Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Letters 405 (2006) 191–195
Neuroscience Letters
www.elsevier.com/locate/neulet

Co-expression of heat shock transcription factors 1 and 2 in rat retinal ganglion cells

Jacky M.K. Kwong [a,*], Maziar Lalezary [b,c], Jessica K. Nguyen [d], Christine Yang [e], Anuj Khattar [f], Natik Piri [a], Sergey Mareninov [a], Lynn K. Gordon [a,g], Joseph Caprioli [a]

[a] *Department of Ophthalmology, University of California Los Angeles, Los Angeles, CA, USA*
[b] *Department of Neuroscience, University of California Los Angeles, Los Angeles, CA, USA*
[c] *School of Medicine, University of California San Diego, San Diego, CA, USA*
[d] *Department of Bioengineering, University of California Los Angeles, Los Angeles, CA, USA*
[e] *Department of Microbiology, Immunology and Molecular Genetics, University of California Los Angeles, Los Angeles, CA, USA*
[f] *Department of Molecular, Cell, and Developmental Biology, University of California Los Angeles, Los Angeles, CA, USA*
[g] *Ophthalmology Section, Greater Los Angeles VA Healthcare System, Los Angeles, CA, USA*

Received 17 May 2006; received in revised form 26 June 2006; accepted 28 June 2006

Abstract

Heat shock protein (HSP) plays an important role in the maintenance of neuronal survival during harmful conditions. Previously, we reported that metabolic stress induces HSP72 in retinal ganglion cells (RGCs) and protects against excitotoxicity, hypoxia and experimental glaucoma. To understand heat shock protein transcriptional mechanisms, we examined the cellular expression of heat shock factors 1 (HSF1) and 2 (HSF2) in the unstressed adult rat retina. Western blotting, immunohistochemistry and RT-PCR showed that mRNA and protein of HSF1 and HSF2 were present in the rat retina and predominantly expressed in RGC layer cells. Western blotting of dissociated RGC suspensions harvested with Thy-1 immuno-labeled magnetic beads confirmed that RGCs expressed HSF1, HSF2 and HSP72. Our findings suggest that both heat shock transcription factors 1 and 2 are linked to the heat shock response in retinal ganglion cells.

Keywords: Heat shock protein; Stress; Rat; Heat shock factor; Retina; Ganglion cell

The heat shock response is a well-conserved response to diverse environmental and physiological challenges, and results in the immediate induction of genes encoding molecular chaperones, known as heat shock proteins (HSPs) [14]. Families of HSPs are classified according to their molecular weights namely HSP100, HSP90, HSP70, HSP60, HSP40 and small HSP (approximately 20 kDa). These proteins function as chaperones under normal, developmental and stressful conditions [1], and they also play a role in disease pathological processes [7]. In the central nervous system, intracellular expression of HSP72 (an inducible form of the HSP70 family) has been demonstrated to protect neurons against heat shock, oxidative stress, apoptotic stimuli, excitotoxic insults, and ischemic-like conditions [4]. Neurons of transgenic mice expressing HSP72 [10,18] or those of rats injected with the herpes virus containing HSP72 genes also have been shown to be resistant to ischemia and seizures [25]. We previously observed that heat shock pre-conditioning increased the production of HSP72 in retinal ganglion cells (RGCs) *in vitro* and *in vivo*, and protected neurons against *N*-methyl-D-aspartate-mediated excitotoxicity and glaucoma [3,9,16]. These findings suggest that regulation of HSP72 is essential to the protection of retinal neurons against noxious insults.

In eukaryotic cells, the regulation of *hsp* genes requires the activation and translocation to the nucleus of a trans-regulatory protein, the heat shock factor (HSF), which recognizes modular sequence elements referred to as the heat shock element (HSE) located within the *hsp* gene promoters [13]. At least three HSFs (HSF1 to 3) have been isolated from the human, mouse and chicken genomes, while an additional factor, HSF4 has recently been described in human cells. HSF1 and HSF2 are believed to be the major members in HSF family and HSF3 is found in avian. The existence of multiple HSFs in vertebrates suggests

* Correspondence to: Department of Ophthalmology, Jules Stein Eye Institute, David Geffen School of Medicine at University of California Los Angeles, Room B-146, 100 Stein Plaza, Los Angeles, CA 90095-7000, USA.
Tel.: +1 310 206 7900; fax: +1 310 206 7773.
E-mail address: kwong@jsei.ucla.edu (J.M.K. Kwong).

0304-3940/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2006.06.070

that different HSFs mediate the response to various forms of physiological and environmental stimuli [11,17]. Recent reports indicated that the DNA binding activities of HSF1 and HSF2 were altered in cell-type- and stress-specific manner [15,19,22]. To implicate the induction of HSP as a therapeutic tool for optic neuropathies, it is fundamental to understanding the transcriptional pathways of HSP in retinal neurons and especially RGCs. Therefore, we performed (i) immunohistochemistry, RT-PCR and western blotting on unstressed rat retinas to examine the localization and expression of HSF1 and HSF2, and (ii) western blotting of dissociated RGC suspension harvested by Thy-1 antibody coated magnetic beads.

All experiments were approved by the Animal Research Committee of the University of California, Los Angeles and were performed in compliance with the Association for Research in Ophthalmic and Vision Research. Consistent with our animal studies [5,8,16], albino male Wistar rats weighing 250–300 g were used. Adult rats were kept in the animal room for 1 week before experiments, and allowed free access to food and water. The animal room was lit with fluorescent lights (330 lux) turned on at 3 a.m. and off at 3 p.m., and was maintained at 21 °C.

To examine the localization of HSFs in the retinas, fluorescence immunohistochemistry was performed as previously described [8]. Animals were deeply anesthetized with intramuscular injections of 0.8 ml/kg of a cocktail containing ketamine, xylazine, and acepromazine, and transcardially perfused with ice cold 4% paraformaldehyde in 0.1 M phosphate buffer. The enucleated eyeballs were fixed for 4 h, incubated with 30% sucrose overnight at 4 °C, and embedded in OCT compound (Sakura Finetec, Torrance, CA, USA). Ten-micrometer thick sections were obtained along the vertical meridian through the optic nerve head. After washing with PBS containing 0.1% Triton X-100, the sections were incubated with blocking serum solution, primary antibody against HSF1 (rat monoclonal at 1:150; Chemicon, Temecula, CA, USA) or HSF2 (rat monoclonal at 1:250; Lab Vision Corporation, Fremont, CA, USA) at 4 °C overnight, and secondary antibody. Anti-biotin antibody conjugated with Cy-3 (Sigma, St. Louis, MO, USA) and 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, OR, USA) were used for fluorescent labeling and nuclear counter-staining respectively. Retinal sections were examined with a fluorescence microscope (Axioplan, Carl Zeiss, Oberkochen, Germany) and imaged. For negative controls, retinal sections were incubated with blocking solution by replacing the primary antibody or with another species secondary antibody by replacing the original secondary antibody.

After euthanasia with inhalation of CO_2, the retinas were dissected immediately and the total retinal RNA was extracted with RNAzol B (Tel-Test, Friendswood, TX, USA) and purified with RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA). After quantification by spectrophotometry at 260 nm and integrity analysis with denaturing agarose gel electrophoresis (1% agarose, 2.2 M formaldehyde), retinal RNA was reverse transcribed to cDNA with SuperScript First-strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The oligonucleotide pairs were as follows: HSF1, 5′-CTGGTGCACTACACGGCTCA-3′(1151–1170) and 5′-GTTGTGCTGGCTTGACCTAG-3′ (1467–1448) (GenBank accession number X83094). HSF2, 5′-GTAAGCTTGTCCGCCTGGAA-3′ (1601–1620) and 5′-ATATGCCTAGTCAGCCAGCC-3′ (1918–1889) (GenBank accession number NM031694). Amplification conditions were as follows: hot start of 2 min at 95 °C; 30 cycles of denaturing (95 °C for 30 s), annealing (60 °C for 15 s), and extension (72 °C for 30 s); and a final extension of 7 min at 72 °C. The PCR products were separated by electrophoresis in a 2% agarose gel, visualized under UV light in the presence of ethidium bromide and photographed.

Four freshly dissected retinas were homogenized in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA) supplemented with protease and phosphatase inhibitor cocktails according to published procedures [4]. One microgram of protein from each sample was subjected to electrophoresis (Mini-Protean II system; Bio-Rad, Hercules, CA, USA) on 12% polyacrylamide gels and transferred to polyvinylidene fluoride membrane (Immobilon-P, Millipore Corporation; Bedford, MA, USA). After blocking with 5% non-fat powdered milk, the membrane was incubated with primary antibodies against HSF1 (1:5000), HSF2 (1:5000), HSP72 (1:5000; Stressgen Biotechnologies, Victoria, BC, Canada) or GFAP (1:10000; Sigma) overnight at 4 °C and followed by incubation with peroxidase-conjugated secondary antibodies. The signals were visualized using an ECL plus Detection Kit (Amersham Biosciences; England). Two independent experiments were performed ($n = 4$ each).

To confirm the expression of HSFs in RGCs, the procedures of RGC isolation using magnetic beads were adopted and modified [20,23]. Four freshly isolated adult rat retinas were dissociated in D-PBS without Ca^{2+} and Mg^{2+} containing 20 U/ml papain, 1 mM L-cystein and 0.005% DNase I (Worthington, Lakewood, NJ, USA) at 37 °C for 30 min. The retinas were gently titrated with 1 mL pipette in a solution containing D-PBS, 0.15% trysin inhibitor, 0.15% BSA (Roche, Indianapolis, IN, USA) and 0.005% DNase I. Single cells were obtained after centrifugation and re-suspended in D-PBS containing 0.1% BSA. Cells were purified using paramagnetic beads with specific antibodies (Dynal, Oslo, Norway) in sequential steps. Macrophages and adherent cells were removed by attachment to CD11b/c monoclonal antibody (BD Pharmingen, San Diego, CA, USA) coated beads. Cells were then selected with magnetic beads coated with the Thy-1.1 monoclonal antibody (Chemicon, Temecula, CA, USA). After multiple washes removing the non-adherent cells, the attached Thy-1 positive cells, referred as RGCs, were released by incubation in DNase buffer and then transferred to Neurobasal A medium (Invitrogen, Carlsbad, CA, USA) containing 0.8% BSA. RGCs were centrifuged and re-suspended in serum-free medium. Approximately 95% of cells were Thy-1 positive as estimated by immunocytochemistry (data not shown). Western blot analysis was performed on the cell suspension as described above. Two independent experiments were performed ($n = 4$ each). To visualize the localization of HSF1 and HSF2 in RGCs, retrograde labeling using Fluorogold (FG; Fluorochrome, Denver, CO, USA) were performed in rats as published elsewhere [6]. A Gelfoam (Upjohn, Kalamazoo, MI, USA) soaked with 6% Fluorogold was applied onto the surface