Hsp27 Upregulation by HIF-1 Signaling Offers Protection against Retinal Ischemia in Rats

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PURPOSE. Previous work from the authors’ laboratory has shown that Hsp27 is specifically upregulated after retinal ischemic preconditioning (IPC), and this upregulation acts as a key cytoprotective factor in preventing retinal ischemic damage. The regulatory mechanisms involved in the upregulation of Hsp27 after IPC are unknown. The purpose of this study was to explore the transcriptional events responsible for the upregulation of Hsp27 after IPC.

METHODS. CoCl2 was used to test for Hsp27 expression after hypoxic stimulus. The promoter and first intron regions of the human Hsp27 gene were cloned by PCR and characterized by deletion analysis by using a reporter assay. In vitro results were then applied to an in vivo model of retinal ischemia to determine whether CoCl2 upregulates rhHsp27 and protects the retina from ischemic injury.

RESULTS. CoCl2 upregulated Hsp27 in cultured retinal neurons. Promoter-intron reporter assays using various DNA deletion constructs indicated that several HIF-1 binding sites were necessary for CoCl2-induced expression of the Hsp27 gene. Furthermore, CoCl2 upregulated Hsp27 in the rat retina and protected the rat retina from ischemic injury.

CONCLUSIONS. These data provide evidence that Hsp27 is regulated by hypoxic signaling through HIF-1 activation and support the idea that an early event in IPC is the activation of HIF-1. These findings are significant, because this is the first time HIF-1 activation has been associated with the protective effects of IPC and with Hsp27 upregulation. (Invest Ophtalmol Vis Sci. 2005;46:1092–1098) DOI:10.1167/iovs.04-0043

Retinal ischemia plays a pivotal role in a number of retinal degenerative diseases such as glaucoma, diabetic retinopathy, central retinal artery occlusion, and retinopathy of prematurity. Understanding the molecular and cellular events associated with retinal degeneration would be valuable in identifying new treatments for these blinding diseases. One endogenous method that has been investigated is ischemic preconditioning (IPC). IPC involves a brief exposure to ischemia followed by a defined period when the retina is protected from subsequent lethal ischemia. Previous studies from this laboratory have shown that Hsp27 is specifically upregulated in the retinal ganglion cell layer after retinal IPC.1 This upregulation of Hsp27 is not accompanied by a similar increase in the other common stress-response heat shock proteins, such as Hsp70 or -90. Recent findings from our laboratory demonstrate that this overexpression of Hsp27 can protect retinal cells from ischemic stress and the calcium overload.2 Furthermore, these studies showed that Hsp27 protects these cells in a caspase-dependent and-independent manner. Through these studies it is evident that Hsp27 plays a role in the development of retinal IPC.

The mechanisms for Hsp27 upregulation after retinal IPC are still unclear. Previous studies have shown that Hsp27 transcription is regulated by activation of the heat shock factor (HSF)-1 transcription factors in response to stress, or by estrogen receptor activation.5–7 However, studies demonstrating that Hsp27 is upregulated when other heat shock proteins are not after retinal IPC indicates that transcriptional control is not through classic HSF-1 activation. In this study we provide evidence that IPC regulates Hsp27 gene expression by hypoxia-inducible factor (HIF)-1. Furthermore, we show that Hsp27 can be specifically upregulated in vivo by the HIF-1 inducer CoCl2, and that this upregulation also protects the retina from ischemic damage.

MATERIALS AND METHODS

Cell Culture

A transformed embryonic rat retinal ganglion cell line (RGC-5) was used to conduct both the ischemic tolerance and promoter experiments. The RGC-5 cells were grown on polypropylene plates and maintained in DMEM (pH 7.4; Sigma-Aldrich, St. Louis, MO) with 1 g/L glucose, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), without phenol red. The cells were passaged every 2 to 3 days. For all experiments, cells were used at a confluence of 60% to 80%.

Protein Purification and Western Blot Analysis of Hsp27 Expression

RGC-5 cells treated with CoCl2 were washed twice with PBS and solubilized with 1% SDS. To evaluate changes in retinal proteins in vivo, animals were euthanatized, eye enucleated, globes bisected at the ora serrata, and retinas dissected from the underlying retinal pigment epithelium (RPE) and choroid. Isolated rat retinas were lysed with cell lysis buffer (50 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl2, 1 mM Na2VO4, 1 mM dithiothreitol (DTT), 1% NP-40, and 1 protease inhibitor cocktail tablet per 10 mL of buffer (Complete Mini; Roche, Indianapolis, IN) and solutions were clarified by centrifugation (15 minutes at 20,000g). Solubilized proteins were then resolved on a 4% to 20% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). After they were blocked with 5% skim milk in Tris-buffered saline (TBS)-Tween, blots were incubated with the following primary antibodies, anti-Hsp25 (StressGen, San Diego, CA), and anti-β-actin antibodies (Sigma-Aldrich). After incubation with secondary antibodies, blots were developed using chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) and visualized...
with a digital imaging system (VersaDoc; Bio-Rad, Hercules, CA). Hsp27 expression levels were normalized to β-actin expression levels. Values are reported as a percentage of the untreated control levels.

**Protein Purification and Western Blot Analysis of HIF-α**

RGC-5 cells were treated with 400 μM CoCl₂ to mimic hypoxia and harvested 1 to 6 hours later. The cells were harvested by quick removal of the growth medium and immediate addition of chilled lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM Na₃VO₄, 10 mM NaF, and 1 protease inhibitor cocktail tablet [Complete Mini; Roche] per 10 mL of buffer) and clarified by centrifugation (15 minutes at 20,000g). Solubilized proteins were then resolved on a 4% to 20% SDS-PAGE gel and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). After blocking with 5% skim milk in Tris-buffered saline with Tween-20, blots were incubated with anti-HIF-1α antibody (Novus Biologicals, Littleton, CO) and visualized in the manner previously mentioned. The blots were then stripped and reprobed with anti-β-actin antibody to control for protein loading. Results are reported as a percentage of the untreated control levels.

**Cloning and Characterization of Hsp27 Promoter and First Intron**

The promoter region and first intron of the human Hsp27 gene were cloned by genomic PCR. The forward (5′) primer used for the promoter was 5′-GGAGTACAGTGTCGACTCCG, and the reverse (3′) primer was 5′-CGAGATCTGTCGGAGGACTGTC. The forward (5′) primer used to create the first promoter deletion mutation at amino acid position 439 was 5′-CGAGATCTCAACCTCTGGGCTCAAAG. A second promoter deletion mutation was made at position 288 using the primer 5′-CTCAGATCTGCGGACCCACAG. The forward (5′) primer used for the first intron was 5′-GTGAGATCTCCTGGACAGTCAAGCA, and the reverse (3′) primer used was 5′-CTGGATCTCTCCTGCTGTTGAC. Promoter sequences were ligated into the pGL3 luciferase reporter vector (Promega, Madison, WI) using BamHI restriction site overhangs incorporated on the sequence during the PCR. The first intron was ligated into the enhancer region of the pGL3 vector using BamHI restriction site overhangs incorporated on the sequence during the PCR. All cloned DNA sequences were sequenced to ensure proper orientation and the lack of sequence mutations.

**Luciferase Promoter Assay**

RGC-5 cells were plated in 24-well culture plates at 3 to 5 × 10⁵ cells per well, allowed to grow overnight, and then transfected with the promoter reporter DNA constructs (Effectene transfection reagent; Qiagen, Valencia, CA). DNA and transfection reagent were removed from the cells after 6 hours, and the cells were allowed to recover for 48 to 72 hours. After 48 hours, select cells were treated with 400 μM CoCl₂ for 24 hours. Four hours before harvest, select cells were exposed to heat-shock treatment (43°C for 1 hour) and then allowed to recover at 37°C for 3 hours. After treatment, cells were washed with PBS and lysed (100 μL Passive Lysis Buffer; Promega). Promoter activity was measured by assaying an aliquot of the cell lysate with a dual luciferase assay (Promega). The promoter activity of a promoter construct after treatment is represented by the percentage increase or decrease of firefly luciferase expression with respect to that of untreated cells with the same promoter construct. Firefly luciferase activity was normalized by the cotransfection and measurement of Renilla luciferase.

**Treatment of Brown Norway Rats with CoCl₂**

Female brown Norway rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 150 to 200 g were injected with (60 mg/kg) CoCl₂ or vehicle (saline) subcutaneously 12 to 160 hours before either retinal ischemia or Western blot analysis of the retinas. Retinal ischemia was induced by raising IOP above systolic blood pressure (155–160 mm Hg). Other studies have shown that the ischemia must be maintained for 40 minutes to create ischemic injury in the rat retina. Brown Norway rats were anesthetized with 75 mg/kg ketamine and 8 mg/kg xylazine, and local anesthetic (0.5% propacaine HCl) was placed on the cornea of the rat. The intraocular pressure was then increased above systolic blood pressure (155–160 mm Hg) by placing a 30-gauge needle connected to an elevated saline reservoir into the anterior chamber of the eye. This ischemic condition was maintained for 45 minutes. The contralateral eye was left untreated for the control. Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and protocols were approved by the Animal Care Committees at the Medical University of South Carolina.

**ERG Analysis**

To quantitate postsischemic functional recovery, we performed ERG. Rats were dark adapted overnight, and ERG recordings were performed under dim red light. For these procedures, rats were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and xylazine (8 mg/kg). Corneal analgesia was achieved using 10 μL of 0.5% proparacaine. Pupillary dilation was maintained with topical administration of 20 μL of a solution containing 0.5% tropicamide, 0.2% cyclopentolate, and 1% phenylephrine HCl. A platinum wire was placed in contact longitudinally with the corneal surface, bilaterally. A reference electrode was placed subcutaneously above the nose, and a grounding electrode was placed subcutaneously in the animal’s tail. Corneal electrical responses to a single 10-μs white-light flash were delivered by a Ganzfeld stimulator. The ERG b-wave amplitude after ischemia was measured and reported as a percentage of the baseline b-wave amplitude taken before ischemia.

**Immunohistochemistry**

The rats were killed by an overdose of pentobarbital, and the eyes were enucleated and fixed for 1 hour in 4% paraformaldehyde in 0.1 M PBS at 4°C. The anterior segment was removed, and the fixation continued for 3 hours. The eyes were then cryopreserved in a solution of 30% sucrose overnight, placed in optimal cutting temperature (OCT) mounting medium, frozen, and parasagittal sections (10 μm) cut and mounted on gelatin-coated slides. The polyclonal antibody SPA-801 (1:200; StressGen) and anti-rabbit IgG conjugated to Alexa Fluor488 (Molecular Probes, Eugene, OR) were used to visualize retinal rHsp27. Sections were viewed and photographed with a confocal microscope (Leica, Heidelberg, Germany).

**Statistical Analysis**

Statistical comparisons were made with the Student’s t-test for non-paired data or ANOVA using the Dunnett posttest (GraphPad Software, Inc., San Diego, CA). P ≤ 0.05 was considered significant.

**RESULTS**

Analysis of 1.1 kb of the Hsp27 promoter and first intron was performed (Fig. 1A, 1B). This analysis revealed all the known promoter elements that had been described in prior publications. It also revealed three possible HIF-1 binding sites and two additional CAT boxes upstream of the previously characterized promoter region. Analysis of the first intron revealed a previously reported heat shock element along with three potential HIF-1 binding sites.

To investigate if the HIF-1 transcription factor could be activated in these RGC-5 cells, we performed Western blot analysis, to confirm that the HIF-1α subunit was stabilized by CoCl₂ treatment, as has been shown in other cells. Figure 2 shows that treatment with 400 μM CoCl₂ produced a significant accumulation of HIF-1α by 6 hours after treatment.
FIGURE 1. Computer analysis of Hsp27 promoter and first intron. The first 1.1 kb of the human Hsp27 promoter sequence (A) and 720 bp of the first intron (B) were analyzed (MATInspector, ver. 2.2; Genomatrix GmbH, Munich, Germany) to identify potential transcription factor binding sites. Results of this analysis confirmed previously reported transcription factor sites, as well as uncharacterized binding sites.
To determine whether the Hsp27 gene was influenced by the HIF-1 transcription factor, RGC-5 cells were treated with the HIF-1 inducer CoCl2 for 24 hours, and Hsp27 expression was evaluated. Results from this experiment revealed a concentration-dependent increase in Hsp27 protein expression (Fig. 3). Figure 4 shows the time course for CoCl2 induction of Hsp27. No increase in Hsp27 protein levels was detected after 6 hours; however, increased Hsp27 levels were detected at 12 hours and peaked by 24 hours after CoCl2 administration.

To determine whether CoCl2 induction of rHsp27 expression results from protein synthesis, RGC-5 cells were treated with 400 μM CoCl2 in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX) for 24 hours. Protein expression was measured by Western blot and densitometry analyses. Pretreatment with 1 μg/mL CHX resulted in complete inhibition of the CoCl2-induced increase in Hsp27 expression (data not shown, n = 4).

To test whether HIF-1 was directly affecting Hsp27 gene activation, a series of promoter assays were performed. The full-length (1.1 kb) human Hsp27 promoter and first intron were cloned by genomic PCR and placed into a luciferase reporter vector. In RGC-5 cells transfected with the full-length promoter and first intron, an increase in luciferase activity was observed after treatment with 400 μM CoCl2 or heat shock (Fig. 5). Removal of the first intron (containing 3 HIF-1 sites) from the reporter construct abolished the CoCl2 response, but did not significantly alter the heat shock response. Replacing the first intron, but deleting the most-distal half of the promoter containing two HIF-1 binding sites, resulted in a similar loss of CoCl2 response. Again, no significant change in heat shock response was measured. A large deletion to include all HIF-1 sites in the promoter region again blocked the CoCl2 response, but did not alter the response to heat shock.

To investigate whether CoCl2 induces rHsp27 expression in vivo, we injected Brown Norway rats subcutaneously with 60 mg/Kg CoCl2. rHsp27 protein levels were measured 12, 24, 72, and 120 hours after injection with either CoCl2 or saline control. No increase in rHsp27 was seen 12 hours after injection. However, increased rHsp27 protein levels were measured at 24 and 72 hours after injection of CoCl2 when compared with saline injected control animals (Fig. 6). Hsp70 protein expression was also measured. There was a slight increase at 12 hours, but expression returned to baseline levels by 24 hours after treatment (data not shown).

Localization of retinal rHsp27 was evaluated by immunohistochemical analysis 24 hours after vehicle or CoCl2 (60 mg/kg) administration (Fig. 7). In vehicle-treated rats, staining for rHsp27 was observed only in the endothelial cells of inner retinal vessels and the choroid. In rats that had received CoCl2, an intense expression of rHsp27 was also observed in the ganglion cell and nerve fiber layers of the retina. Light staining of the cell bodies of the inner and outer nuclear layer was occasionally observed in these animals.

To assess whether this upregulation of Hsp27 in the retina after CoCl2 pretreatment offers any functional protection, animals were subjected to complete retinal ischemia 12, 24, 72,
or 120 hours after CoCl₂ injection, and retina function was estimated by ERG analysis. Complete retinal ischemia in saline-injected rats resulted in a 50% reduction in b-wave amplitudes, when measured 3 days after the ischemic insult. Rats injected with CoCl₂ showed a significant increase in b-wave recovery after retinal ischemia, when the ischemic event was 24 to 72 hours after injection (Fig. 8). No protection was evident at 12 and 120 hours after CoCl₂ treatment.

**DISCUSSION**

IPC of the retina offers significant protection against ischemic insults. Previous studies have shown that the protective response in the retina requires protein synthesis. Understanding what these proteins are and the signaling pathways that regulate their expression is vital to the rational development of new neuroprotective strategies. We have shown that Hsp27 is specifically upregulated in the retina after IPC. Furthermore, it has been demonstrated that the overexpression of Hsp27 in cultured retinal ganglion cells can protect them from SOGD as well as intracellular calcium overload. The purpose of this study was to identify the signaling mechanism associated with retinal IPC that was responsible for the specific upregulation of Hsp27.

Under stressful conditions, such as ischemia, it is not uncommon to see the upregulation of several stress responses and heat shock proteins. Usually, these responses are mediated by the stress-related transcription factor HSF-1. The activation of this transcription factor induces a global heat shock response that includes the upregulation of a number of heat shock proteins including Hsp90, -70, and -27. Because IPC increases Hsp27 mRNA and protein, but not the other common heat shock proteins (i.e., Hsp70 and -90), mechanisms other than classic HSF-1 transcription factor activation probably regulate Hsp27 gene after retinal IPC. Our examination of a broader region of the promoter sequence (up to 1.1 kb) for additional transcription elements uncovered three possible HIF-1 binding sites in both the promoter and first intron. The sites located in the promoter were located upstream of the previously characterized Hsp27 promoter. The overwhelming presence of these possible binding sites led us to investigate whether Hsp27 gene expression is regulated by HIF-1 activation after IPC. This type of transcriptional control may explain the differential expression of this heat shock protein over the...
the RGC-5 cells and treated with either CoCl\textsubscript{2} or heat shock, an ischemia. Rats were treated with 60 mg/kg CoCl\textsubscript{2} or an equal volume of saline vehicle (control) 12, 24, 72 or 120 hours before complete retinal ischemia. Retinal function was estimated 3 days after ischemia through ERG b-wave analysis. The data are expressed as the mean ± SE of results in four to eight rats per treatment group. Significant increase (P < 0.05) in b-wave recovery in rats treated with CoCl\textsubscript{2} versus saline-treated (control) animals.

other common heat shock proteins that have been observed in the retina.

CoCl\textsubscript{2} is a well-characterized hypoxia mimicker that also activates the HIF-1 transcription factor.\textsuperscript{10–17,22} CoCl\textsubscript{2} activates HIF-1 by stabilizing the HIF-1\textalpha subunit. Stabilization of the HIF-1\textalpha subunit allows it to translocate to the nucleus and bind with HIF-1\textbeta to form the active transcription complex.\textsuperscript{23–26} Treatment of these RGC-5 cells with CoCl\textsubscript{2} resulted in a significant increase in HIF-1\textalpha after 6 hours of treatment. After activation of HIF-1, an increased level of Hsp27 protein was observed after 12 hours of treatment, and the level peaked at 24 hours of CoCl\textsubscript{2} treatment. The kinetics of the CoCl\textsubscript{2} induction of Hsp27 is similar to the induction of other HIF-1-mediated genes using CoCl\textsubscript{2}.\textsuperscript{10,14,23,24,26}

To further confirm that HIF-1 activation mediates Hsp27 expression, a series of reporter assays were performed on both the full-length promoter and first intron of Hsp27 as well as on a series of deletion mutations of the promoter. The first intron was included in these analyses, because other studies on the heat shock regulation of Hsp27 identified an active heat shock element within this DNA sequence, and our computer analysis of this sequence revealed numerous potential HIF-1 binding sites.\textsuperscript{4} When the full-length constructs were transfected into the RGC-5 cells and treated with either CoCl\textsubscript{2} or heat shock, an increase in the luciferase reporter was observed. Deletion of the first intron resulted in the loss of CoCl\textsubscript{2} induction, but heat shock induction was maintained. Deletion of the promoter region containing the two most distal HIF-1 sites (bases −1098 through −439) resulted in the loss of CoCl\textsubscript{2} induction, but again heat shock induction remained unchanged. Identical results were seen when a larger deletion (bases −1098 through −288) was placed with the first intron and analyzed. These data further support the idea that the Hsp27 gene is regulated by HIF-1 activation.

Another study has shown that the administration of CoCl\textsubscript{2} before cerebral ischemia can reduce the amount of ischemic damage as measured by histologic analysis.\textsuperscript{10} Recent studies from our laboratory have shown that the overexpression of Hsp27 in the RGC-5 cell line is protective against ischemic stress.\textsuperscript{2} To determine whether the upregulation of Hsp27 by CoCl\textsubscript{2} can protect the retina from ischemic injury, Brown Norway rats were treated with 60 mg/kg (ip). Results from these experiments demonstrate that Hsp27 was upregulated in the retina 24 to 120 hours after CoCl\textsubscript{2} treatment (Fig. 6). To confirm that Hsp27 was not upregulated simply by a heat shock response, expression of the inducible Hsp70 was measured in these same protein samples. Although these results demonstrate that 12 hours after the administration of CoCl\textsubscript{2} the protein level of Hsp70 had increased, we did not measure any increase in Hsp70 at later time points when a sustained increase in Hsp27 was observed. These data provide additive evidence for the differential regulation of Hsp27 expression from the larger heat shock proteins.

This upregulation of Hsp27 in the retina by CoCl\textsubscript{2} injection also had a protective effect against complete retinal ischemia. In control (saline injected) rats, 45 minutes of retinal ischemia reduced b-wave amplitudes 44% to 58%. However, in rats treated with CoCl\textsubscript{2} 24 to 72 hours before the creation of retinal ischemia b-wave amplitudes were significantly elevated, then compared with saline-treated animals. In previous studies when retinal ischemia was achieved by raising the intraocular pressure above the systolic blood pressure, the inner retina appeared to be affected more than the outer retina.\textsuperscript{6} ERG analysis performed after retinal ischemia generally resulted in a reduction in b-wave amplitudes more so than a-wave amplitudes. For this reason, recovery of b-wave amplitudes served as a good index of retinal protection.\textsuperscript{27} These results are consistent with a previous report\textsuperscript{4} demonstrating that upregulation of Hsp27 can protect the retina from acute ischemic injury. However, the limited expression of Hsp27 in the retina indicates that other factors also contribute to the neuroprotective actions of Hsp27. Other potential candidates include Hsp60 and -90, as these proteins have been shown to be upregulated in the ocular hypertensive eye.\textsuperscript{27,28}

The data presented in this study provide evidence that Hsp27 expression is regulated by HIF-1. Being classified as an HIF-1-mediated protein has several implications, not only for retinal ischemia, but for cancer therapy. We have also shown that HIF-1 signaling may be a vital aspect of IPC in vivo—that is, the HIF-1 inducer CoCl\textsubscript{2} offers protection in the rat retina against ischemia. Taken together, these data support the conclusion that on the initial preconditioning ischemic event, HIF-1 is activated, and over the required IPC recovery period, Hsp27 is upregulated. This upregulation of Hsp27 then offers protection against the subsequent ischemic insult.

References


