likely to involve an intermediate regulatory step for a yet unidentified, transcription factor that transcribes Shh given that increased gene expression is not seen within the first 4 hours. Another possibility includes an increase in Shh mRNA stability under ischemic/hypoxic conditions. We have assumed that Shh protein upregulation seen in neural progenitors in culture and within the hippocampus is largely due to increased translation, because we observed increased Shh mRNA. However, we should not rule out the possibility that the Shh protein increase is due to altered stability or degradation.

It is interesting to note that despite evidence of in vitro upregulation of Shh in NPCs, we were unable to detect Shh by immunohistochemistry in proliferating neuronal progenitors. One possibility is that the in vitro conditions do not accurately reflect conditions of subgranular zone progenitors. Another possibility is that NPCs express such low levels of Shh that we are unable to detect such an increase with immunohistochemical investigation. Another in vitro versus in vivo discrepancy exists with the very high levels of Shh in proliferating glial cultures. However, we were unable to detect any Shh signal in GFAP-stained cells. It is likely that the proliferative conditions of 10% fetal bovine serum must alter this level of expression. This is contrasted to both neuronal and NPC cultures, which are maintained in serum-free defined media.

We note that the highest levels of Shh expression in the hippocampus both at baseline and after ischemia occur in the subgranular zone and the CA3 region. It is unclear why Shh is limited to these 2 regions. It is possible that Shh produced in the CA3 region might play a role in regulating axonal guidance from the newborn granular neurons of the dentate, because the CA3 region receives the mossy fiber output from the granular neurons of the dentate. Another possibility is that the CA3–dentate system is similar to the cerebellar system, where the Purkinje neuron releases Shh as a mitogen for the cerebellar granular neurons. If the latter proposal were true, then CA3 neurons would also release Shh as a mitogen for the subgranular progenitors. A final possibility is that the subgranular neurons transport Shh down their axons to the CA3 region, similar to the system of retinal ganglion cells and supracollicular neurons. All of these hypotheses remain to be tested.

Summary

We have provided evidence that Shh plays a role in regulating ischemia-induced neural progenitor proliferation. This response can be blocked in vitro and in vivo and can be enhanced, at least in vitro. Modulation of ischemia-induced proliferation, survival, differentiation, and reconnection through small molecule modulators or proteins requires further work in vivo if augmented progenitor proliferation will serve as a mechanism for future stroke recovery therapy.

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Disclosures

None.

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