DNA microarray analysis of gene expression in human optic nerve head astrocytes in response to hydrostatic pressure

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Yang, Ping, Olga Agapova, Amy Parker, William Shannon, Paula Pecen, Jill Duncan, Mercedes Salvador-Silva, and M. Rosario Hernandez. DNA microarray analysis of gene expression in human optic nerve head astrocytes in response to hydrostatic pressure. Physiol Genomics 17: 157–169, 2004. First published January 27, 2004; 10.1152/physiolgenomics.00182.2003.—There is clinical and experimental evidence that elevated intraocular pressure (IOP), a mechanical stress, is involved in the pathogenesis of glaucomatous optic neuropathy. The mechanism by which astrocytes in the optic nerve head (ONH) respond to changes in IOP is under study. Gene transcription by ONH astrocytes exposed either to 60 mmHg hydrostatic pressure (HP) or control ambient pressure (CP) for 6, 24, and 48 h was compared using Affymetrix GeneChip microarrays to identify HP-responsive genes. Data were normalized across arrays within each gene. A linear regression model applied to test effect of time and HP on changes in expression level identified 596 genes affected by HP over time. Using GeneSpring analysis we selected genes whose average expression level increased or decreased more than 1.5-fold at 6, 24, or 48 h. Expression of selected genes was confirmed by real-time RT-PCR; protein levels were detected by Western blot. Among the genes highly responsive to HP were those involved in signal transduction, such as Rho nucleotide exchange factors, Ras p21 protein activator, tyrosine kinases and serine threonine kinases, and genes involved in transcriptional regulation, such as c-Fos, Egr2, and Smad3. Other genes that increased expression included ATP-binding cassettes, solute carriers, and genes associated with lipid metabolism. Among the genes that decreased expression under HP were genes encoding for dual activity phosphatases, transcription factors, and enzymes involved in protein degradation. These HP-responsive genes may be important in the establishment and maintenance of the ONH astrocyte phenotype under conditions of elevated IOP in glaucoma.

DNA microarray; differential gene expression; glaucoma

ASTROCYTES ARE THE MOST ABUNDANT cell type in the adult central nervous system (CNS). Under normal conditions, astrocytes participate in metabolic and structural support of neurons and in homeostatic maintenance and detoxification of the extracellular space in the CNS. Astrocytes are the major glial cell type in the nonmyelinated optic nerve head (ONH) in most mammals and provide cellular support functions to the axons, forming the interface between connective tissue surfaces and surrounding blood vessels. In the lamina cribrosa, astrocytes form lamellae oriented perpendicular to the axons (28). Quiescent astrocytes are terminally differentiated cells, and several populations are found in different regions of the CNS (11). Astrocytes of the ONH have many of the same functions as astrocytes in the white matter. Astrocytes supply energy substrate to axons in the optic nerve and maintain extracellular pH and ion homeostasis in the periaxonal space. Astrocytes maintain the extracellular matrix (ECM) in the lamina cribrosa consisting of collagens, elastic fibers, and glycoproteins such as laminin and proteoglycans. In the normal ONH, astrocytes express a wide variety of growth factors and receptors, many of which serve as trophic and survival factors (88).

Adult, quiescent astrocytes become “reactive” after injury and in disease and participate in the formation of a glial scar, which does not support axonal survival or growth. The major hallmark of the glial scar is the accumulation of enlarged astrocyte cell bodies and a thick network of processes with increased expression of glial fibrillary acidic protein (GFAP) and vimentin. Reactive astrocytes increase expression of various cell surface molecules that play important roles in cell–cell recognition and in cell adhesion to substrates, as well as various growth factors, cytokines, and receptors. Reactive astrocytes express many new ECM proteins such as laminin, tenascin C, and proteoglycans. The expression of TGF-α and TGF-β, ciliary neurotrophic factor (CNTF), fibroblast growth factor 2 (FGF-2), platelet-derived growth factor (PDGF), and their receptors has been reported to induce the transition of quiescent astrocytes into the reactive phenotype or to modulate the function of reactive astrocytes (65, 77). Reactive astrocytes are thought to play major roles in the pathogenesis of chronic neurodegenerative diseases including Alzheimer disease, amyotrophic lateral sclerosis (ALS), Parkinson disease, multiple sclerosis, and glaucomatous neuropathy (2, 17, 73, 78). These diseases have different pathogenic mechanisms, suggesting that quiescent astrocytes become reactive in response to a wide variety of stimuli including inflammation and oxidative and mechanical stress.

Primary open angle glaucoma (POAG) is a sight-threatening optic neuropathy characterized by loss of the axons of the retinal ganglion cells and cupping of the optic disc. POAG affects an estimated 67 million people worldwide (38). In human glaucoma and in experimental models of glaucoma, irreversible damage to the axons occurs at the level of the lamina cribrosa in the ONH (28, 62). Elevated intraocular pressure (IOP) is the most important risk factor associated with POAG (33). Other important risk factors for glaucoma include age, race, and genetic background.
In normal individuals in vivo, astrocytes in the lamina cribrosa are exposed to a hydrostatic pressure (HP) gradient between the intraocular compartment and the retrolaminar tissue pressure (49). In glaucoma, there is elevated IOP as well as daily fluctuations and spikes of IOP. Under such conditions, the lamina cribrosa undergoes significant deformation in response to changes in IOP, which generates mechanical stress on astrocytes and other cell types of the lamina cribrosa (6, 90). In human glaucoma, there is marked remodeling of the ECM and changes in astrocyte phenotype from quiescent to reactive (28). In glaucoma, in response to elevated IOP, astrocytes migrate to the nerve bundle area and synthesize neurotoxic mediators such as nitric oxide (NO) and tumor necrosis factor-α (TNF-α), which may be released directly in the vicinity of the axons, causing damage to the retinal neurons (41, 76).

Physical forces, such as HP, shear stress, stretch, and fluid flow, are major players in the physiological responses of various cell types (4, 72, 83). The most important functions that astrocytes play in the optic nerve and in the rest of the CNS, such as metabolic and structural support of neurons, maintenance of the blood-brain barrier, and immune and inflammatory responses, are dependent and/or influenced by transcriptional activity to physical (mechanical, thermodynamic, electrical) or by biological events (neurotransmitters, hormones, growth factors) to various stimuli. Expression of HP-responsive genes has been identified using traditional methods, such as Northern blot and reverse transcription polymerase chain reaction (RT-PCR), in various cell types including ONH astrocytes (30, 64). Recently, oligonucleotide microarray analysis has been used to determine transcriptional responses of brain astrocytes to lead neurotoxicity, oxidative stress, and lipopolysaccharide stimulation (7, 37, 57). Oligonucleotide microarray analysis was used to demonstrate differential expression of a large number of genes that promote cell mobility and migration, downregulate cell proliferation, are associated with structural tissue changes, and contribute to neural degeneration in cultured glaucomatous ONH astrocytes compared with normal ONH astrocytes (29). Here we examined the effects of sustained HP on the global transcriptional profile of normal ONH astrocytes in vitro by microarray analysis. This study allowed us to identify and characterize HP-responsive genes involved in signal transduction, transcriptional regulation, and metabolic activity in ONH astrocytes exposed to pressure.

MATERIAL AND METHODS

Human Eyes

Two pairs of normal human eyes from male Caucasian donors (age 15 and 19 yr) with no history of eye disease, diabetes, or chronic CNS disease were obtained from Mid-America Transplant Services (St. Louis, MO). ONHs were dissected within 24 h of death and processed to generate ONH astrocytes (type 1B) (36, 92).

Astrocyte Cultures

 Cultures of human ONH astrocytes were generated as previously described in detail (36). Briefly, four explants from each lamina cribrosa were dissected and placed into 25-cm² Primaria tissue culture flasks (Falcon; Becton-Dickinson, San Jose, CA). Explants were maintained in DMEM-F12 supplemented with 10% FBS (BioWhittaker, Walkersville, MD) and PSFM (10,000 U/ml penicillin, 10,000 µg/ml streptomycin, and 25 µg/ml amphotericin B; GIBCO-BRL, Invitrogen, Carlsbad, CA). Cells were kept in a 37°C, 5% CO₂ incubator. After 2–4 wk, primary cultures were purified by using a modified immunopanning procedure (48, 92). Purified cells were expanded after characterization by immunostaining for astrocyte markers GFAP and neural cell adhesion molecule (NCAM) as described (36, 92). Second passage cell cultures were stored in DMSO in liquid nitrogen until use in these experiments. Then, for each set of experiments, the cells were thawed and cultured for one more passage, so that sufficient cells from the same batch were available to use in each set of experiments.

Hydrostatic Pressure Experiment

Astrocytes were seeded at a density of 200,000 cells per 100 dish. Cells were maintained in DMEM-F12 with 5% FBS to 75% confluence. Cells were then transferred to HP chambers. HP chambers consisted in closed chambers equipped with a manometer. Elevated HP was created by compression of the gas phase in the closed chamber, as described previously (32, 70). Because the solubility of gases changes with changing pressurization of the gas phase, in these experiments we used 8% CO₂ instead of the standard 5% CO₂, to maintain the pH at ~7.3–7.4 (91). Partial oxygen pressure, PO₂, was not altered in the conditions of our experiments, as described earlier (27, 64). Pressure was raised inside the chamber to 60 mmHg (8.6 kPa) above ambient pressure by filling the chambers with a gas mixture of 92% air and 8% CO₂. Chambers were placed in a tissue culture incubator and maintained at 37°C for 6, 24, and 48 h. Culture dishes were placed on a rack inside the chambers with sterile water in the bottom to maintain 98% relative humidity. Control groups consisted in sister cell cultures seeded at the same density and placed inside similar chambers, but at 0.15 mmHg ambient pressure (CP). Experiments, in triplicate, were designed so that control and experimental cultures were harvested at the same time to account for variations. At each time point, RNA and protein were extracted for further experiments.

Oligonucleotide Microarray Analysis

Total RNA was extracted from each time point using Qiagen RNeasy mini kit (Qiagen, Valencia, CA). RNA was then purified and quantified by measuring absorbance at 260 nm. cDNA was synthesized from 5–10 µg purified RNA by using SuperScript Choice system (GIBCO-BRL, Invitrogen) and T7-(dT)24 primer (Genset, La Jolla, CA). Using the Bioassay High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY), we carried out in vitro transcription by using the cleaned double-stranded cDNA as a template in the presence of biotinylated UTP and CTP. Purified biotin-labeled cRNA was fragmented before the hybridization. Hybridization of the labeled cRNA to Human Genome U95Av2 GeneChips (Affymetrix, Santa Clara, CA) was carried out by using the GeneChip Instrument System (Affymetrix) at the GeneChip Core Facility of Washington University. A total of 44 GeneChips were generated and distributed as follows: seven for control pressure (CP) at time 0, four CP at 6 h, seven for HP at 6 h, eight for CP at 24 h, eight for HP at 24 h, five for CP at 48 h, and five for HP at 48 h. Astrocyte cultures from the two donors were equally represented at all time points. The arrays were washed and stained with streptavidin-phycocerythrin (Molecular Probes, Eugene, OR) followed by scanning on an Affymetrix GeneArray Scanner. Data was analyzed by the Affymetrix Microarray Suite (version 5.0), linear regression analysis, and GeneSpring Expression Analysis Software (version 5.0; Silicon Genetics, Redwood City, CA).

The study was conducted according to standards developed by the Microarray Gene Expression Data Society (MGED), and data complying with the “minimum information about microarray experiments” (MIAME) have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database.
Data Analysis

Linear regression model. Affymetrix spiked probes and probes that were absent in all GeneChips (n = 5,466) were deleted, leaving 7,092 probes for subsequent analyses. The data were normalized as follows: for a specific gene the mean and standard deviation of the gene was calculated over 44 GeneChips. Each GeneChip expression value then had the mean of the gene subtracted from it and then was divided by the standard deviation of the gene. A linear regression model of the log expression value as a function of time, pressure, and the time x pressure interaction term \[ \log(\text{signal}) = \beta_0 + \beta_1 \text{Time} + \beta_2 \text{Pressure} + \beta_3 \text{Pressure x Time} \] was applied to each gene to test the effect of time and pressure on changes in expression level. Significance was set at \( P < 0.05 \) with no adjustment made for multiple testing. All analyses were performed using SAS (Cary, NC) statistical software.

GeneSpring analysis. Raw data of 44 GeneChips, scaled to the same target intensity, were loaded into GeneSpring software. Data were normalized using the median of each gene across all GeneChips as the reference. Changes in expression level were calculated for 596 genes selected by the linear regression model, comparing HP and CP samples at each time point. Using GeneSpring, we selected genes whose average expression level increased more than 1.5-fold or decreased more than 1.5-fold at least at one time point. For these two groups hierarchical gene clustering (gene tree) was performed according to the software manufacturer’s instructions. Briefly, GeneSpring analyzed the standardized expression values for 38 HP-upregulated and 24 HP-downregulated genes using average linkage algorithm. Standard correlation was used for similarity measure.

RNA Isolation for Real-Time PCR

Independent pressure experiments were performed in triplicate using human ONH astrocytes from the same donors at CP and HP for 0, 6, 24, and 48 h, and total RNA was isolated in TRIzol (Invitrogen Life Technologies, Carlsbad, CA) (64). After isolation, RNA was precipitated and resuspended in 10 μl nuclease-free water. RNA absorbance at 260 nm and absorbance ratios at 260/280 nm were measured, and 1 μg RNA was treated with RNase-free DNase (Ambion, Austin, TX) before reverse transcription.

Real-Time Quantitative RT-PCR

Randomly-primed cDNA was synthesized from 1 μg total RNA using SuperScript II reverse transcriptase (Invitrogen). Five microliters of 1:5 to 1:20 diluted cDNA was used for reaction with 2× Bio-Rad SYBR Green Supermix in 50 μl, and quantitative RT-PCR was performed by monitoring in real time the increase of fluorescence of SYBR Green using the iCycler (Bio-Rad Laboratories, Hercules, CA). Custom primers were designed using the Primer Express program (PE Applied Biosystems, Foster City, CA); sequences of primers are available upon request. Amplicons crossed exon-exon boundaries to prevent genomic DNA amplification. There is no detectable signal in RT− (sample without RT) and H2O negative controls. Primer quality (lack of primer-dimer amplification) was confirmed by melting curve analysis. Relative quantitation of gene expression was performed using the standard curve method (user bulletin 2 of the ABI Prism 7700 Sequence Detection System, PE Applied Biosystems). Serial dilutions (1:2.5, 1:10, 1:40, 1:160) of a mix of all samples were used for standard curves. In each experiment the relative amounts of mRNA for target genes were calculated from the standard curves and normalized to the relative amounts of reference gene RNA (β-actin mRNA and 18S RNA), which were obtained from a similar standard curve. All experiments were carried out in triplicates. The results were expressed as the means ± SE of the relative amount of normalized mRNA at time 0 and after 6, 24, and 48 h exposure to HP. Significant differences between the means were set at \( P < 0.05 \) (Student’s t-test).

Western Blot

Cells were washed twice in cold 1× PBS and lysed in 200 μl of cold IP buffer [10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.25% Triton X-100, Roche protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN)]. After 15 min, cells were scraped with disposable cell lifters and centrifuged at 4°C and 14,000 rpm for 15 min, and the supernatant was collected for cytoplasmic proteins. Cell pellets were washed twice with lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM MgCl2, 0.3% Nonidet P40), and nuclear proteins were extracted at 4°C overnight in 50 μl lysis buffer with 0.5 M NaCl. Protein concentration was determined using the Bio-Rad Protein Assay Kit (Bradford method), and cytoplasmic and nuclear proteins were stored at −80°C until further use.

Samples from different time points (5–20 μg per lane) were run on 4–15% gradient SDS polyacrylamide gel under reduction conditions and transferred to a Bio-Rad nitrocellulose membrane. The membrane was blocked for 1 h in blocking solution (5% skim milk in TTBS buffer) and incubated with primary antibody diluted in 2.5% skim milk in 2:1 TTBS:TBS solution for 1 h at room temperature. The following primary antibodies were used: rabbit anti-human c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1/500 dilution; rabbit anti-human Cdc42 (Santa Cruz) at 1/250; rabbit anti-human Dbl (MF2) at 1/500 (Santa Cruz); rabbit anti-human VEGF-C (Abcam, UK) at 1/100; goat anti-human vimentin (Sigma) at 1/2,000; mouse anti-human tubulin (NeoMarkers) at 1/5,000; and rabbit anti-human MKP-1 (DUSP1) at 1/100 (Santa Cruz). Membranes were washed in TTBS and then incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature. For detection of membrane-bound primary antibodies, we used the enhanced chemiluminescence ECL Western blotting detection system (Amersham Pharmacia, Piscataway, NJ). For c-Fos, both nuclear and cytoplasmic proteins were isolated and processed for Western blot detection.

Activated Rho and Ras Affinity Precipitation Assay

ONH astrocytes were exposed to HP for 0, 10, 30, and 60 min, and activated Rho and Ras were detected using Rho and Ras Activation Assay Kits (Upstate, NY) according to manufacturer’s protocols. Briefly, cells were lysed in 400 μl of ice-cold Mg2+ lysis/wash buffer (MLB) with protease inhibitors (Roche Molecular Biochemicals), and protein concentration was determined using the Bio-Rad Protein Assay Kit (Bradford method). Then, 100 μg of cell lysates was incubated with Rhotekin RBD (“Rho binding domain”) or Raf-1 RBD (“Ras binding domain”) agarose 45 min at 4°C. Activated Rho (Rho-GTP) bound to Rhotekin RBD agarose beads and activated Ras (Ras-GTP) bound to Raf-1 RBD agarose beads were precipitated by centrifugation and washed three times in MLB. Agarose beads were boiled in reducing sample buffer, resolved by electrophoresis, transferred to nitrocellulose membrane, and probed with anti-Rho or anti-Ras antibodies. For positive and negative controls we used samples loaded with GTPγS/GDP and cell lysates from astrocytes stimulated 10 min with EGF (200 ng/ml). Three independent experiments were performed for each assay. Films were scanned and relative band density was determined using AlphaEase imaging system (Alpha Innotech). Data (optical density) were expressed as means ± SE. Significant differences between the means were set at \( P < 0.05 \) (Student’s t-test).
RESULTS

Global Gene Expression Analysis by High-Density Oligonucleotide Arrays

To identify changes in gene expression in human ONH astrocytes exposed to sustained HP of 60 mmHg, we performed gene expression profiling using Human Genome U95Av2 GeneChips from Affymetrix. We obtained high-quality arrays in all 44 GeneChips. The expression level and detection call for 12,588 probes were obtained from Affymetrix Microarray Suite 5.0. Affymetrix spike probes and probes absent across 44 GeneChips (n = 5,466) were deleted, leaving 7,092 probes for subsequent analyses.

Data Analysis

Data were normalized by a z-statistics transformation (i.e., for each gene expression value, subtract the mean expression for that gene and divide by that gene’s standard deviation). After normalization, statistical analysis using the linear regression model was used to rank the genes in order of the differential expression under pressure over time. This statistical analysis identified 596 genes whose expression was influenced by HP and time (Supplemental Table 3, available at the Physiological Genomics web site1). 338 genes exhibited positive interaction between pressure and time on the level of gene expression, and 258 genes exhibited negative interaction between pressure and time on the level of expression (P < 0.05) (Supplemental Tables 4 and 5). We confirmed by real-time RT-PCR expression of few genes positively affected by HP according to the linear model, such as vimentin, c-Jun, and elastin (Supplemental Table 4 and Fig. 2C). Western blots of protein products of vimentin and tubulin positively affected by HP are shown in Fig. 3B.

To assign a “fold change cutoff” threshold, we used GeneSpring analysis, to select genes whose average expression level increased or decreased more than 1.5-fold at 6, 24, or 48 h with significance set at P < 0.05. As shown in Tables 1 and 2, the effect of HP was transient in many genes during the time course of the experiments. For clarity, the genes are grouped according to their involvement in specific cellular functions.

Under exposure to HP, expression of 38 genes increased either at 6, 24, or 48 h (Table 1). Among genes increased under HP, nine genes are involved in signal transduction pathways, five genes are involved in transcriptional regulation, and other genes belong to functional groups of ion channels, cancer markers, lipid metabolism, cytoskeleton proteins, immune response, etc. Four genes have unknown function.

Expression of 24 genes decreased at 6, 24, or 48 h (Table 2). Among genes that exhibit decreased expression under HP, four genes are involved in signal transduction pathways, five genes are involved in transcriptional regulation, and other genes belong to functional groups of enzymes, tumor suppressors, cytoskeleton protein, and nuclear and mitochondrial proteins, and one gene has unknown function (Table 2).

Using GeneSpring software for hierarchical clustering, we grouped differentially expressed genes according to their expression over time. Figure 1 shows the heat map of up- and downregulated genes after 6, 24, and 48 h of exposure to HP and CP. The columns correspond to the samples, and the rows correspond to the genes upregulated or downregulated. Red corresponds to high expression, green to low expression, and black to an intermediate level of expression. The dendrograms represent the average linkage tree for the selected genes. Visual inspection identified two major groups in the upregulated genes (Fig. 1A). The first group of 18 genes displayed an increase in gene expression after exposure to HP for 24 and 48 h, and the second group of 20 genes displayed an increase in gene expression after 6 h HP. In the second group, the dendrogram identified seven genes that grouped into two clusters of three genes each and one individual gene. In the group of genes that were downregulated under HP, almost all genes exhibited lower expression after 6 h of exposure to HP compared with CP (Fig. 1B).

Hydrostatic Pressure Modulates Expression of Genes Involved in Signal Transduction

HP, like other forms of mechanical stress, induces signaling pathways in astrocytes that may be relevant to the functions of these cells in vivo. Our microarray data set shows early upregulation of expression of Dbl, a prototype guanine nucleotide exchange factor, Rho-GEF, which modulates the activity of the Rho family of small GTPases. Expression of Cdc42, a Rho GTPase, and FARP1 mRNA, another Rho-GEF, are upregulated after exposure to HP (Table 1). Real-time RT-PCR confirmed upregulation of Cdc42 and Dbl mRNA in astrocytes exposed to HP (Fig. 2A). By Western blots, protein levels of the corresponding gene products were also increased under HP (Fig. 3A).

To determine Rho activation upon exposure to HP, a Rho binding domain (RBD) assay was performed on ONH astrocytes utilizing the RBD fragment from the Rho effector, Rhoetkin. The RBD fragment binds only active Rho-GTP and does not bind inactive Rho-GDP. Figure 4A is a representative Western blot and densitometry analysis of specific bands illustrating the effects of HP on Rho activity. Cultures were exposed to HP for 10, 30, and 60 min, and Rho was immunoprecipitated as outlined in MATERIAL AND METHODS. Stimulation with HP for 30 min induced a rise in activity. Rho activity remained upregulated under exposure to HP over the duration of the experiment (Fig. 4A).

The mRNA encoding for Ras p21 protein activator 2 (RASA2), a member of the Ras family of small GTPases, was upregulated after 24 and 48 h of exposure to HP by microarray (Table 1). Real-time PCR confirmed RASA2 mRNA increase (Fig. 2A). In addition, a functional pull-down assay for the detection of activated Ras (Ras-GTP) was performed to demonstrate that the amount of Ras-GTP increased after short-term exposure to HP compared with CP. There was three times more Ras-GTP after 10 min exposure to HP and approximately six times more after 30 and 60 min than in control (Fig. 4B). In addition, expression of GRP1, a Ras GDP/GTP exchange factor, was upregulated in ONH astrocytes exposed to HP (Table 1).

Two genes encoding for tyrosine kinases, TEK and AXL, were upregulated 6 h after exposure to HP by microarray analysis (Table 1). TEK encodes for a receptor tyrosine

1The Supplementary Material for this article (Supplementary Tables 3, 4, and 5) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00182.2003/DC1.

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A kinase that induces signal transduction pathways upon angiopoietin-1 (Ang1) stimulation. AXL encodes for a receptor tyrosine kinase, which is activated by the ligand growth arrest-specific 6 (Gas6). From the linear model analysis, HP and time positively influenced expression of the Axl ligand, Gas6 (Supplemental Table 4). However, differential expression of Gas6 was not significant by GeneSpring analysis.

Table 1. Genes upregulated in ONH astrocytes exposed to hydrostatic pressure

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<td>ATP5S</td>
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<td>PMP34</td>
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<td>ABCA12</td>
<td>ATP-binding cassette (ABC1) member 12</td>
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Data represent the fold increases of 38 genes, whose average expression level increased more than 1.5-fold at each time point (P < 0.05) selected by GeneSpring. The GenBank accession number, symbol, product, and chromosomal position (Map) are given. Genes were separated in functional categories. ONH, optic nerve head.
Expression of two novel genes encoding for serine-threonine kinases was increased after exposure to HP: PRKWNK1 at 48 h and CDKL2 (p56 KKIAMRE) at 6 h (Table 1).

Hydrostatic Pressure Regulates Expression of Transcription Factors

Mechanical stress is known to upregulate transcription of the proto-oncogenes c-Fos and c-Jun (34). Expression of c-Fos (FOS) mRNA was increased after 48 h exposure to HP by microarray and was further confirmed by real-time RT-PCR (Fig. 2B). c-Fos protein levels were visualized in nuclear and cytoplasmic fractions of cell lysates of astrocytes exposed to HP (Fig. 3C). The antibody recognized three bands at 43, 52, and 63 kDa, respectively (Fig. 3C), corresponding to c-Fos in the cytoplasmic fraction (53). The level of c-Fos decreased over time in the cytoplasm in astrocytes exposed to HP, whereas the levels of the nuclear c-Fos at 43 kDa was high throughout the experiment. Detection of nuclear c-Fos in control astrocytes reflects normal transcriptional activity. Expression of Smad3 (MADH3) mRNA was increased in cells exposed to HP by microarray (Table 1). Real-time RT-PCR confirmed increased expression of Smad3 (MADH3) mRNA at 6 h HP as observed on the microarray (Fig. 2B). EGR2 increased in astrocytes exposed to HP by microarray (Table 1).

Expression of three repressors of cell cycle, ZFP95, ZNF75, and CBFA2T1, was upregulated by HP (Table 1). Among the genes encoding for transcription factors down-regulated by HP is the cAMP response element-binding protein (CREB1) (Table 2). Real-time RT-PCR confirmed the decrease of CREB1 mRNA after 6 h of exposure to HP (Fig. 5A).

Hydrostatic Pressure Regulates Expression of Growth Factors

Expression of vascular endothelial growth factor-C (VEGF-C) mRNA was significantly decreased by GeneSpring analysis at 6 h of exposure to HP (Table 2). Real-time RT-PCR confirmed decreased expression at 24 h (Fig. 5A). Western blots showed the presence of the protein in the cytoplasm of astrocytes, but no difference was evident between CP and HP (Fig. 5B).

Table 2. Genes downregulated in ONH astrocytes exposed to hydrostatic pressure

<table>
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<tr>
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<td>Dual specificity phosphatase 5</td>
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- **Signal transduction pathways**
- **Transcription factors**
- **Growth factors**
- **Transport**
- **Enzymes**
- **Tumor suppressors**
- **Cytoskeleton and related proteins**
- **Unknown and others**

Data represent the fold decrease of 24 genes, whose average expression level decreased more than 1.5-fold at each time point (P < 0.05) selected by GeneSpring. The GenBank accession number, symbol, product and chromosomal position are given (Map). Genes were separated in functional categories.
Linear regression analysis identified mRNAs encoding four growth factors positively affected by HP: fibroblast growth factor 1, FGFA (acidic); fibroblast growth factor 2, FGF-2 (basic); connective tissue growth factor, CTGF; and bone morphogenetic protein 4, BMP4 (Supplemental Table 4); however, differential expression did not reach significance by GeneSpring analysis.

**Hydrostatic Pressure Regulates Genes Involved in Cellular Metabolism**

Exposure to HP for 6 h upregulated expression of mitochondrial ATP synthase regulatory component factor B mRNA (ATP5S), an enzyme that has a key role in energy production (16). Of interest is the upregulation of thioredoxin-like protein 2 (Txl-2) mRNA, a member of the thioredoxin family, after 6 h exposure to HP. Increase in gene expression of the peroxisomal membrane protein (34 kDa) was detected after 48 h of exposure to HP. Other genes involved in lipid metabolism, such as the very low lipoprotein receptor (VLDLR) and the apolipoprotein B RNA editing deaminase (APOBEC1), were also upregulated after exposure to HP (Table 1).

**Hydrostatic Pressure Induces Downregulation of Genes Involved in Protein Degradation**

Expression of two genes encoding for dual specificity phosphatases, DUSP1 and DUSP5, decreased in response to HP by microarray analysis (Table 2). The expression of DUSP1 and DUSP5 was confirmed by real-time RT-PCR at all times; however, the relative levels of expression did not match the decrease in the microarray results (Fig. 5A). DUSP1 mRNA decreased at 6, 24, and 48 h of exposure to HP compared with control cells at ambient pressure. DUSP5 mRNA decreased after 6 h of exposure to HP. Western blot analysis identified a band at 39 kDa corresponding to DUSP1; however, there was no apparent change in the level of protein in astrocytes exposed to HP over time (Fig. 5B). Astrocytes exposed to PMA for 1 h served as positive controls for DUSP1 expression (not shown). In addition, HP downregulated expression of UBE2D1, a ubiquitin-conjugating enzyme E2D1, and of SMURF2, a ubiquitin protein ligase E3 (Table 2).

**DISCUSSION**

Mechanical forces such as HP can modulate gene and protein expressions and affect cellular functions by activating mechanoreceptors in the membrane and intracellular signaling pathways (4). Oligonucleotide microarray analyses of differential gene expression demonstrated that exposure of cultured cells to mechanical stresses such as fluid flow, cyclic and stationary pressure, and shear stress led to differential expression of transcripts encoding proteins involved in signal transduction, regulation of transcription, antioxidants, and many other cell functions (10, 25, 59, 71). The changes in global gene expression to HP appear to be cell-type-specific rather than stimulus-specific; for example, chondrocytes respond to elevated HP with upregulation of heat-shock proteins (32), vascular endothelium responds to cyclic pressure with upregulation of VEGF-C (59), whereas ONH astrocytes respond to static HP with downregulation of VEGF-C and upregulation of...
genes encoding to small GTPases such as Ras and Rho (this study). On the other hand, the upregulation of intermediate early genes such as c-Fos and c-Jun that occur in response to various types of stress in different cell types probably represents a general response to stress.

The functional class of genes most responsive to exposure to HP in astrocytes is that of genes involved in signal transduction. This most likely reflects the increased activity of pathways that were activated following initial exposure to HP. Our results indicate that the reprogramming of gene expression in response to HP involves genes such as tyrosine kinases and serine threonine kinases that modulate the cellular response to a variety of stresses.

Rho Signaling Pathway

Members of the Rho family GTPases (Rho, Rac, and Cdc42) regulate a variety of cellular activities such as cell-cycle progression, migration, or axonal guidance by controlling actin cytoskeletal rearrangements or gene expression (66). Activa-
tion of Rho family GTPases is catalyzed by their guanine nucleotide exchange factors (GEFs), which share a Dbl homology domain and an adjacent pleckstrin homology domain (95). In this study, the mRNA encoding for Dbl, a prototype Rho-GEF, increased by microarray and by real-time PCR; protein levels were also increased by Western blot in ONH astrocytes exposed to HP. Cdc42 is a member of the Rho family of GTPases that controls the organization of the actin cytoskeleton and formation of filopodia (5, 47). Previous studies have shown that in cultured astrocytes, Cdc42 is key to cellular orientation in the direction of migration (22). Cdc42 mRNA and protein were also increased in astrocytes exposed to HP in this study. A pull-down assay confirmed and quantified the increase of GTP-bound Rho GTPase in astrocytes exposed to HP for 10–60 min. Taken together, our results indicate that morphological changes in astrocyte cell shape and motility induced by HP are mediated by the Rho signaling pathway.

Ras Signaling Pathway

The microarray data suggested that molecular cascades leading to the activation of Ras p21 might be involved in responses to HP. HP can activate astrocyte membrane receptors such as growth factors, G proteins, and integrin receptors to initiate the signal transduction involving the Ras signaling pathway (86). Alternatively, it is possible that HP-induced changes in fluidity of cellular membrane lipid bilayer may affect the conformation and/or interaction of membrane proteins that can be transduced into Ras activation (35). In this study, HP induced Ras activity in ONH astrocytes as detected by a pull-down assay 10 min after exposure to HP. Ras p21 activates the extracellular signal-regulated protein kinase 1/2 (ERK1/2), a member of the mitogen-activated kinase family (MAPK) in reactive astrocytes in brain injury and in cultured astrocytes exposed to various forms of mechanical injury (45, 52). Ras p21 activation mediates induction of nitric oxide synthase (iNOS) in primary astrocytes (56). Reactive astrocytes express iNOS in the ONH with glaucoma in vivo and after exposure to HP in vitro (41, 54). Our results indicate that RASA2 mRNA, a member of the GAP1 family of GTPase-activating proteins (15), is upregulated under HP. RASA2 gene product stimulates the GTPase activity of normal Ras p21 but not of its oncogenic counterpart. Acting as a suppressor of Ras function, the protein enhances the weak intrinsic GTPase activity of Ras proteins, resulting in the inactive GDP-bound form of Ras, thereby allowing regulation of cell proliferation and differentiation. In addition, increased expression of GRP1, a Ras GDP/GTP exchange factor (Table 1), suggests the generation of strong Ras pathway signaling in response to HP (9).

**Fig. 4.** HP induces activation of Rho and Ras pathways in ONH astrocytes. Cells were exposed to HP for 0, 10, 30, and 60 min. GTP-loading assays for Rho and Ras activity were performed as described in MATERIAL AND METHODS. Representative examples of Western blot detection of affinity precipitated GTP-Rho (A) and GTP-Ras (B) and densitometry analysis of specific bands. Bars show mean fold increase in density ± SE of 3 determinations in cells exposed to HP compared with CP. *P < 0.05.

**Fig. 5.** A: real-time RT-PCR of selected genes identified by microarray analysis as decreased under hydrostatic pressure (Table 2). Each bar represents mean ± SE of mRNA relative expression level at 0, 6, 24, and 48 h HP. *P < 0.05 HP vs. 0 h control. B: Western blots show expression of DUSP1 and VEGF-C protein in ONH astrocytes exposed to control and to HP for 6, 24, and 48 h. Note that there is no detectable difference in protein levels between controls and HP. Cell lysates were prepared and separated by SDS-PAGE gel electrophoresis as described in the MATERIAL AND METHODS.
Receptor Tyrosine Kinases

In this study, HP affected early expression of two genes (AXL and TEK) encoding for receptor tyrosine kinase receptors, Axl and Tek. Axl is a transmembrane protein receptor tyrosine kinase that appears to be involved in normal mesenchyma and in CNS development (61). In vivo, Axl and Gas6, the specific ligand for Axl, have been localized to articular cartilage (43), smooth muscle cells (24), and in the neointima of arteries after balloon injury (46), suggesting that Axl might function in regulating cell-cell or cell matrix interactions in astrocytes exposed to HP. Tek tyrosine kinase receptor has been studied in astrocytomas as an angiogenic factor, but its function is unknown in normal astrocytes (19).

Serine-Threonine Kinases

PRKWNK1 is a novel serine-threonine kinase that is expressed predominantly in the kidney and plays a general role in the regulation of epithelial Cl− flux (85). In this study, expression of PRKWNK1 increased after 48 h of exposure to HP; however, it is unknown whether PRKWNK1 participates in the regulation of Cl− flux in astrocytes or in astrocyte swelling after injury (44). Interestingly, KCNN4, an intermediate-conductance calcium-sensitive potassium channel, is upregulated in astrocytes exposed to HP for 6 h (Table 1). KCNN4 is thought to increase chloride conductance in secretory epithelial cells (14). Reactive astrocytes in the injured CNS may regulate and control the K+ and Cl− concentration in the extracellular space resulting in persistent astrocyte swelling (58, 81). However, it is unknown whether this mechanism is responsible for reactive astrocyte hypertrophy in glaucomatous astrocytes in vivo.

The cyclin-dependent kinase-like 2 (p56 KKIAMRE, CDKL2) is a member of a large family of CDC2-related serine/threonine protein kinase (26). The human p56 KKIAMRE phosphorylates c-myc and EGFR in vitro, suggesting that KKIAMRE might regulate gene expression by phosphorylation of transcription factors (74).

Expression of ONH astrocytes to elevated HP resulted in an increase in the levels of expression of transcription factors. Of the 38 genes differentially increased in cells exposed to HP, 6 genes encoded for transcription factors, some of which have previously been described as immediate early genes whose expression is induced by a range of stimuli. These transcription factors include Smad3, c-Fos, Egr2, CBFA2T1 (AML1/ETO), and two members of the zinc finger family of transcription factors.

Smad3 is phosphorylated by activated activin/TGF-β receptors (50). Smad3 signaling pathway is essential for the increase in Rho activity, induction of stress fiber formation, and increased production of all collagen types in response to TGF-β1 stimulation (42). Smad3 interacts with c-Jun and c-Fos to mediate TGF-β-induced immediate early transcriptional activation of target gene promoters (94). Expression of Smurf2 was decreased under exposure to HP. Smurf2 is a Smad-ubiquitin ligase that induces the ubiquitination and degradation of the TGF-β-Smad2/3 pathway to regulate TGF-β signaling (40).

Expression of the immediate early genes c-Fos and c-Jun was increased after exposure to HP as demonstrated earlier in responses to mechanical stress (34, 39). c-Fos is a leucine zipper protein that forms dimers with proteins of the Jun family in the nucleus, thereby forming the transcription factor complex AP-1. In these experiments using Western blot analysis, we show that under elevated HP, 43 kDa c-Fos protein levels decreased in the cytoplasm and increased in nuclear extracts of ONH astrocytes, suggesting that HP induces c-Fos protein translocation from the cytoplasm into the nuclear compartment. We show here that Egr2 (Krox-20) is upregulated at 24 and 48 h in astrocytes exposed to HP. Egr2 belongs to the Egr family of zinc-finger transcription factors, which is involved in myelination in the CNS and PNS and mutations on this gene produce various neuropathies (51). Egr-2 can stimulate the transcriptional activity of c-Fos and c-Jun; however, the downstream events of these interactions are unknown. The induction of c-Fos, c-Jun, and Egr-2 in response to HP suggests that a cocktail of immediate early genes might be required for the responses of ONH astrocytes to pressure in vivo and in vitro.

Our previous data indicated that HP did not stimulate ONH astrocyte proliferation (64), and this is reflected by our data here by increased expression of ZFP95, ZNF75, and CBFA2T1, which carry the zinc finger motif and function as repressors of transcription in cell growth arrest by inhibiting DNA synthesis (8, 20, 79). Increased cell proliferation is characteristic of reactive astrocytes after CNS injury; in contrast, to date there is no experimental evidence of reactive astrocyte proliferation in response to elevated pressure in human or in animal models of ocular hypertension. Moreover, human glaucomatous astrocytes in culture exhibit a very low proliferation rate similar to normal astrocytes and express several genes that downregulate cell division (29).

One of the early responses to mechanical stress is production of cAMP in various cell types including vascular endothelial cells, fibroblasts, and ONH astrocytes (12, 31, 84). The decrease in CREB1 expression, a cAMP effector, after 6 h of exposure to pressure suggests an inhibition of the cAMP pathway after initial activation by HP.

In this study, the gene encoding for thioredoxin-like 2 (TXNL2) was upregulated in response to HP. Thioredoxin expression was induced as in brain astrocytes by exposure to oxidative stress (37). Thioredoxin reductase mRNA was upregulated in vascular endothelia by laminar shear stress presumably in response to oxidative stress (83). A recent report demonstrates that Txl-2 is associated with the microtubule cytoskeleton (68), suggesting a protective function for the microtubule cytoskeleton in ONH astrocytes exposed to HP. Upregulation of the mitochondrial ATP-synthase regulatory component B mRNA suggests an increase in ATP demand in ONH astrocytes in response to HP (16). Metabolic regulation in response to HP may allow the adjustment of astrocytes to changes in a mechanical stress. In astrocytes, ATP is a major factor mediating intercellular communication in the CNS via the activation of specific ligand-gated purinergic receptors and G-protein-coupled metabotropic purinergic receptors (1). Moreover, activation of ERK in stressed astrocytes involves extracellular ATP and purinergic receptors (52).

Expression of several genes involved in lipid metabolism and lipid transport was increased in ONH astrocytes after exposure to HP. Among the cellular components that participate in cellular mechanotransduction are the cellular membrane and the cytoskeleton (23). Lipids are key components of the
membrane lipid bilayer and of integral membrane proteins such as ion channels and phospholipases A and C. Expression of ABCA12, a member of the ATP-binding cassette (ABC) transporter superfamily (18) that functions in cellular lipid trafficking, increased in astrocytes exposed to HP. In the CNS, members of the ABCA family of transporters influence cholesterol metabolism (87). PMP34 expression was upregulated after 48 h of exposure to HP. PMP34, an adenine nucleotide transporter (80), belongs to the mitochondrial solute carrier family, which supplies ATP for the activation of fatty acids in the peroxisomal matrix. Other genes involved in lipid biosynthesis and metabolism upregulated by HP were the very-low-density lipoprotein receptor (VLDLR) (75), apolipoprotein B RNA editing deaminase (APOBEC1) (60), and N-acylsphingosine amidohydrolase (acid ceramidase)-like (ASAH1L) (55).

In light of the fact that long-term exposure of ONH astrocytes to HP results in changes in cell shape and increased mobility (30, 64, 69, 70), our microarray data indicates upregulation of genes that encode proteins involved in cytoskeletal structure. For example, tubulin and the microtubule-associated protein (MAP), EML1 (21), were upregulated by HP. Coordinated regulation of tubulin and MAP suggests extensive reorganization of cytoskeleton following exposure to HP, confirming previous reports in ONH astrocytes (70). DOCK3 expression was upregulated in response to HP. DOCK3 (MOCA) is a member of the CDM (for CED-5, DOCK180, and Cdc42) family that regulates cell adhesion by forming complexes with other proteins to activate small GTPases such as Rho and Cdc42 (13). Thus increased expression of DOCK3 (MOCA) in ONH astrocytes may participate in the reorganization of the cytoskeleton under HP.

Among the genes downregulated by HP are dual specificity phosphatases (DUSP1 and DUSP5), key regulatory components in signal transduction pathways (82). DUSP1 has dual specificity for tyrosine and threonine kinases and specifically inactivates MAPK in vitro (89). The HP-induced decrease in the amounts of expression of dual specificity phosphatases may allow a prolonged response capability by maintaining kinases in an active state. In addition, expression of UBE2D1, a ubiquitin-conjugating enzyme (67), was downregulated in astrocytes exposed to HP for 6 h, suggesting inhibition of ubiquitin-dependent degradation of proteins.

Some genes that are known to be induced by HP in ONH astrocytes, including NOS2 (41), elastin (30), and 3α-hydroxysteroid dehydrogenase (AKR1C2) (3), were not identified as differentially expressed using microarray analysis, indicating some limitations to the application of microarrays. This may be attributed to the design and selection of the oligonucleotide probes and to lack of sensitivity of the detection system. Real-time RT-PCR confirmed the regulation several of selected genes; however, there were discrepancies with microarray data. Microarrays tend to have a low dynamic range, which can lead to misrepresentations of fold changes in gene expression. This might arise either from overestimating the noninduced genes due to nonspecific hybridization or from underestimating the level of induction due to target saturation effects (63). As real-time RT-PCR has a greater dynamic range, it is often used to validate the observed trends rather than duplicate the fold changes obtained by microarray experiments (93). The discrepancies between the protein levels and gene expression microarray data for genes such as vimentin and tubulin may be due to saturation level intensities for these probes resulting from their high-level expression.

In summary, we describe here the transcriptional response of ONH astrocytes to HP using cDNA microarrays. We identified a number of HP-responsive genes including transcription factors, signal transduction proteins, and genes involved in cellular metabolism, whose modulation may reflect the key nuclear signaling events that lead to the changes in cell physiology exhibited by ONH astrocytes exposed to pressure. The results presented here will serve as candidate genes to test for relevance to the glaucomatous process in vivo. Characterization of the coordinated regulation of pressure-responsive gene families will help further elucidate the mechanisms governing ONH astrocyte response to elevated IOP in glaucomatous optic neuropathy.

GRANTS

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