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Increased intraocular pressure alters the cellular distribution of HuR protein in retinal ganglion cells – A possible sign of endogenous neuroprotection failure

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ABSTRACT

The RNA-binding protein, HuR, modulates mRNA processing and gene expression of several stress response proteins i.e. Hsp70 and p53 that have been postulated to be involved in the pathogenesis of glaucoma, a chronic optic neuropathy leading to irreversible blindness. We evaluated HuR protein expression in retinas and optic nerves of glaucomatous rats and human primary open angle glaucoma patients and its possible impact on stress response mechanisms. We found that the cytoplasmic content of HuR was reduced more extensively in glaucomatous retinas than in optic nerves and this was linked with a declined cytoplasmic Hsp70 level and p53 nuclear translocation. In the optic nerve, the p53 content was decreased as a feature of reactive gliosis. Based on our findings, we conclude that the alteration in the HuR content, observed both in rat glaucoma model and human glaucoma samples, affects post-transcriptionally the expression of genes crucial for maintaining cell homeostasis; therefore, we postulate that HuR may be involved in the pathogenesis of glaucoma.

1. Introduction

Glaucoma is a group of progressive optic neuropathies that lead to irreversible loss of retinal ganglion cells (RGC); the disease can be characterized by several clinical symptoms with a common feature i.e. the specific pattern of the visual field loss [1,2]. Until now, various risk factors of glaucoma development have been identified, but the detailed biological basis of this disease has remained unclear [3,4]. Thus, one of the major goals of glaucoma research has been to elucidate the mechanism leading to RGC death. It has been postulated that the efficiency of cellular endogenous neuroprotective systems can be one of crucial factors affecting the RGC's apoptotic susceptibility [5,6].

In general, neurons are non-proliferating cells i.e. in mature individuals, the population of these cells should be sufficient to last the lifespan. Thus, it is crucial that these long-living cells possess specific mechanisms to protect them from both intracellular and environmental toxicity [7]. The regulation of gene expression in neurons, whose dendrites and axons can extend for long distances, is recognized as being a complex and dynamic process [8]. Changes in gene expression in response to cellular stress play an important role in endogenous physiological neuroprotective mechanism. In addition to the transcriptional control, there are crucial post-transcriptional mechanisms which can modulate gene expression by regulating protein expression level and localization to changing external conditions. Changes in RNA-binding proteins (RBPs) have been shown to associate with various neurodegenerative disorders at the post-transcriptional level [9–11]. ELAV/Hu is a well-known family of RBPs. The acronym ELAV (embryonic lethal abnormal vision) originates from the discovery that this

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gene is fundamental for the development and the maintenance of the visual system in the fruit fly *Drosophila* [12]. All ELAV members preferentially interact with 3′-untranslated regions of a subset of mRNAs containing adenylate/uridylate (AU)-rich elements (ARE) [13,14]. In vertebrates, HuR (Human antigen R, ELAVL1) is expressed broadly in multiple tissues, whereas HuB, HuC and HuD, grouped together as neuronal ELAV (nELAV), are the neuron-specific members of the ELAV family [15,16]. In particular, HuR protein positively regulates the expression of many well-known stress response genes (Hsp70, p53, SOD-1) [10,17–19], anti-inflammatory cytokines (IL-10) and pro-inflammatory factors (such as TNFα, IL-1, IL-6, IL-8) [20]. Hsp70 and p53 proteins are both potentially involved in neurodegeneration processes by regulating the cell cycle and the cellular response to proteotoxic and genotoxic stress [21,22].

We report here, for the first time, the connection between chronically elevated intraocular pressure and HuR protein content *in vivo* and HuR trafficking in retinal neurons, including RGC and the optic nerve of glaucomatous rats and corresponding changes in human primary open glaucoma samples. Since gene expression of the critical proteins that are involved in maintaining cellular homeostasis can be regulated by HuR, any alterations in this process can have detrimental effects on cell survival. Therefore, we postulate that HuR protein may be involved in the pathogenesis of glaucoma.

2. Materials and methods

2.1. Experimental glaucoma

All experiments involving animals have been approved by the Finnish National Animal Ethics Committee in the State Provincial Office of Southern Finland adherent to the European Communities Council Directive (86/609/EEC) and comparable to the guidelines published by the Institute for Laboratory Animal Research. Forty-four 16-week old male Wistar rats (Laboratory Animal Center, University of Eastern Finland, Kuopio, Finland) were deeply anesthetized with an intraperitoneal injection of a mixture of ketamine (50 mg/kg; Ketalar, Pfizer Oy Animal Health, Finland) and medetomidine (0.4 mg/kg; Domitor, Orion Oy, Finland) and intraocular pressure was increased unilaterally up to approximately 50 mm Hg, by intracameral injection of polystyrene microbeads, as previously described [23,24]. Ocular hypertension was monitored for 1 day, 1 week, 4 weeks or 8 weeks after the beads were injected. The fellow eye was used as a healthy control. IOP was measured using a laboratory tonometer (TonoLab, Icare, Finland).

2.2. Study groups and ocular tissues analyses

All 44 rats were sacrificed by CO_2 inhalation; eyes with approximately 6 mm stump of retrobulbar optic nerve were enucleated. In the 8-week group 26 animals were analyzed. Samples from 4 animals (retina, optic nerve – divided into proximal and distal stump) were preserved in RNA-later buffer (Qiagen, Hilden, Germany) for further gene expression analysis, tissues from another 6 animals were placed in Nuclear Extract buffer (Nuclear Extract kit, Active Motif, Carlsbad, CA, USA), then fractionated and processed for western blots. Two animals from this group were transcardially perfused with 100 ml of 0.9% NaCl, followed by perfusion with 100 ml of 4% paraformaldehyde solution using a half-perfusion method (with clumping main vessels below the level of the liver). Eyeballs with optic nerves were then enucleated, post-fixed and processed for immunohistochemistry. Eyes from six animals were used for Mass Spectrometry analysis. Eight animals were utilized for the glaucoma model evaluation in electron microscopy, retinal cell counting and western blots. Tissues from the animals in the other follow-up groups (i.e. 1 day, 1 week, 4 weeks – $n = 6$ animals per group) were analyzed by immunostainings.

2.3. Proteomic analysis by mass spectrometry

Retinas and optic nerves from 6 Wistar rats (6 healthy and 6 eight-week glaucoma eyes) were collected similarly as described above and processed for mass spectrometric analysis. Tissue from three animals were pooled together to obtain a sufficient protein amount. Retinas and optic nerves were homogenized, pH was adjusted, proteins were reduced, alkylated and digested sequencing grade modified trypsin (Promega, WI, USA). The trypsinized samples were purified with C18 silica microspin columns (The Nest Group, Southborough, MA, USA) and analyzed on a hybrid LTQ Orbitrap Elite Mass Spectrometer (Thermo Scientific, Rockford, IL, USA) coupled to an Easy nLCII (liquid chromatography) nanoflow system (Thermo Scientific, Rockford, IL, USA) via a nanoelectrospray ion source (Thermo Scientific, Rockford, IL, USA). The RAW files were analyzed using Thermo Scientific™ Proteome Discoverer™ 1.4 software connected to the SEQUEST® search engine. Spectra were searched against the Protein Knowledgebase (UniProtKB reviewed completed *Rattus norvegicus* database).

2.4. Cluster and gene ontology analysis

The hierarchical clustering of the identified proteins was constructed using Gene Cluster 3.0. The clusters were visualized with Tree View 1.1.6 and the matrix2png web server (http://www.chibi.ubc.ca/matrix2png/). Gene ontology (GO) enrichment analysis was performed with GOrilla and REVIGO for subsequent visualization [25–27]. The control sample list (healthy retinal homogenate) was used as a background to search enriched GO terms with respect to biological process, molecular function and cellular components. The *p*-values associated with each annotation terms were $p < 0.001$. Each rectangle in the TreeMap represents a single cluster and the size relates to the p-value. The joined cluster shows related terms.

2.5. Western blots

Tissue extracts for western blotting – nuclear and cytoplasmic fractions (from retinas and optic nerves), were prepared using the Nuclear Extract Kit (Active Motif), according to the manufacturer's instructions. To improve sensitivity of protein detection in the retina and the optic nerve, we pooled tissues from two animals together. In this way, there were three biological samples that were replicated two times. A total of 10–30 μg of protein concentrate was separated on 10% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham, GE Healthcare, UK) at 17 V, 80–120 mA overnight. After transfer, the nitrocellulose membranes were blocked with 3% milk/0.3% Tween – PBS buffer and incubated with the following primary antibodies: mouse ELAVL1/HuR (dilution 1:10,000; Santa Cruz, Dallas, TX, USA), rabbit p53 (dilution 1:10,000; Sigma Aldrich, Saint Luis, MO, USA), mouse β3-tubulin (dilution 1:10,000; Abcam, Cambridge, UK), mouse GAPDH (dilution 1:10,000; Abcam), rabbit Hsp70 (dilution 1:1000; Enzo Life Sciences, Farmingdale, NY, USA). The nitrocellulose membranes signals were detected by chemiluminescence. Protein bands were quantified using ImageJ software (http://imagej.nih.gov/ij/).

2.6. Quantitative PCR analysis

Rat tissue samples were stored in RNAlater (Qiagen) to stabilise the RNA which was then extracted using RNAeasy mini kit (Qiagen) and treated with DNAse (DNA free, Ambion, Austin, TX, USA). cDNA was synthetized by reverse transcription using M-MuLV (Fermentas, Hanover, MD, USA). Amplification and detection were performed in ABI Prism 7500 (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems) and following primers: *Elavl1* (NM_001108848.1 *Rattus norvegicus* ELAV like RNA binding protein 1), - forward. *5′-TCGGGATAAAGTTGCAGGACA-3′*, reverse. *5′-TGATGATTCGCCCAAA CCGA-3′*; *GAPDH* (NC_005103.4 *Rattus norvegicus* glyceraldehyde-3-