Proteomics Analyses of Human Optic Nerve Head Astrocytes Following Biomechanical Strain*§

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We investigate the role of glial cell activation in the human optic nerve caused by raised intraocular pressure, and their potential role in the development of glaucomatous optic neuropathy. To do this we present a proteomics study of the response of cultured, optic nerve head astrocytes to biomechanical strain, the magnitude and mode of strain based on previously published quantitative models. In this case, astrocytes were subjected to 3 and 12% stretches for either 2 h or 24 h. Proteomic methods included nano-liquid chromatography, tandem mass spectrometry, and iTRAQ labeling. Using controls for both stretch and time, a six-plex iTRAQ liquid chromatography- tandem MS (LC/MS/MS) experiment yielded 573 proteins discovered at a 95% confidence limit. The pathways included transforming growth factor β1, tumor necrosis factor, caspase 3, and tumor protein p53, which have all been implicated in the activation of astrocytes and are believed to play a role in the development of glaucomatous optic neuropathy. Confirmation of the iTRAQ analysis was performed by Western blotting of various proteins of interest including ANXA 4, GOLGA2, and αB-Crystallin. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.012302, 1–17, 2012.

Glaucoma is the world’s most common neurodegenerative disease, affecting an estimated 60 million people, double the number affected by all other neurodegenerative diseases combined. It is also the second leading cause of blindness worldwide (1). Primary open angle glaucoma, the most common type of glaucoma, can be characterized by the slow and irreversible apoptotic death of retinal ganglion cells, a unique optic nerve neuropathy and loss of visual function (2). Intraocular pressure (IOP) is a major risk-factor (3, 4) for the development of glaucoma, and reducing the IOP has been shown to be unequivocally beneficial in the clinical management of patients with the disease (4–6). Nickells (7) proposed a 5-stage model of glaucoma that unifies much of the clinical, animal, and cell based research. The primary stage is described as the “elevation of IOP and the activation of optic nerve glia in the lamina cribrosa” and includes disruption of both retrograde and anterograde axonal transport, including neurotrophins and motor proteins. The research presented here is intended to contribute to our knowledge of stage 1 of this disease, the “activation of the optic nerve glia in the lamina cribrosa” (7).

Astrocytes are the cell type of interest in this study as they are the major glial cell within the optic nerve head (ONH), providing a supportive role to the surrounding axons, while communicating with connective tissues and surrounding blood vessels (8). Normally the astrocytes remain “quiescent,” but following insult through injury or disease, they become reactive and can either reduce or exacerbate the damage to the neural tissue (9). They support the tissue through the release of neurotrophic factors and antioxidants, and through the degradation of abnormal extracellular protein deposits (10). The role they play in the degeneration of surrounding tissue is believed to occur by release of reactive oxygen species, proteases, cytokines, and nitric acid (11–17) for review see (18–22)). Glial fibrillary acidic protein (GFAP) has been shown to be up-regulated with astrocyte activation (23), and is associated with an increase in cell surface molecules important to cell-cell interactions, as well as cell adhesion substrates, cytokines, and growth factors (8, 17, 24).

To evaluate the effects of IOP on ONH biology, understanding the forces and deformations experienced by cells in the ONH is of the utmost importance. This is problematic as the tissue of primary interest, the laminar cribrosa (LCr), is small,
relatively inaccessible, and difficult to visualize. Moreover, it is a relatively compliant (mechanically weak) tissue that is surrounded by the much stiffer sclera, making it difficult to isolate the mechanical properties of the LCr. Our research (25–36), and that of others (37–44) has therefore applied finite element modeling to better understand the biomechanical environment within the ONH. We know that astrocytes, and other cells, are sensitive to mechanical stretch, and that the viability of retinal ganglion cells depend on normal astrocyte function (24, 45–48).

From these numerical models, we developed cell culture models to replicate the conditions experienced by astrocytes within the LCr. In these models we mimic the in vivo biomechanical environment in the LCr by growing human ONH astrocytes on flexible, silastic membranes and subjecting the cells to deformation. A similar approach has been used previously on LCr cells (48, 49). Other studies have analyzed the protein regulation of cells from the ONH using hydrostatic pressure (45, 50–53). However, this is the first time that equi-axial stretch has been applied to human ONH astrocytes. Lei et al. (54) recently investigated the effects of hydrostatic pressure and the resulting changes in oxygen tension on cell migration, morphology, and α-tubulin architecture. They reported that an increase in hydrostatic pressure had no effect, and that the biological effects previously reported were most likely artifacts caused by hypoxia within the medium. We are confident that our approach of inducing biomechanical strain using equi-axial stretch is a more realistic model of the conditions found within the human lamina cribrosa. For proteomics, the proteins from the cell lysate were analyzed in collaboration with the Ontario Cancer Biomarker Network (OCBN). Previous eye related proteomic research have been reviewed by Steely and Clark (55) and Tezel (14). Steely and Clark (56) characterized the human trabecular meshwork (TM) proteome through the use of a transformed TM cell line, which was compared with healthy cells. Zhao et al. (57) and Fuchshofer et al. (58) investigated the role of TGF-β, proposing its importance in the pathogenesis of glaucoma. Bhatthacharya et al. (53) found a positive link between the presence of PAD2 and glaucoma and in more recent research this group has outlined the occurrence of retinal deamination (59, 60), further confirming the post-translational effects and their role in glaucoma. Tezel and colleagues (14, 61, 62) have used proteomics to determine the role of oxidative stress on retinal proteins in glaucoma, the influence of hemoglobin expression and regulation, (16) and the importance of the complement pathway (62). Tezel (14) has recently proposed a unifying theory of oxidative stress and its importance to the immune response in the pathogenesis of glaucoma.

We present the total protein analysis of ONH astrocytes, stressed in a manner similar to that modeled to occur following raised IOP, with the ultimate goal of better understanding astrocyte activation and how this may lead to the loss of retinal ganglion cells. Proteins of interest are isolated and presented based on gene ontology (GO) analysis, including the role that they play in relating pathways from physical stress to transcriptional changes.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The LCr from 4 healthy human donors (Eye Bank of Canada, Ontario Division) were dissected into explants and grown in Dulbecco Modified Eagle Medium (DMEM) Nutrient Mixture F12 (4 mM l-glutamine; 1/100,000 insulin; 1.5 g/L sodium bicarbonate; 10% FBS; penicillin/streptomycin) until confluent in accordance with the human biosafety requirements of the University Health Network, Toronto, Canada. The astrocytes were isolated from other cell types using a technique previously described (63). Briefly, explants were split into serum-free astrocyte growth media (AGM -serum, Lonza, Switzerland) for a period of 2 weeks, when the media was replaced with Dulbecco Modified Eagle Medium /F12. The cells were grown to confluence and split in this media until there were enough viable cells to conduct an experiment. A fourth cell line was grown to be used for validation purposes using Western blotting. Cultures were maintained in sterile incubators at 37 °C and 5% CO2, and media was changed twice a week. Morphologically, the astrocytes were cultured in a monolayer and were similar to those previously reported (23, 64, 65). See Fig. 1 for cell characterization and morphology. All cultures are of at least 95% purity based on morphology and staining.

**Immunohistochemistry**—Cells from each cell line were grown on 35-mm plates after the third passage and allowed to grow to confluence after which they were washed twice with Dulbecco’s Phosphate Buffered Saline (½ Mg and Ca). The cells were then fixed in formalin, permeabilized in Triton-X and the specific primary and secondary antibodies. Primary human astrocytes were characterized by positive staining for glial fibrillary acidic protein (GFAP), desmin, vimentin, S-100, neural cell adhesion molecule (NCAM), and negative staining for A2B5, α-smooth muscle actin (α-smA) and Pax-2. (Fig. 1).

**Stretch**—For the stretch experiments, cells at the fourth passage were seeded onto 6-well flexcell plates (Flexcell International Corporation, Hillsborough, NC) and allowed to grow to confluence. Using the Flexercell® Tension Plus FX-4000T system, a programmable amount of equi-axial strain was applied to the cells through the use of a vacuum pump and a custom base-plate. The astrocytes were seeded onto pre-coated collagen type IV 6-well plates, which were additionally coated with collagen type I (Rat Tail Collagen, BD Biosciences, Franklin Lakes, NJ). Four plates per experiment were stretched whereas four control plates were placed in the same incubator. All cells were serum deprived for 24 h prior to stretching, which was performed at a 1 Hz cycle of 0% to 3%, or 0% to 12%, for either 2 h or 24 h. This gave rise to a total of six experimental conditions for each cell line.

**Protein Isolation**—Total protein was isolated from three experimental cell lines and combined to be used in proteomics analysis through the use of a radioimmunoprecipitation assay buffer. A volume of radioimmunoprecipitation assay buffer was added to each well (300 μl) and allowed to sit at room temperature for 15 min. The six wells from each plate were scraped down, combined into an Eppendorf tube, aspirated, and centrifuged at 10,000 rpm for 10 min. The cleared protein lysate was then prepared using the Total Protein Clean-up Kit (Norgen Biotek Corp., Thorold, ON). The protein required for the western validation was collected through the use of a RPN/Protein purification kit (Norgen), and kept at ~8 °C until required.

**Proteomics Analysis**—Proteomic screening was performed by the Ontario Cancer Biomarker Network (OCBN, Toronto, ON), using six astrocyte cell lysates; a 2 h control, a 24 h control, a 12% for 2 h stretch, a 12% for 24 h stretch, a 3% for 2 h stretch, and a 3% for 24 h stretch (each lysate was a combination of the respective treatment from the three cell lines).
Digestion and Labeling—Protein was extracted from cell lysates using a Norgen Total Protein Clean-up kit. The concentration was determined using a micro-BCA assay kit (Thermo Scientific). One hundred micrograms of protein from each condition was processed for isobaric Tag for Relative and Absolute Quantitation (iTRAQ) (66, 67) labeling. Briefly, the proteins were denatured, reduced, alkylated, trypsin digested, and then labeled with the appropriate iTRAQ tags. After labeling, the 100 µg aliquots were pooled.

Strong-Cation Exchange (SCX) Chromatography—Each pooled sample was fractionated using SCX chromatography and a Thermo BioBasic SCX column, 0.2 mm internal diameter and 10 cm long. Each sample was diluted with the loading buffer (15 mM KH2PO4 in 25% acetonitrile, pH 3.0) to a total volume of 2 ml and the pH adjusted to 3.0 with phosphoric acid. Samples were filtered using a 0.45 µm syringe filter (Millipore, Canada) before loading onto the column. Two milliliters of diluted sample were injected into the SCX system. Separation was performed using a linear binary gradient where solvent A is 98% H2O: 2% CH3CN and 0.1% formic acid and solvent B is 2% H2O : 98% CH3CN and 0.1% formic acid, and at a flow-rate of 300 nL/min with a 60 min gradient to 30% B. The equivalent of 2 µg of protein was injected.

Reversed-Phase Chromatography—The Nano LC-Ultra (Eksigent Technologies) consists of a trap column (300 µm ID) and an analytical column (75 µm ID) packed with 5 µm, 300Å Zorbax SB-C18 beads. The analytical column is made at OCBN. Separation was performed using a linear binary gradient where solvent A is 98% H2O: 2% CH3CN and 0.1% formic acid and solvent B is 2% H2O : 98% CH3CN and 0.1% formic acid, and at a flow-rate of 300 nL/min with a 60 min gradient to 30% B. The equivalent of 2 µg of protein was injected.

LC/MS/MS—The eluant from the Nano LC was coupled to a quadrupole time-of-flight mass spectrometer (QSTAR® Elite, AB Sciex, Foster City, CA), through an electrospray ionization source equipped with a 15 µm ID emitter tip, for use in a single-run iTRAQ analysis. After each survey scan, from m/z 400 to m/z 1500, three of the most intense ions with charge state 2 to 4 were selected for MS/MS analysis. These ions were then placed in a dynamic exclusion list for 3 min in order to avoid further selection of the same ions. For this proposed project, each protein will be quantified by a minimum of two peptides and each peptide will be monitored by a minimum of three transitions. The iTRAQ workflow on Analyst® QS software was employed.

Western Blotting—The protein was purified using the Total Protein/RNA Clean-up Kit (Norgen Biotek Corp., Thorold, ON). The targeted proteins were visualized using Western Blotting. Fig. 1. This characterization panel shows that these cells are astrocytes through positive staining for the intermediate filaments GFAP, desmin and vimentin, S-100, the paired box gene Pax-2, and the neural cell adhesion molecule (NCAM). Negative staining for A2B5, and a-smA is also shown. (40 × magnification on a Zeiss Axioplan deconvolution microscope).
proteins were ANXA 4 (Proteintech Group, Inc., rabbit anti-human polyclonal, 1:2000), GOLGA2 (Abcam, Cambridge, MA; rabbit monoclonal, 1:2000) and αB-Crystallin (Crystallin, Abcam, mouse monoclonal, 1:750). Equal volumes of protein are resolved on SDS-PAGE gels and then transferred to polyvinylidene difluoride membrane by electrophoretic transfer at 4 °C. Membranes were blocked with 3% bovine serum albumin in PBST and antibodies were diluted in 1% bovine serum albumin in PBST. Incubation with the primary antibody proceeded overnight at 4 °C. After washing, blots were incubated with HRP-conjugated secondary antibody incubated for 1 h at room temperature (Cedarlane’s anti-rabbit IgG HRP (Cat # 12–348; 1:1000) or anti-mouse IgG HRP (Cat # 12–349; 1:2000)). Antigens were visualized using the West Pico System (Pierce, Waltham, MA) and the Bio-Rad Fluor-S Max Multimager. Densitometry was performed using both ImageJ and Quantity One Software (Fig. 8) and the bands were equilibrated to the protein concentration in the samples.

Data Processing and Analysis—Relative quantification and protein identification were performed with the ProteinPilot™ software version 2.0 (AB SCIEX) using the Paragon™ algorithm as the search engine (68). Each MS/MS spectrum was searched against a concatenated forward and reverse database of human protein sequences containing >500,000 sequence entries comprising ~190 million amino acids abstracted from ~200,000 references (Swiss-Prot, 22/07/2008). The search parameters allowed for 8-plex iTRAQ, QSTAR Elite ESI, trypsin digestion, cysteine modification by methyl methanethiosulfonate, homo sapiens, and biological modifications programmed in the algorithm (which include phosphorylations, amidations, and semitryptic fragments). The detected protein threshold (unused protscore [confidence]) in the software was set to 0.05 to achieve 10% confidence, and identified proteins were grouped by the ProGroup algorithm (AB SCIEX) to minimize redundancy. The bias correction option was executed (68). Proteins without quantitative information, i.e. only one iTRAQ ratio, were deleted from the list of identified proteins. Differentially expressed proteins were defined as those showing an absolute fold-change of at least 1.5 relative to time-matched controls. Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, www.ingenuity.com, version 8.7) was used to determine pathways and functions implicated in stretching the cells for the prescribed amount of time. For each condition, the IPA Core Analysis feature was used to construct molecular interaction networks based on relationships between observed proteins and with other molecules, as annotated in the Ingenuity Knowledge Base. The resulting networks were then merged (using the Merge Networks feature) through the introduction of additional relationships. This analysis also provided a mapping of observed proteins to known cellular functions and processes; a p value based on a right-tailed Fisher Exact Test was associated with each functional category found to be enriched in the expression data set. Gene Ontology categorizing was also used on the resulting proteins. This type of analysis allowed for the grouping of proteins into relevant functional groups that are pertinent to our research.

Fig. 2. ROC plot analysis indicating a high number of true positives and a relatively low level of false positives. Note the unequal scales on each axis. This demonstrates that the samples had a high degree of sensitivity and specificity.

Fig. 3. Values (-log (P)) representing trends in major protein pathways discovered by Ingenuity pathway Analysis. A, shows the top functional group for the 3% 2 h stretch, metabolic activity. B, shows the top group for 3, 24 h, which was cellular activity and organization. C, shows cell cycle was at the highest level in the 3% stretch for 24 h, whereas DNA replication, recombination and repair was the highest at the 12% for 24 h stretch.
### Table I

Top proteins differentially expressed through the 3% 2 hour stretch and time parameters found within IPA networks. Gene symbol and accession are listed with the protein name. The number of peptides found, and confidence levels for those peptides are listed. The percent coverage (95%) (%Cov95) is calculated by dividing the number of amino acids of peptides identified with 95% confidence by the total number of amino acids in the protein.

| Gene Symbol | Accession | Protein Name | Number of Peptides | Peptide Conf. | Cellular Location | %Cov95 | Fold Ratio |
|-------------|-----------|--------------|--------------------|---------------|------------------|--------|-----------|
| CALML3      | P27482    | Calmodulin-like 3 | 1                  | 99            | Cytoplasm        | 30.2   | 3.015     |
| TMSL3       | A8MW06    | Thymosin-like 3  | 1                  | 91            | Unknown          | 70.5   | 2.597     |
| TALDO1      | P37837    | Transaldolase    | 2                  | 76, 86        | Cytoplasm        | 54     | 2.45      |
| YWAH5       | P63104    | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polyopeptide | 7 | 86 to 99 | Cytoplasm | 82.9 | 2.223    |
| CALM1       | P62158    | Calmodulin      | 3                  | 99            | Nucleus          | 51.7   | 2.04      |
| TUBB3       | Q13509    | Tubulin beta-3 chain | 4                | 99            | Cytoplasm        | 69.8   | 2.03      |
| S100A13     | Q99584    | Protein S100-A13 | 1                  | 99            | Cytoplasm        | 45.9   | 2.0       |
| ANXA4       | P09525    | Annexin A4      | 1                  | 99            | Plasma membrane  | 42.0   | 1.99      |
| RPS12       | P25398    | 40S ribosomal protein S12 | 1 | 99 | Cytoplasm | 28.8  | 1.81      |
| TFIP11      | Q9UBB9    | Tufefin-interacting protein 11 | 1 | 99 | Cytoplasm | 25.8  | 1.8       |
| LDHAL6B     | Q9BYZ2    | L-lactate dehydrogenase A-like 6B | 1 | 99 | Unknown | 4.5 | -4.75     |
| TUBB2C      | P68371    | Tubulin, beta 2C | 5 | 99 | Cytoplasm | 43.8 | -2.37     |
| NUFIP2      | Q7Z417    | Nuclear fragile X mental retardation-interacting protein 2 | 1 | 95 | Unknown | 1.6 | -1.87     |
| EIF4E       | P06730    | Eukaryotic translation initiation factor 4E | 1 | 99 | Cytoplasm | 6.5 | -1.59     |
| EEF1A1      | P68104    | Elongation factor 1-alpha 1 | 1 | 99 | Cytoplasm | 45.02 | -1.57    |
| PCNP        | Q8WW12    | PEST proteolytic signal-containing nuclear protein | 1 | 78 | Nucleus | 56.7 | -1.54     |
| Gene Symbol | Accession | Protein Name                                                                 | Number of Peptides | Peptide Conf. | Cellular Location | %Cov95 | Fold Ratio |
|-------------|-----------|--------------------------------------------------------------------------------|--------------------|---------------|-------------------|--------|-----------|
| PSME1       | Q06323    | Proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)              | 2                  | 71            | Cytoplasm         | 67.8   | 2.189     |
| COR1C       | Q9ULV4    | Coronin-1                                                                      | 1                  | 98            | Cytoplasm         | 30.4   | 1.76      |
| UGDH        | O60701    | UDP-glucose 6-dehydrogenase                                                    | 1                  | 94            | Nucleus           | 48.6   | 1.71      |
| SPCS2       | Q15005    | Signal peptidase complex subunit 2                                             | 1                  | 99            | Cytoplasm         | 23.5   | 1.70      |
| DNJB4       | Q9UDY4    | DnaJ homolog subfamily B member 4                                               | 1                  | 99            | Nucleus           | 47.8   | 1.69      |
| HDGR3       | Q9Y3E1    | Hepatoma-derived growth factor-related protein 3                               | 1                  | 81            | Nucleus           | 17.7   | 1.61      |
| FRIL        | P02792    | Ferritin light chain                                                           | 2                  | 99            | Cytoplasm         | 70.3   | 1.59      |
| SH3L1       | O75368    | SH3 domain-binding glutamic acid-rich-like protein                              | 1                  | 85            | Unknown           | 47.4   | 1.54      |
| FBLN2       | P98095    | Fibulin-2                                                                      | 2                  | 99            | Extracellular matrix | 22.6     | 1.51      |
|             |           |                                                                                 |                    |               |                   |        |           |
| H2B1H       | Q93079    | Histone H2B type 1-H                                                           | 2                  | 99            | Nucleus           | 61.1   | 2.91      |
| RABP1       | P29762    | Cellular retinoic acid-binding protein 1                                       | 2                  | 99            | Cytoplasm         | 30.7   | 2.01      |
| ROA0        | Q13151    | Heterogeneous nuclear ribonucleoprotein A0                                    | 1                  | 99            | Nucleus           | 48.5   | 1.97      |
| CA123       | Q8NW4     | UFP0587 protein C1orf123                                                       | 1                  | 98            | Unknown           | 18.125 | 1.93      |
| RBM27       | Q9P2N5    | RNA-binding protein 27                                                          | 1                  | 92            | Unknown           | 26.6   | 1.72      |
| CO1A1       | P02452    | Collagen alpha-1(l) chain                                                      | 3                  | 99            | Extracellular matrix | 81.1     | 1.69      |
|             |           |                                                                                 |                    |               |                   |        |           |
| 2ABA        | P63151    | Serine/threonine-protein phosphatase 2A                                         | 1                  | 99            | Cytoplasm         | 21.9   | 1.68      |
| SMAP        | O00193    | Small acidic protein                                                           | 1                  | 99            | Unknown           | 19.7   | 1.68      |
| TBB5        | P07437    | Tubulin beta chain                                                             | 6                  | 99            | Cytoplasm         | 87.4   | 1.68      |
| PRS6B       | P43886    | 26S protease regulatory subunit 6B                                              | 1                  | 99            | Nucleus           | 30.9   | 1.65      |
### TABLE III

Top proteins differentially expressed through the 12% 2 hour stretch and time parameters found within IPA networks. Gene symbol and accession are listed with the protein name. The number of peptides found, and confidence levels for those peptides are listed. The percent coverage (95%) (%Cov95) is calculated by dividing the number of amino acids of peptides identified with 95% confidence by the total number of amino acids in the protein.

| Gene Symbol | Accession | Protein Name                                                                 | Number of Peptides | Peptide Conf. | Cellular Location | % Cov | % Cov (95) | Fold Ratio |
|-------------|-----------|------------------------------------------------------------------------------|--------------------|---------------|-------------------|-------|-----------|------------|
| **Up-regulated** | | | 12% for 2 Hours | | | | | |
| HMGN2 | P05204 | Non-histone chromosomal protein HMG-17 | 1 | 82 | Nucleus | 80 | 36.7 | 1.62 |
| MYH11 | P35749 | Myosin-11 | 1 | 80 | Cytoplasm | 51.3 | 2.9 | 1.60 |
| ANXA4 | P09525 | Annexin A4 | 1 | 99 | Plasma membrane | 42.0 | 4 | 1.59 |
| SH3K1 | Q96897 | SH3 domain-containing kinase-binding protein 1 | 1 | 99 | Cytoplasm | 26.6 | 2.1 | 1.57 |
| HTRA1 | Q92743 | Serine protease HTRA1 | 1 | 76 | Extracellular matrix | 25 | 2.3 | 1.57 |
| S10AD | Q99584 | Protein S100-A13 | 1 | 99 | Cytoplasm | 45.9 | 12.2 | 1.52 |
| PEA15 | Q15121 | Astrocytic phosphoprotein | 1 | 87 | Cytoplasm | 56.2 | 22.3 | 1.5 |
| CRYAB | P02511 | Alpha-crystallin B chain | 1 | 99 | Nucleus | 53.7 | 6.3 | –2.77 |
| CX4NB | O43402 | Neighbor of OX4 | 1 | 99 | Cytoplasm | 26.7 | 7.6 | –2.21 |
| PSA3 | P25788 | Proteasome subunit alpha type-3 | 1 | 99 | Cytoplasm | 36.6 | 5.5 | –1.93 |
| SKP1 | P63208 | S-phase kinase-associated protein 1 | 1 | 99 | Nucleus | 41.1 | 8.6 | –1.81 |
| AT1B3 | P54709 | Sodium/potassium-transporting ATPase subunit beta-3 | 1 | 95 | Plasma membrane | 22.6 | 10.8 | –1.75 |
| PTN1 | P18031 | Tyrosine-protein phosphatase non-receptor type 1 | 1 | 95 | Cytoplasm | 35.6 | 5.1 | –1.69 |
| NEK7 | Q8TX7 | Serine/threonine-protein kinase Nek7 | 1 | 99 | Nucleus | 31.1 | 5.3 | –1.67 |
| RSSA | P08865 | 40S ribosomal protein SA | 1 | 99 | Plasma membrane | 56.9 | 19.0 | –1.56 |
| **Down-regulated** | | | | | | | | |

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### Table IV

Top proteins differentially expressed through the 12% 24 hour stretch and time parameters found within IPA networks. Gene symbol and accession are listed with the protein name. The number of peptides found, and confidence levels for those peptides are listed. The percent coverage (95%) (%Cov95) is calculated by dividing the number of amino acids of peptides identified with 95% confidence by the total number of amino acids in the protein.

| Gene Symbol | Accession | Protein Name | Number of Peptides | Peptide Conf. | Cellular Location | %Cov | %Cov (95) | Fold Ratio |
|-------------|-----------|--------------|--------------------|---------------|------------------|------|-----------|------------|
| Up-regulated 12% for 24 Hours |
| FA44A | Q8NFC6 | Protein FAM44A | 1 | 71 | Unknown | 21.3 | 0 | 5.79 |
| SFRS6 | Q13247 | Splicing factor, arginine/serine-rich 6 | 1 | 85 | Nucleus | 47.7 | 0 | 3.46 |
| LMAN2 | Q12907 | Vesicular integral-membrane protein VIP36 | 1 | 99 | Cytoplasm | 24.7 | 2.5 | 2.61 |
| HTRA1 | Q92743 | Serine protease HTRA1 | 1 | 76 | Extracellular matrix | 25.0 | 3.0 | 2.45 |
| CP2C9 | P11712 | Cytochrome P450 | 1 | 97 | Cytoplasm | 19.0 | 3.3 | 1.93 |
| RSSA | P08865 | 40S ribosomal protein SA | 2 | 99 | Plasma membrane | 57.0 | 19.0 | 1.90 |
| COR1C | Q9ULV4 | Coronin-1C | 1 | 98 | Cytoplasm | 30.4 | 5.1 | 1.87 |
| TWF1 | Q12792 | Twinfilin-1 | 1 | 81 | Cytoplasm | 25.8 | 7.9 | 1.83 |
| DNJB4 | Q9UDY4 | DnaJ homolog subfamily B member 4 | 1 | 99 | Nucleus | 47.8 | 3.6 | 1.77 |
| ANXA4 | P09525 | Annexin A4 | 1 | 99 | Plasma membrane | 42.0 | 3.4 | 1.59 |
| Down-regulated |
| ES1_H | P30042 | ES1 protein homolog, mitochondrial | 1 | 97 | Cytoplasm | 36.9 | 3.7 | -4.34 |
| EF1A3 | Q5VTE0 | Putative elongation factor 1-alpha-like 3 | 8 | 99 | Unknown | 78.6 | 38.3 | -2.61 |
| COPB | P53618 | Coatomer subunit beta | 1 | 76 | Cytoplasm | 30.3 | 2.0 | -2.42 |
| PRS6B | P43686 | 26S protease regulatory subunit 6B | 1 | 99 | Nucleus | 30.9 | 3.3 | -2.12 |
| K1543 | Q9PY5 | Uncharacterized protein | 1 | 88 | Unknown | 20.9 | 0.0 | -1.96 |
| CA123 | Q9NNV4 | UFP0587 protein C1orf123 | 1 | 98 | Unknown | 18.1 | 10.0 | -1.87 |
| GNA13 | Q14344 | Guanine nucleotide-binding protein alpha-13 subunit | 1 | 69 | Plasma membrane | 49.1 | 2.9 | -1.75 |
| SCFD1 | Q8WWM8 | Sec1 family domain-containing protein 1 | 1 | 99 | Cytoplasm | 24.8 | 2.6 | -1.70 |
| RBM27 | Q9PN5 | RNA-binding protein 27 | 1 | 92 | Unknown | 26.6 | 0.0 | -1.53 |
| NNMT | P40261 | Nicotinamide N-methyltransferase | 1 | 99 | Cytoplasm | 18.2 | 7.2 | -1.52 |
specifically, apoptosis, activation, neurodegeneration, DNA damage/repair, cellular remodelling, or stress responses.

RESULTS

Prior to samples being injected, a 5 fmol bovine serum albumin digest was used to determine the quality of the liquid chromatography-tandem MS (LC/MS/MS) system. The sample had to record at least 20% sequence coverage with scores per peptide > 20. The identification of the protein was performed using ProteinPilot against a database with concatenated target and decoy sequences. Using this method, a false discovery rate was estimated to identify proteins with a probability of being correct 95% or more of the time. Based on the results of this search, a numeric receiver operating characteristic (ROC) plot (Fig. 2) showing absolute numbers of correct and incorrect protein identifications indicated how well proteins were correctly discriminated from false positives. The ROC curve showed a steep initial slope, angling toward the top left of the graph, indicating a high degree of sensitivity and specificity. ProteinPilot reports a $p$ value and an error factor associated with each protein ratio, and an error percentage associated with each peptide ratio. Because the protein ratio statistics were not provided for all proteins, we report the maximum peptide ratio error percentage associated with each protein as an estimate of protein quantification error. The peptide error percentage is a measure of the error in the calculated peptide ratio, derived from the error for each of the reporter ion peak areas used in the ratio calculation.

The database search yielded 573 proteins identified at a 95% confidence limit. (All proteins provided in supplemental material, provided on web page.) Differentially expressed proteins for each experimental condition were processed by IPA in order to determine the pathways and functions involved in a specific stretch. The distribution of the fold-changes for the top differentially expressed proteins associated with known molecular interactions and functions according to IPA bioinformatics are listed in Tables I–IV. Table V lists proteins that were found in more than one stretch/time condition, and met the GO criteria; involved in apoptosis, activation, neurodegeneration, DNA damage/repair, cellular remodelling or stress responses. Table VI presents the proteins that were differentially expressed proteins for each experimental condition were processed by IPA in order to determine the pathways and functions involved in a specific stretch.

| Name                          | Accession | Number of peptides | Confidence % | Cellular Location | Stretch %Cov (95) | Fold Ratio |
|-------------------------------|-----------|--------------------|--------------|-------------------|-------------------|------------|
| Annexin A4                    | P09525    | 1                  | 99           | Cytoplasm         | 3% for 2 hour     | +1.99      |
| Asterotic phosphoprotein PEA-15 | Q15121    | 3                  | 1 at 98      | Cytoplasm         | 3% for 2 hour     | +1.57      |
| Serine protease HTRA1         | Q92743    | 2                  | 1 at 99      | Secreted          | 12% for 2 hour    | +1.56      |
| 26S protease regulatory subunit 6B | P43686    | 1                  | 98           | Cytoplasm         | 3% for 24 hours   | +2.44      |
| NEDD8 ultimate buster 1      | Q9Y5A7 505| 1                  | 97           | Nucleus           | 12% for 24 hours  | +1.54      |

| Name                          | Accession | Number of peptides | Confidence % | Cellular Location | Stretch %Cov (95) | Fold Ratio |
|-------------------------------|-----------|--------------------|--------------|-------------------|-------------------|------------|
| Protein S100-A13              | Q99584    | 1                  | 99           | Cytoplasm         | 3% for 2 hour     | +2.000     |
| Serine/threonine-protein kinase Nek10 | Q6ZWH5    | 1                  | 99           | Unknown           | 3% for 24 hours   | +1.506     |
| Probable transcription factor PML | P29590    | 1                  | 73           | Nucleus           | 3% for 2 hour     | +1.516     |
| Serine/threonine-protein kinase Nek7       | Q8TDX7    | 1                  | 99           | Nucleus           | 3% for 24 hours   | +1.616     |
| L-lactate dehydrogenase A-like 6B | Q9BYZ2    | 1                  | 69           | Unknown           | 3% for 24 hours   | +1.923     |

| Name                          | Accession | Number of peptides | Confidence % | Cellular Location | Stretch %Cov (95) | Fold Ratio |
|-------------------------------|-----------|--------------------|--------------|-------------------|-------------------|------------|
| Annexin A4                    | P09525    | 1                  | 99           | Cytoplasm         | 3% for 2 hour     | +1.99      |
| Asterotic phosphoprotein PEA-15 | Q15121    | 3                  | 1 at 98      | Cytoplasm         | 3% for 2 hour     | +1.57      |
| Serine protease HTRA1         | Q92743    | 2                  | 1 at 99      | Secreted          | 12% for 2 hour    | +1.56      |
| 26S protease regulatory subunit 6B | P43686    | 1                  | 98           | Cytoplasm         | 3% for 24 hours   | +2.44      |
| NEDD8 ultimate buster 1      | Q9Y5A7 505| 1                  | 97           | Nucleus           | 12% for 24 hours  | +1.54      |
tially expressed in three different stretch groups, but did not meet the GO criteria.

Interaction networks from the 3% for 2 h stretch have TP53, TNF, and TGFβ (Fig. 4) as primary hubs. Of the differentially expressed proteins observed in this condition, 17 were specific to this stretch condition alone, six were found in one other stretch condition, and three were found in three stretch conditions (Tables I, V, and VI).

The cells that were stretched at 3% for 24 h produced merged networks again showing the same three principle hubs; TP53, TNF, and TGFβ (Fig. 5). Fifteen proteins were found to be specific to this stretch condition alone, six were found in one other stretch condition, and three were found in three stretch conditions (Tables I, V, and VI).

The cells that were stretched at 3% for 24 h produced merged networks again showing the same three principle hubs; TP53, TNF, and TGFβ (Fig. 5). Fifteen proteins were found to be specific to this stretch condition alone, six were found in one other stretch condition, and three were found in three stretch conditions (Tables I, V, and VI).

When the cells were stretched at 12% for 2 h the resulting networks created by the proteins detected in these samples again had hubs at TP53, TNF, and TGFβ. In addition, there was a greater role played by specific caspases (CASP3 and CASP8) (Fig. 6). Eight proteins were found in this stretch condition, with five in two conditions, and two were found in three conditions (Tables I, V, VI).

When the cells were stretched at 12% for 24 h, as in the previous conditions, the primary hubs involved in these networks were TGFβ, TNF, and TP53 (Fig. 7). In this final group, 18 of the differentially expressed proteins were unique to this condition, 14 were present within two conditions, while two were observed in three conditions (Tables I, V, and VI).

The top cellular functions that were discovered from the pathways of each time/stretch experiment identified various cellular reactions to biomechanical insult (Fig. 3). The relationship of the specific function charts showed change at a cellular level as time or intensity was increased.

Western blotting of lysate using the 12%, 2 h stretch condition for ANXA4, GOLGA2, and β-Crystallin (Fig. 8) were performed to validate the protein screening results. These proteins were chosen for validation purposes as they demonstrated regulation and antibodies were readily available. Western blots showed changes in regulation that closely matched those found using iTRAQ: ANXA4 was up-regulated 1.5-fold as compared with iTRAQ’s 1.99-fold increase; GOLGA2 was up-regulated 2.3-fold compared with iTRAQ’s...
2.58; and αB-Crystallin was downregulated 1.7-fold compared with a 2.77-fold decrease with iTRAQ. A further Western blot was performed to determine the presence of glial fibrillary acidic protein (GFAP). Normally, the astrocytes remain in a “quiescent” mode, however when insulted through injury or disease, they can become “reactive,” as evidenced by changes in cell morphology and increased staining of marker proteins, most prominently the intermediate filament component GFAP (8, 14, 69). The results show an increase of 1.9 times the level of GFAP as compared with the control (Fig. 9).

**DISCUSSION**

iTRAQ-based proteomics analysis enables the investigation of the mechanisms of disease using a multiplexed approach (53, 55, 57, 61, 70). This is the first time that the iTRAQ technique has been used to explore optic nerve head glial cell activation, believed to be involved in the very earliest stages of the development of glaucomatous optic neuropathy (7, 17). In this study we investigated the differential protein expression of ONH astrocytes that were stretched by either 3% or 12%, for 2 or 24 h. The stretch magnitudes were based on finite element models from our previous research (25–36). These models directly contribute to the various stretch and time combinations that were chosen for our experiments. Sigal et al. (35) predicted a peak strain within the lamina cribrosa approaching 15% when IOP was raised to 50 mmHg. The 3% strain was used to represent a much lower insult, and may in future be considered as a baseline level of strain. Our use of the 2-hour and 24-hour time points was to compare the cellular reactions following different exposure to insult, a model which has been used previously (47, 48). Because of the experimental design, the resulting proteins were not expected to be similar across all conditions (Tables V, VI). Wanner et al. (71) stretched brain astrocytes in order to investigate glial scar formation and axonal growth inhibition. They used rat cortical astrocytes and stretched the cells for 50 ms. This is very different to our own models that use human optic nerve head astrocytes stressed over periods of hours using differ-
ential levels of strain. However our work is similar with respect to the level of cellular activation and GFAP expression. Further refining this work through proteomic research we have been able to detect biomarkers for glial cell activation.

Over 1500 total proteins were found in each stretch/time combination, of which 573 were discovered at a 95% confidence limit. The ROC plot, depicting true and false positive rates for the iTRAQ technique, indicated a high level of sensitivity and specificity, supporting the use and experimental design of this technique in our study.

A number of observed proteins were potential markers of astrocyte activation. These proteins were chosen primarily based upon their ontology, as well as for their potential role in mediating the response of the physical stress on the cellular membranes to actual transcriptional changes within the nucleus. This included astrocytic phosphoprotein (PEA15) which was up-regulated 1.5-fold in the 12%, 2 h stretch condition. PEA15 was first identified by Araujo et al. (72). Renault et al. (73) described its role in the control of apoptosis and cell cycle within astrocytes. It has also been shown to play a significant role in mitogen-activated protein (MAP) kinases, which respond to extracellular stimuli. Specifically, it activates the extra-cellular signal receptor -activated kinases (ERK1/2) (74). This is an indicator that PEA15 is an early response protein to physical stretch. It is predominantly expressed within the central nervous system and, considering its role within cells targeted for apoptosis and the fact that it was up-regulated within our experiments, it may provide an important target of future research.
UDP-glucose dehydrogenase was characterized by Spicer et al. (75) and is noted for its role in converting UDP-glucose to UDP-glucoronate, which is an essential component to the glycosaminoglycans, such as hyaluronans which are needed for cell proliferation and migration. It was found up-regulated 1.72-fold in the 3% for 24 h stretch. It is known that in the absence of this enzymes activity, embryogenesis fails to occur (76). Further research has indicated that defects in the production, activity or expression of this protein may lead to more general defects in proteoglycan or glycosaminoglycan function (75). It has also been shown that cells that are treated with TGFβ gave an increase in production of UDP-glucose dehydrogenase (77). Similar results were reported following hypoxia (78). Clarkin et al. (79) went on to implicate the role of UDP-glucose dehydrogenase in mitogen-activated protein kinase (MAPK) pathways and demonstrated how it may be a good therapeutic target because of its role in the conservation of the extracellular matrix.

Annexin A4 (ANXA4) was up-regulated 2.0- and 1.6-fold in 3 and 12% stretch for 2 h, and 1.58-fold in the 12% stretch for 24 h. This protein is a member of the lipocortin family of calcium-dependent phospholipid-binding proteins (80) and is interesting as other members of its family have previously been reported to be up-regulated in animal models of glaucoma (81). In this latter study, ANXA1 and ANXA3 were proposed to play a role in membrane repair or in the aggregation of vesicles known to occur in axonal transport blockade. ANXA2 has also been shown to play a role in angiogenesis through colocalization and binding with a member of the s100 family (82). Although ANXA4 function is not well documented, it may play a role similar to others in its family. It has been shown to have numerous other cellular functions including cell division, Ca2+ signaling, growth regulation and inflammation (83), and to colocalize on cell membrane surfaces (84), which may indicate a role in the reaction to physical stress. It also plays an important role in modulating the NF-κB signaling pathway (85), which is important in regulating numerous genes involved in the immune response, cell proliferation, differentiation, survival, and apoptosis (86, 87).

**FIG. 7.** Integration of the identified proteins into the canonical pathways for astrocyte cell stretch of 12% for 24 h using Ingenuity Pathway Analysis. Proteins were identified as being within the nucleus, the cell membrane, extracellular, or intracellular. Solid lines indicate direct interaction. Dashed lines indicate indirect interactions. Red molecules were up-regulated and green molecules were down-regulated. White molecules were not user specified, but were incorporated into the network through relationships with other molecules. Of particular note were the network hubs centered on TP53, TNF, TGFβ1, and CASP3.
Another protein of interest belongs to the family of s100 proteins and is known to regulate the progression of inflammation, innate immunity, tissue damage and wound healing (88–90). In particular, s100-a13, is a protein that has been reported to participate directly in the angiogenic process, particularly of cancerous tissue types (82, 91). This protein was up-regulated 2.00-fold and 1.52-fold in the 3% for 2 h and 12% for 2 h stretch respectively. Previous research on this protein in the eye showed an up-regulation following an inflammation-associated corneal neovascularization (88). S100-a13 has also been shown to play a role in damage-associated molecular patterns, which interact with the NF-κB pathway and various inflammatory cytokines such as IL-1β and IL-8, (92) which implicates this important pathway in our model.

The functional pathways in Fig. 3 describe how an increase in strain percentage or duration can affect the regulation of the pathway. Normal protein patterns are associated with low percentage strain, with cellular repair and DNA repair associ-

**Fig. 8. Validation of iTRAQ proteomic results by Western blot for specific markers.** A. Cultured human ONH astrocytes were submitted to 0%, 3%, OR 12% stretch for 2 h. Total proteins were collected in lysis buffer, concentrated, and quantified by Bradford assay. Equal amounts of protein from each condition were probed by Western blot for three markers that were differentially regulated in the iTRAQ results; GOLGA2, ANXA4, and CRYAB. Detection and band densitometry was performed with a Li-Cor infrared imager and marker bands were normalized to actin. B. Fold change was calculated from four separate cell lines by dividing the normalized result from each condition by the 0% stretch result and averaged, as alongside the original iTRAQ stretch results for comparison. (WB: Western blot, iTRAQ: preliminary iTRAQ results).

**Fig. 9. Increased staining of GFAP in ONH astrocytes submitted to increasing amounts of stretch.** A. Cultured human ONH astrocytes were submitted to 0%, 3%, OR 12% stretch for 2 h. Total proteins were collected in lysis buffer, concentrated with ultrafiltration columns, and quantified by Bradford assay. Equal amounts of protein from each condition were probed by Western blot for GFAP and actin. Detection and band densitometry was performed with a Li-Cor infrared imager and GFAP bands were normalized to actin. Fold increase was calculated by dividing the normalized result from each condition by the 0% stretch result.
ated with the higher percentage strain and longer exposure. In Fig. 3A metabolic activity is the most regulated pathway in the 2 h stretch models, with the highest levels seen following 3% stretch. This would indicate that at 3%, 2 h, the cells are adjusting to the initial insult. There was no major increase of other diseased or damaged cell functional pathways following 3% stretch. In cellular assembly and organization (Fig. 3B), the largest change was seen following the 3% for 24 h stretch. Regulated proteins, such as golgi proteins (GOLGA2) and collagens (COL1A1, COL1A2), are associated with normal cellular processes such as cellular division (93) and cellular structure of the eye (94). Cell cycle functions (Fig. 3C) following 12% strain resulted in proteins that are associated with both normal and diseased cellular processes. Normal functioning proteins include kinesin family 14 (KIF14) (95) and kinase anchor proteins (AKAPs), both of which are important in microtubule organization (96). However AKAPs are also associated with cancer (97–99) and is an important anaphase promoting complex (100). Fig. 3D demonstrated that following 12% stretch for 24 h, DNA replication, recombination and repair showed the greatest functional response. Of the top proteins involved in DNA replication, recombination and repair, promyelocytic leukemia is one of the most important and has been shown to play a role in apoptosis, senescence and cell death. It has recently been proposed as a possible target for therapeutic approaches to a variety of diseases (101) and has also been associated with single nucleotide polymorphisms in glaucoma (102). Together Fig. 3, demonstrates an increasing response to increasing biomechanical strain.

In summary, we investigated multiple stress interactions of time and stretch on the activation of human optic nerve head astrocytes. Lysates from each time/stretch condition were investigated proteins involved in cellular differentiation and morphogenesis that may have a role in the early activation of astrocytes, eventually leading to the apoptotic death of retinal ganglion cells in glaucoma. The proteomics strategy used has been previously validated to identify proteins of interest and potential biomarkers (103–105). We have identified a number of proteins of potential interest, including PEA15, UDP-glucose dehydrogenase, ANXA4, and s100a13.

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