Quantitative Proteomic Analysis of Glaucomatous Rat Retinal Ganglion Cells

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SUPPORTED BY Research to Prevent Blindness

National Eye Institute, NATIONAL INSTITUTES OF HEALTH

Abstract

Purpose: We seek to quantify proteome changes in retinal ganglion cells (RGCs) associated with elevated intraocular pressure for insights into the molecular mechanisms of glaucoma pathology.

Methods: Experimental glaucoma was laser-induced unilaterally in rats. Retinas were isolated after elevated intraocular pressure was demonstrated for 4-6 weeks and RGCs were rapidly isolated by panning. Proteins were extracted from RGCs in SDS, digested with trypsin, peptides were labeled with iTRAQ tags, glucomatous and control digests combined in equal amounts and fractionated by strong cation exchange chromatography. Proteins were identified by LC MS/MS using a Mascot search engine and the Swiss-Protein sequence database. Relative protein quantification from iTRAQ labeling utilized code written in the statistical program R. Bioinformatic analysis was performed with GeneGo Metacore and Ingenuity Pathways Analysis 5.0.

Results: Relative protein quantitation has been obtained from RGCs pooled from 22 glaucomatous and 22 contra lateral control rat eyes. The LC MS/MS analyses yielded quantification of 273 proteins from two or more unique peptides per protein. Proteins with ratios ≥ 2 SD above the mean included aconitase hydratase, endoplasmic reticulum protein ERp29 and poly(U)-binding-splicing factor PUF60. Proteins with ratios ≥ 2 SD below the mean included scaffold attachment factor B1, prothymosin alpha, and calreticin. Pathway analysis suggest alterations in several networks including those involving cell death, immune response, RNA post-translational modification, gene expression and cellular assembly and organization.

Conclusions: These preliminary results demonstrate the feasibility of quantitative proteomic analysis of rapidly isolated RGCs. More proteins were detected in glaucomatous RGCs as decreased rather than increased relative to control RGCs, suggesting suppressed biosynthetic activity and downregulation of select functional networks in challenged neurons. Further studies with multiple analyses are required to identify disease mechanisms.

Commercial Relationships: J.S. Crabb, None; X. Yuan, None; G. Dvoriantchikova, None; D. Ivanov, None; V.I. Shestopalov, None; J.W. Crabb, Allergan Inc. C.

Support: NEI-EY07961, EY10147, EY14229, EY15538, Ohio SRTT 05-29, RPB Challenge Grant to Cole Eye Institute, RPB Sr Investigator Award, RPB grant to Bascom Palmer Eye Institute, and a Steinbach Award

Introduction

Quantitative proteomic analysis of rat retinal ganglion cells (RGCs) was pursued for insights into the molecular mechanisms of glaucoma. Laser-induced experimental glaucoma was used to generate ocular hypertension in one eye and the contra lateral eye was used as a control. RGCs were isolated by immunopanning. iTRAQ quantitative mass spectrometric analyses have yielded preliminary proteomic results from a very small amount of protein.

Methods

Glaucoma Animal Model. Experimental glaucoma due to chronic ocular hypertension was induced in Wistar rats (9 months old, 250-300 g) by unilateral laser photocoagulation of anterior angle tissue (1). Contralateral eyes were used as controls. IOP was monitored in both eyes using a TONOLAB retractable tonometer. A second laser treatment was performed 1-2 weeks after the initial treatment if IOP elevation was not sustained. Retinas were harvested 4-6 weeks post initial treatment from animals (n = 22) with integral IOP in the range of 300-350 mm Hg-days.

Isolation of Retinal Ganglion Cells (RGCs). RGCs were isolated from rat retina by immunopanning using a modification of published methodology (2). Briefly, whole retinas were incubated in the digestion solution containing papain (0.7 U/ml; Worthington, Lakewood, NJ) and L-cysteine (0.3 mg/ml; Sigma, St. Louis, MO) in Neurobasal-A medium (Invitrogen, CA) for 30 min (37°C; CO2 incubator), rinsed twice in 5 ml of medium, triturated in 4 ml of the medium containing 1:50 B27 supplement (Invitrogen, CA). Macrophage and endothelial cells were removed from the cell suspension by panning with anti-macrophage antiserum (Axell Accurate Chemical Corp, Westbury, NY). RGCs were specifically bound to the panning plates containing anti-Thy1.1 antibody, and unbound retinal cells were removed by washing with BSS media. RGCs were then released by trypsin incubation. Approximately 22 x 103 RGCs/retina were obtained from laser-treated eyes and 28 x 103 RGCs/retina from control eyes.

Sample Preparation for Proteomic Analysis. All isolated cells from n = 22 animals were combined into two pooled samples, yielding one glaucomatous and one control preparation. RGCs were homogenized in 100 mM triethylammonium bicarbonate (TEAB) containing 2% SDS and soluble protein was precipitated with four volumes of acetone and redissolved in 500 mM TEAB containing 0.1% SDS. Protein was quantified by phenylthiocarbamyl amino acid analysis (3) and each pool found to contain ~4 µg protein. Protein was reduced with tris-(2-carboxyethyl) phosphine (TCEP), cysteines alkylated with methyl methanethiosulfonate and the protein was digested with trypsin (4).

iTRAQ labeling and SCX Chromatography. Glucomatous RGC tryptic peptides were labeled with iTRAQ tag 117 and mixed with an equal amount of control peptides labeled with iTRAQ tag 115. The peptide mixture was fractionated by strong cation exchange (SCX) chromatography and fractions were collected at 1 min intervals and vacuum dried.

Protein Identification. SCX fractions were further fractionated on a 75 µm x 15 cm PepMap100 C18 column (3 µm particle size, 100 Å pore size, Dionex) using a Ultimate 3000 LC system (LC Packings), aqueous trifluoroacetic acid/acetonitrile solvents (5). The eluted peptides were mixed 1:2 (v/v) with matrix [4.5 mg/ml a-cyano-4-hydroxycinnamic acid (CHCA) in 0.56 mM ammonium citrate] and were spotted with a Probot target spotter (LC Packings) on a MALDI target at 30s interval for 36 min. Samples were analyzed with a model 4800 MALDI TOF TOF mass spectrometer (Applied Biosystems) and identified using GPS Explorer 3.6 software (Applied Biosystems), Mascot 2.1, and the Swiss Protein database version 56.2. Database searches were restricted to tryptic peptides of rat sequences (7180 sequence entries) and mouse sequences (15980 sequence entries) with 2 missed tryptic cleavage sites allowed, precursor ion mass tolerance = 0.8 Da, and fragment ion mass tolerance = 0.8 Da. Fixed modifications were selected for iTRAQ N-terminal and e-Lys modifications and S-methyl Cys from SCX fractions. Met oxidation was selected as a variable modification.

Protein Quantitation. Quantitation required a minimum of two unique peptides per identified protein, a minimum 95% confidence interval for the Mascot protein score and peptide Mascot ion scores ≥ 25. After deleting outliers and multiple copies of the same peptide, peptide ratios were normalized and SD and p values were determined. Protein ratios were determined in log space and transformed for reporting.

Bioinformatic Analyses. Functional classification of quantified proteins in rat ganglion cell was performed with The Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System (available at www.pantherdb.org). For PANTHER analysis, SwissProt accession numbers were converted to Entrez gene identification numbers using UniProt (available at http://www.uniprot.org). Analysis of possible pathway networks involving the quantified proteins in ganglion cells was performed with Ingenuity Pathways Analysis 7.0 (Ingenuity® Systems).

Results

Table 1
Mean IOP per Experimental Glaucoma Group

Glaucomatous Group	Number Animals	Mean IOP Integral Hg-days
1	6	382 ± 43
2	6	387 ± 49
3	6	369 ± 59
4	4	338 ± 41

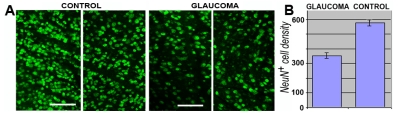


Figure 1. RGCs in Glaucomatous vs. Control Rat Retinas.
Unilateral laser photocoagulation of anterior angle tissue was used to induce elevated IOP in one eye and the contralateral eye was used as a control. **A.** Representative micrographs are shown of control and glaucomatous rat retinas taken at the same eccentricity from the optic nerve. Neurons in the ganglion cell layer (GCL) are labeled with anti-NeuN antibodies (green). Bar, 50 mm. **B.** RGC loss approximated 40% in experimental relative to control retinas (n=17) based on quantification of the NeuN-positive cells in the ganglion cell layer. Mean IOP for the experimental animals is shown in Table 1.

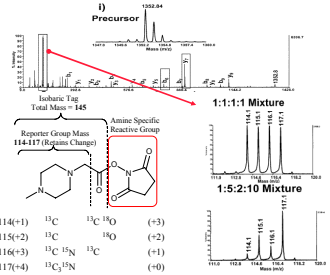


Figure 2. iTRAQ Quantitative Proteomics Technology.
iTRAQ = isobaric tags for relative and absolute quantitation

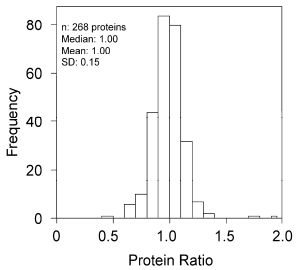


Figure 3. Distribution of Glaucomatous/Control Protein Ratios from Rat RGCs.
Quantitative proteomic analysis of glaucomatous rat RGCs was performed using iTRAQ technology, two dimensional chromatography, and MALDI TOF TOF tandem mass spectrometry. A total of 268 proteins were quantified with two or more peptides from a small amount of RGC protein (~8 µg total). The distribution of protein ratios for the 268 quantified proteins is shown, along with the median and mean values and the standard deviation (SD). The relative abundance of all proteins quantified with two or more peptides is presented in Table 2.

The above distribution of protein ratios is near-to-normal but with some spread below 1.0, suggesting reduced abundancy of several proteins in glaucomatous relative to control RGCs.

Table 2
Relative Abundance of Proteins in Glaucomatous Rat Retinal Ganglion Cells

Total Proteins Quantified = 268; Median protein ratio= 1.00; Mean protein ratio= 1.00; SD=0.15

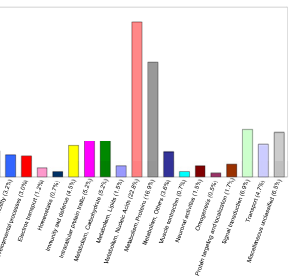
SwissProtein Accession	Protein	Protein Ratio Glaucoma/Control	SD	P value	Unique Peptides	Sequence Coverage
P07942	Aconitate hydratase, mitochondrial	1.95*	0.58	NA	2	3.3%
P52555	Endoplasmic reticulum protein ERp29	1.79*	0.04	NA	2	9.6%
Q9WV25	Poly(U)-binding-splicing factor PUF60	1.53*	0.02	NA	2	2.7%
P41516	DNA topoisomerase 2-alpha	1.28	0.24	NA	2	1.2%
P11030	Acyl-CoA-binding protein	1.26	0.07	NA	2	18.4%
P06376	Acylphosphatase-1	1.25	0.29	NA	2	20.2%
P84089	Enhancer of rudimentary homolog	1.23	0.12	NA	2	16.3%
P11232	Thioredoxin	1.21	0.02	NA	2	21.0%
Q921F4	Heterogeneous nuclear ribonucleoprotein L-like	1.19	0.02	NA	2	2.5%
P06685	Sodium/potassium-transporting ATPase subunit alpha-1	1.18	0.03	NA	2	2.1%
P81155	Voltage-dependent anion-selective channel protein 2	1.23	0.17	0.034	6	24.1%
P07943	Aldose reductase	1.20	0.02	0.006	3	8.5%
P62989	Ubiquitin	1.20	0.12	0.009	7	77.6%
Q9ER34	Aconitate hydratase, mitochondrial	1.95*	0.58	-	2	3.3%
P52555	Endoplasmic reticulum protein ERp29	1.79*	0.04	-	2	9.6%
Q9WV25	Poly(U)-binding-splicing factor PUF60	1.53*	0.02	-	2	2.7%
P41516	DNA topoisomerase 2-alpha	1.28	0.24	-	2	1.2%
P11030	Acyl-CoA-binding protein	1.26	0.07	-	2	18.4%
P06376	Acylphosphatase-1	1.25	0.29	-	2	20.2%
P84089	Enhancer of rudimentary homolog	1.23	0.12	-	2	16.3%
P11232	Thioredoxin	1.21	0.02	-	2	21.0%
Q921F4	Heterogeneous nuclear ribonucleoprotein L-like	1.19	0.02	-	2	2.5%
P06685	Sodium/potassium-transporting ATPase subunit alpha-1	1.18	0.03	-	2	2.1%

Protein ratios (Glaucoma/Control), SD, p values, unique peptides and % sequence coverage for proteins exhibiting ratios ≥ mean + 1 SD with p values < 0.05. Total Proteins Quantified = 268, Median protein ratio= 1.00, Mean protein ratio= 1.00, SD=0.15, Table 1). SD = Standard deviation; p values are from two sided T tests for the null hypotheses that the peptide mean = 1.0. Asterisks denote ratios ≥ 2 SD above the mean.

Table 3
Abundant Proteins in Glaucomatous Rat Retinal Ganglion Cells

SwissProtein Accession	Protein	Protein Ratio Glaucoma/Control	SD	P value	Unique Peptides	Sequence Coverage
Abundant in Glaucomatous RGCs						
P81155	Voltage-dependent anion-selective channel protein 2	1.23	0.17	0.034	6	24.1%
P07943	Aldose reductase	1.20	0.02	0.006	3	8.5%
P62989	Ubiquitin	1.20	0.12	0.009	7	77.6%
Possibly Abundant in Glaucomatous RGCs						
Q9ER34	Aconitate hydratase, mitochondrial	1.95*	0.58	-	2	3.3%
P52555	Endoplasmic reticulum protein ERp29	1.79*	0.04	-	2	9.6%
Q9WV25	Poly(U)-binding-splicing factor PUF60	1.53*	0.02	-	2	2.7%
P41516	DNA topoisomerase 2-alpha	1.28	0.24	-	2	1.2%
P11030	Acyl-CoA-binding protein	1.26	0.07	-	2	18.4%
P06376	Acylphosphatase-1	1.25	0.29	-	2	20.2%
P84089	Enhancer of rudimentary homolog	1.23	0.12	-	2	16.3%
P11232	Thioredoxin	1.21	0.02	-	2	21.0%
Q921F4	Heterogeneous nuclear ribonucleoprotein L-like	1.19	0.02	-	2	2.5%
P06685	Sodium/potassium-transporting ATPase subunit alpha-1	1.18	0.03	-	2	2.1%

Protein ratios (Glaucoma/Control), SD, p values, unique peptides and % sequence coverage for proteins exhibiting ratios ≥ mean + 1 SD with p values < 0.05. Total Proteins Quantified = 268, Median protein ratio= 1.00, Mean protein ratio= 1.00, SD=0.15, Table 1). SD = Standard deviation; p values are from two sided T tests for the null hypotheses that the peptide mean = 1.0. Asterisks denote ratios ≥ 2 SD above the mean.


25
20
15
10
5
0
%
Accretion (1.2%)
Cell cycle (5.0%)
Cell Death and Survival (5.7%)
Cell Morphology and Differentiation (3.7%)
Cellular Movement (2.0%)
Connective Tissue Disorders (0.7%)
Developmental Disorder (2.2%)
Endocrine Disorders (0.7%)
Gene Expression (18.5%)
Hematological System Dev. and Function (0.7%)
Hematological Disease (0.7%)
Hematological System Dev. and Function (0.7%)
Immune Response (1.5%)
Immunological Disease (1.5%)
Inflammatory Response (1.5%)
Lipid Metabolism (1.5%)
Macromolecular Synthesis (1.5%)
Metabolic Disease (2.2%)
Molecular Transport (1.5%)
Nucleic Acid Metabolism (2.2%)
Protein Synthesis (6.6%)
Protein Trafficking (2.2%)
Protein Synthesis (6.6%)
Protein Trafficking and Modification (1.5