Neuroprotective Effect of Upregulated Sonic Hedgehog in Retinal Ganglion Cells Following Chronic Ocular Hypertension

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PURPOSE. To determine sonic hedgehog (Shh) expression and whether it exerts neuroprotective effects on retinal ganglion cells (RGCs) in a rat chronic ocular hypertension model.

METHODS. Intraocular pressure (IOP) elevation in adult rat was induced by episcleral vein cautery. Retinal expression of Shh protein and mRNA was determined by immunohistochemistry, Western blot analysis, and real-time PCR. Exogenous Shh and its inhibitor cyclopamine were intravitreally injected to examine their effects on RGC survival after ocular hypertension by the counting of retrograde Dil-labeled RGCs. Shh pathway components mediating neuroprotective effects were characterized using Western blot analysis and real-time PCR.

RESULTS. Shh was mainly detected in the RGCs in normal adult rat. Retinas from the elevated IOP group had 2.1- to 4.4-fold greater Shh expression than control retinas (P < 0.05). Shh promoted RGC survival at 2 and 4 weeks after IOP elevation in a dose-dependent manner, resulting in a loss of only 4.54% ± 0.56% RGCs at 2 weeks (P < 0.01; vs. PBS-treated groups). In contrast, cyclopamine increased RGC loss. Protein and mRNA levels of the Shh signal transducer Smo and the downstream transcription factor Gli1 were significantly upregulated in RGCs after chronic ocular hypertension or intravitreal injection of Shh.

CONCLUSIONS. Shh and Smo are upregulated in a time-dependent manner in retinas exposed to ocular hypertension, and Shh has neuroprotective effects on damaged RGCs in a rat chronic hypertension model. Shh may exert neuroprotective effects by relieving the inhibition of Smo and subsequently activating Gli1. (Invest Ophthalmol Vis Sci. 2010;51:2986–2992) DOI: 10.1167/iovs.09-4151

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Glaucoma is a leading cause of irreversible vision loss and is characterized by progressive retinal ganglion cell (RGC) death.1,2 Both the number of surviving RGCs and their axons are important determinants in the extent of visual impairment in glaucoma patients.3,4 One major goal in the research and clinical management of glaucoma is to prevent RGC degeneration. Glaucoma induces a variety of molecular responses, involving both beneficial and cytotoxic factors that may influence the survival of damaged RGCs.5–7 Thus, identifying the beneficial factors that promote RGC survival in glaucoma is important for the successful development of clinical strategies that prevent RGC death in glaucoma patients. Currently, glaucoma models of elevated intraocular pressure in the rat have been used extensively to investigate the mechanisms underlying RGC survival and to search for possible cures.8–10

Sonic hedgehog (Shh) is a soluble signaling protein that plays an important role in both embryonic development and adult stem cell function.11–15 The Shh pathway is closely associated with development of the eye, with this factor inducing the formation of glia from precursors, guiding RGC axon projections, promoting retinal progenitor proliferation, and determining retinal cell fate.14–17 Shh may also act as a mitogen to induce the ciliary marginal zone or the retinal margin cells to regenerate into retinal neurons.18 Increasing evidence suggests that Shh is not only an important regulator of neurogenesis of CNS, it also has neuroprotective effects in adult organisms.19 In vivo studies have demonstrated that Shh is neuroprotective in models of stroke and malone excitotoxicity.20 Shh was recently found to be significantly upregulated after facial nerve axotomy, which promotes neuronal survival.21

However, it has not been elucidated whether Shh continues to function in the adult retina under pathologic conditions. In this study, we investigated retinal Shh expression and the neuroprotective effects of Shh on RGCs in a well-established rat model of chronic ocular hypertension. Our results provide the first evidence that Shh is upregulated in a time-dependent manner in retinas exposed to ocular hypertension and that Shh has neuroprotective effects on damaged RGCs under these conditions. Our findings suggest that activation of the Shh signaling pathway has potential therapeutic value in chronic glaucoma.

MATERIALS AND METHODS

Animals

Experiments were conducted with adult male Sprague-Dawley (SD) rats weighing approximately 250 g. Animals were maintained under a 12-hour light/12-hour dark cycle (7:00 am–7:00 pm), and standard rodent chow was provided ad libitum. All experiments and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the University of Fudan Institutional Animal Care and Use Committee. Unilateral IOP
elevation was induced through episcleral vein cauterization (EVC). Rats (n = 20) not undergoing this procedure served as controls. Before surgery, 0.5% alcaine (Alcon-Couvreur, Puurs, Belgium) was applied to the eyes, and 0.3% tobramycin (Tobres; Alcon-Couvreur) was administered to prevent infections after treatment. In each experimental group, 12 animals were used for RGC counting, Western blot analysis, immunohistochemistry, and real-time PCR. Animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.36 mL/100 g), and they were humanely killed at the indicated time through an overdose of anesthesia.

Recombinant Mouse Shh and Shh Antagonist

Recombinant mouse Shh-N (R&D systems, Minneapolis, MN) was freshly prepared at a concentration of 10, 50, or 100 μg/mL in 0.1 M phosphate-buffered saline (PBS). Cycloheximide (Toronto Research Chemicals, North York, ON, Canada) or tomatidine (Sigma-Aldrich, St. Louis, MO) was dissolved at a concentration of 1.0 or 5.0 μg/mL in 45% (wt/vol) 2-hydroxypropyl-cyclodextrin (HBC; Sigma-Aldrich, St. Louis, MO) in PBS.

Experimental Rat Ocular Hypertension Model

IOP elevation was induced in the right eye by cauterizing two episcleral veins, as previously described.24,25 Briefly, a 2-mm-long incision was made through the conjunctiva and Tenon’s capsule at the limbal periphery of the dorsal eye. Two dorsal episcleral veins located near the superior rectus muscle were isolated from the surrounding tissues. A cautery was then precisely applied to the selected vein, taking care to avoid thermal damage to the neighboring tissues. The contralateral eyes, which served as controls, were sham-operated by separating the veins in a similar manner without any cauterization. A subset of animals was compared with that in the left eye (percentage left eye, mean ± SD). The contralateral eye was compared with its ipsilateral eye.

Immunohistochemistry

Anesthetized rats were perfused with 4% PFA as described. Both eyes of each rat were enucleated and then immersion fixed in 4% PFA for 2 hours after removal of the cornea and lens. The eyecups were cryoprotected in graded sucrose solutions (20%-30% in PBS) at 4°C, and they were humanely killed at the indicated time through an overdose of anesthesia.

Western Blot Analysis

Western blot analysis was performed as described previously.27 Briefly, the retinas were separated from the eyes, and retinal proteins were extracted using cell lysis buffer (Cell Signaling Technology Inc., Beverly, MA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Rehovot, Israel). Ten micrograms of each protein were separated by SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane. The membranes were incubated with goat anti-Shh (1:2000), rabbit anti-Ptc (1:2000), rabbit anti-Smo (1:2000), or goat anti-Gli1 (1:1000) in 5% nonfat milk. Membranes were then incubated with the appropriate HRP-conjugated secondary antibody (1:2000 in TBS-Tween; Santa Cruz Biotechnology), and bound secondary antibodies were visualized using ECL Western blot analysis detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Western blot analysis data were subjected to densitometric analysis with Scan analysis software for Macintosh ( Biosoft).

Quantitative PCR

For mRNA detection, animals were decapitated at the time of the Western blot analyses. The eyes were quickly enucleated and chilled in ice-cold PBS. Retinas were dissected on ice, quickly frozen in dry ice or liquid nitrogen, and stored at −80°C. Total RNA was isolated (RNeasy Kit; Qiagen, Valencia, CA). The quantity and quality of the RNA were assessed by determining the 260/280 nm absorbance ratio (Genequant II; Pharmacia, Düebendorf, Switzerland). For each sample, 250 ng RNA was reverse transcribed into cDNA, using the reverse transcription portion of a qRT-PCR kit (SuperScript III Platinum Two-Step qRT-PCR; Invitrogen, Carlsbad, CA). Real-time PCR was carried out using SYBR Green PCR Master Mix (Invitrogen) and the following primers: Shh: forward, 5'-ATGCTGCTGCTGGCTGGCCAGA-3'; reverse, 5'-TCAGTCTTGACCTGACGTCCAT-3'; Ptc: forward, 5'-ATGCTGATAAAGCCGGAGT-3'; reverse, 5'-CAGAGGCTCACAGGGCG-3'; Smo: forward, 5'-CTCCGGGACTATGTGCTAT-3'; reverse, 5'-GAAGTCCGAGCCTGCTC-3'; and glyceraldehyde phosphate dehydrogenase (GAPDH): forward, 5'-CATCAAGAAGGTTGATGAGG-3'; reverse, 5'-CCTCCGGGACTATGTGCTAT-3'. Real-time PCR reactions were performed (Prism 7000; Applied Biosystems, Foster City, CA). Retinal expression of the housekeeping gene GAPDH did not significantly correlate with IOP. Relative target gene expression was normalized with a calibrator (normal retinas). The final result, expressed as the N-fold difference relative to GAPDH and the calibrator, was determined using the following formula: 

\[ N_{\text{target}} = 2^{-\Delta\Delta Ct} \]

Statistical Analysis

Statistical analysis was performed using statistical software (SPSS, version 14.0 for Windows; SPSS, Chicago, IL). Two groups were compared using Student’s t-tests and paired t-tests. Multiple groups were compared using a one-way ANOVA with Duncan’s multiple pairwise comparison tests. Data were shown as mean ± SD. P < 0.05 was considered significant.
**RESULTS**

**Shh Upregulation in the Rat Ocular Hypertensive Retina**

Previous studies have demonstrated that normal mouse RGCs express Shh.28 Here, we examined Shh protein expression in both normal and hypertensive rat eyes using immunohistochemistry. In the normal and sham-operated control eyes, Shh was mainly detected in the RGCs. Weak Shh staining was also present in the inner plexiform layer (IPL), consistent with either the Shh protein being present in the ganglion cell processes or the Shh protein having diffused to IPL. Analysis of retinas at 2 weeks after IOP elevation revealed that Shh was present at much higher levels in the RGCs and in the IPL and outer plexiform layer (OPL) (Fig. 1A). Shh staining in the OPL may be attributed to the diffusion of Shh, which is initially synthesized and expressed in the RGCs, since RGC processes are not present in this layer. No staining was detected in control experiments in which the primary antibody was excluded (data not shown). We confirmed these findings through Western blot analysis (Fig. 1B). Hypertensive retinas had 2.1- to 4.4-fold greater Shh expression than control retinas, with Shh expression reaching a peak at 2 weeks after IOP elevation (P < 0.05). To quantify the Shh transcripts, we performed real-time quantitative analysis of retinal Shh mRNA expression after ocular hypertension. Shh mRNA expression was upregulated at 1 week after IOP elevation and reached a peak at 2 weeks. In contrast, no differences were detected in Ptc expression between hypertensive and control retinas. Real-time quantitative PCR analysis of Smo, Ptc, and Gli1 expression revealed that Smo and Gli1 mRNA expression increased in a time-dependent manner after IOP elevation. However, Ptc mRNA upregulation was not detected (Fig. 2C).

**Shh Promotion of RGC Survival after Ocular Hypertension**

To determine whether upregulated Shh expression influences RGC survival, we counted Dil-labeled RGCs in flatmounted retinas. Retrograde Dil labeling marked not only RGCs but also debris and microglial cells. Microglia take up Dil during the process of ganglion cell death and can be identified based on their small size and highly branched nature. Therefore, we counted only those RGCs of certain sizes (>10 μm) and shapes (circular or oval, with an aspect ratio of 0.8–1.2) to discriminate non-RGCs from RGCs.32,33 As shown in Figure 3A, 15.26% ± 1.57% of RGCs were lost 2 weeks after IOP elevation in the PBS-treated groups. In contrast, Shh-treated retinas lost only 4.54% ± 0.36% of RGCs (P < 0.01 compared with PBS-treated groups). To investigate the effect of Shh on long-term RGC survival, we injected Shh intravitreally once a week for 4 weeks after ocular hypertension. Similar to RGC loss after 2 weeks of injury, RGC loss after 4 weeks after IOP elevation was significantly reduced by Shh (P < 0.01). The protective effect of Shh on RGC survival was dose dependent. Cyclopamine exacerbated RGC loss after ocular hypertension, whereas tomatidine had no effect. Representative photomicrographs of retinal flat-
mounts at 4 weeks after ocular hypertension are shown in Figure 3B. Quantitative real-time PCR and Western blot analysis revealed that intravitreal Shh administration increased retinal expression of both Gli1 and Smo protein (Fig. 3C) and mRNA (Fig. 3D). These results suggest that Shh exerts a significant neuroprotective effect by activating Gli1 in the retina after ocular hypertension.

IOP Profile

EVC resulted in sustained increases in IOP, as previously reported.24,34 The IOP in control eyes was 13.4 ± 0.27 mm (mean ± SD) Hg and remained constant throughout the experiment. The IOP of eyes undergoing EVC was 27 ± 2.31 mm Hg and was significantly greater than the IOP of control eyes at all time points studied (P < 0.01). Importantly, IOPs of eyes treated with Shh or cyclopamine were comparable to those of PBS-treated eyes (P > 0.05) (Fig. 4). Therefore, Shh has a neuroprotective effect on RGCs without affecting IOP.

DISCUSSION

The results presented here show that Shh and its signal transducer Smo are upregulated in chronic hypertensive retinas in a time-dependent manner. Moreover, either exogenous or endogenous Shh promoted the survival of damaged RGCs in a well-established model of rat chronic hypertension. Our findings suggest that the Shh signaling pathway might exert neuroprotective effects by relieving the inhibition of Smo and subsequently activating transcriptional factor Gli1.

Glioma is a chronic and progressive optic nerve neuropathy involving the death of RGCs. In humans, elevated IOP is a major risk factor for glioma. The model used in this study mimics the slow, progressive neuropathy produced by elevated IOP in humans. Consistent with a previous report,24 neither alteration of retinal blood flow nor attenuation of blood vessels was observed in our study of this animal model. Retrograde labeling of RGCs from the superior colliculus with fluorescent dyes such as Dil and 4Di-10-ASP Dil has often been used for the quantification of RGCs in retinal flatmounts.35,36 Microglia, which are activated after IOP elevation, can also be visualized with these dyes because they take up the fluorescent RGC debris.35 For this reason, we used certain morphologic criteria to distinguish RGCs from other cell types in the retinal flatmounts, as reported previously.32,33

Our results revealed that Shh is expressed at a low level in the adult rat retina and is upregulated after IOP elevation. Shh is an extracellular signaling molecule that has been studied in great detail in relation to vertebrate development.13 It determines the fates of different cell types in the ventral neural tube during early development.11 RGC-derived Shh plays a critical role in the cell-cell and axon-glial interactions necessary for normal glial precursor cell diversification in the mammalian optic nerve.15 During retinal development, Shh expression at the chiasm border is thought to define a constrained pathway within the ventral midline that guides the progression of RGC axons.57 Here, we present the first evidence that chronic ocular hypertension upregulates Shh and Smo, which exert neuroprotective effects on RGCs. The coordinated expression of this ligand and its signal transducer in adult RGCs strongly suggests that Shh functions as a survival factor in an autocrine manner. Alternatively, Shh may act on the surrounding cells, such as astrocytes, microglial cells, and Müller cells, to release molecules that modulate RGC survival. Although the precise molecular pathway remains unclear, our findings indicate that Shh has a novel neuroprotective function in mature RGCs because its expression level was altered after IOP elevation.
Interestingly, in addition to functioning in retina or optic nerve development, Shh may play a role in response to ocular hypertension in the adult retina. Additional studies will be necessary to understand how widely this key molecule is operative not just in the embryo but in the adult retina as well.

In this study, we found that exogenous Shh prevented RGC loss associated with increased IOP. However, this finding does not shed light on whether endogenous Shh protects damaged RGCs. Shh appears to have dual effects in cardiac ischemia, with high exogenous levels fostering tissue repair and endogenous levels deleterious.38 We subsequently used the steroidal alkaloid cyclopamine, which inhibits Shh signaling by binding to Smo.39 In ocular hypertensive adult rats, the intravitreal alkaloid cyclopamine, which inhibits Shh signaling by binding just in the embryo but in the adult retina as well.

Our results confirmed that IOP was indeed significantly greater in eyes undergoing EVC than in sham-operated eyes. Although elevated IOP impairs axonal transport, no differences were detected in the IOP between Shh- and cyclopamine-treated groups and the control groups. Any effect of increased IOP on axonal transport can be ruled out through comparison of these groups. Thus, the neuroprotective effect of Shh, as shown by an increase in Dil-labeled RGCs, is not an artifact of changes in dye transport associated with alterations in IOP.

The molecular mechanism by which Shh protects damaged RGCs remains elusive. The Shh receptor system consists of Ptc, a 12-transmembrane protein that acts as the Shh receptor, and Smo, a 7-transmembrane protein that serves as the Shh transducer.40,41 Binding of Shh to Ptc abrogates inhibition of Smo and induces extensive phosphorylation of the Smo cytoplasmic tail by protein kinase A and casein kinase I. This phosphorylation then triggers an intracellular cascade that ultimately activates the transcription factor Gli1.42–44 Our study revealed that, in hypertensive retinas, the upregulation of Shh was accompanied by an elevated expression of Smo and Gli1, suggesting that both factors participate in Shh-induced RGC survival. Moreover, cyclopamine, an inhibitor of the Shh signaling pathway that binds directly to Smo, significantly increased RGC loss after ocular hypertension. These results im-

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933456/)
May be interesting to test whether Shh overexpression or downregulation of Smo and the subsequent activation of Gli1 play critical roles in Shh-induced neuroprotection after IOP elevation. Notably, we found that elevations in neither exogenous nor endogenous Shh induced detectable changes in Ptc mRNA or protein levels, a finding that is consistent with a report that Shh upregulation in motor neurons is not followed by Ptc upregulation after facial nerve axotomy.21 How Shh may work in the local pathway underlying chronic glaucoma is not fully understood. In unpublished experiments, we found that, in the ocular hypertensive retinas, upregulation of Shh, Smo, and Gli1 were followed by upregulation of brain-derived neurotrophic factor (BDNF), glia-derived neurotrophic factor, fibroblast growth factor, and nerve growth factor. Moreover, of these factors, only Gli1 and BDNF expression were downregulated by cyclopamine. Future studies should investigate whether Shh-mediated neuroprotection involves BDNF (and, if so, to what extent) and the mechanism(s) by which Shh regulates BDNF transcription. Understanding such a mechanism in glaucoma is important for finding ways to stimulate endogenous neuroprotection.

Our findings suggest that Shh is upregulated as a molecular response to chronic hypertension and that Shh has neuroprotective effects on damaged RGCs in a well-established model of rat chronic ocular hypertension. These findings also suggest that, in addition to having an early role in directing cell fates during retinal or optic nerve development, Shh functions as a survival factor after chronic ocular hypertension. An additional interesting aspect of our findings is the potential therapeutic value of activation of Shh signaling in chronic glaucoma. It would be interesting to test whether Shh overexpression or activation of its signaling components, both of which are novel therapeutic approaches, might provide an attractive strategy to prevent the neurodegeneration associated with glaucoma.

References


