

Gene Expression Changes in Steroid-Induced IOP Elevation in Bovine Trabecular Meshwork

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PURPOSE. To determine whether gene expression changes occur in the trabecular meshwork (TM) of cow eyes with steroid-induced intraocular pressure (IOP) elevation.

METHODS. Adult female Braford cows ($n = 4$) were subjected to unioocular prednisolone acetate treatment for 6 weeks. IOP was monitored with an applanation tonometer. At the conclusion of the experiment, animals were euthanized, eyes were enucleated, and the TM was dissected and stored in an aqueous nontoxic tissue storage reagent. RNA was extracted and subjected to microarray analysis using commercial oligonucleotide bovine arrays. Some of the genes differentially expressed between control and experimental eyes were confirmed by quantitative RT-PCR and some of the respective proteins were studied by immunoblotting.

RESULTS. IOP began to increase after 3 weeks of treatment, reaching a peak 2 weeks later. IOP differences between corticosteroid-treated and fellow control eyes were 6 ± 1 mm Hg (mean $\pm$ SD) at the conclusion of the study. Microarray analysis revealed that expression of 258 genes was upregulated, whereas expression of 187 genes was downregulated in the TM of eyes with steroid-induced IOP elevation. Genes identified to be differentially expressed include genes coding for cytoskeletal proteins, enzymes, growth and transcription factors, as well as extracellular matrix proteins and immune response proteins. A number of relevant gene networks were detected by bioinformatic analysis.

CONCLUSIONS. Steroid-induced IOP elevation alters gene expression in the bovine TM. Identification of genes with changing expression in this model of open-angle glaucoma may help elucidate the primary changes occurring at the molecular level in this condition. (*Invest Ophthalmol Vis Sci.* 2011;52:8636–8645) DOI:10.1167/iops.11-7563

Glucocorticosteroids are known to cause intraocular pressure (IOP) elevation.^{1–3} Steroid-induced ocular hypertension generally occurs within weeks in susceptible individuals

but is usually reversible with discontinuation of steroid treatment.³ However, if steroid treatment is continued for prolonged periods of time it can lead to glaucomatous optic neuropathy.⁴ The open-angle glaucoma so produced resembles primary open-angle glaucoma not only in the optic neuropathy but also in the mechanism by which IOP is elevated, that is, a decrease of trabecular meshwork (TM) outflow facility.⁵ Even though some of the cellular and molecular mechanisms underlying this decrease in outflow facility have been previously studied in tissue and/or organ culture, the exact pathophysiology of this condition remains elusive. We have shown that 100% of bovine eyes tested respond with a significant IOP elevation when cows were given daily topical corticosteroid treatment for a period of more than 4 weeks.⁵ The consistency of the steroid response in this species, in conjunction with the size of the bovine eye and its similarities to the human eye, make it an ideal animal model for understanding open-angle glaucoma caused by decreased TM outflow facility. We have also shown that the TM of steroid-treated cows exhibits histopathologic alterations that may be responsible for IOP elevation in this species.⁶ These changes are highly similar to changes observed in the TM of humans with glaucoma.^{7,8} We thus tried to determine gene expression changes that occur in the bovine TM shortly after IOP elevation. We hypothesized that some of these changes in gene expression would be responsible for the histopathologic changes detected and thus the reason for IOP elevation. In this report we explore the differences in gene expression induced in the TM of cows by treatment with corticosteroids of sufficient duration to cause IOP elevation.

MATERIALS AND METHODS

Animals

Animal experiments were performed in Corrientes, Argentina under the supervision of a local veterinarian. All animal experimentation protocols have been previously approved by the local Institutional Animal Care and Use Committee of the Universidad Nacional del Nordeste School of Medicine in Argentina. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and all animal experiments were performed according to the ARVO guidelines.

Four healthy Braford (female) cows (between 3 and 5 years of age, weighing 350 to 420 kg) were selected for this study from a local ranch. The cows were tagged for individual identification on their ear lobes. They were herded from pasture whenever it was necessary to instill the drops or to measure the IOP; IOP measurement and drop instillation were performed with the animals restrained in a loosely fitting yoke positioned at the end of a funnel corral. This allowed movement and holding of the head by one person while another cowboy instilled the drops and one of the investigators measured the IOP. This procedure took approximately 4 minutes per cow; otherwise, the cows were free to pasture.

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At the conclusion of the study all animals were euthanized and the eyes were removed and placed on ice.

Steroid-Induced IOP Elevation and Monitoring

IOP elevation was induced as previously described.⁵ Specifically, after two baseline measurements of IOP at least 3 days apart, prednisolone acetate 0.5% (Ultra-cortenol; Novartis Ophthalmics, Hettlingen, Switzerland) was instilled in one eye of each animal. Artificial tears were instilled in the contralateral eye. Both control and experimental instillations consisted of 2 drops, administered three times daily at 8:00 AM, 2:00 PM, and 6:30 PM for the duration of intervention. Investigators (who measured IOP) and cowboys (who instilled the drops) were masked to the study protocol. IOP measurements were performed (by RG) with an applanation tonometer (Clement Clarke Perkins MK2; World Precision Instruments, Sarasota, FL) under topical anesthesia with 0.5% proparacaine and fluorescein. Two measurements were obtained per eye and averaged. Tonometer readings were recorded and converted off-line to IOP values using a previously constructed ex vivo calibration curve. All IOP measurements were performed between 8:30 AM and 10:00 AM.

RNA Isolation and Microarray Gene Expression Profiling

On euthanization, the aqueous humor was exchanged with a solution of nontoxic tissue storage reagent (RNAlater; Qiagen, Valencia, CA) using two needles simultaneously inserted in the anterior chamber (one used for removal of aqueous and the other for injection of RNAlater). Eyes were then opened anterior to the equator using a razor blade. The lens was removed from the anterior part, together with the bulk of the ciliary body and iris by gentle traction, and the anterior eye cups were placed in tissue storage reagent solution. Tissues remained at 4°C in the tissue storage reagent while being transported to the United States. On arrival to the United States, tissues were further dissected to isolate the TM. Dissection was performed as previously described⁹ by creating cuts anteriorly and posteriorly to the TM and removing it using a jewelers forceps.

RNA was extracted from TM tissue using a commercial reagent (TRIzol; Qiagen) after washing carefully with diethylpyrocarbonate-treated water to remove traces of tissue storage reagent (RNAlater). Briefly, the tissue was homogenized (in TRIzol) and chloroform was added to separate proteins from RNA. After being centrifuged, the RNA-containing supernatant was aspirated and the organic phase was used for protein extraction. The RNA was precipitated with isopropanol, washed in 70% ethanol, and column purified using a commercial kit (RNAeasy Mini Kit; Qiagen) in accordance with the manufacturer's instructions and subjected to microarray analysis (MA). Proteins were extracted from the organic phase by dialyzing in cellulose dialysis tubing against three changes of 0.1% SDS at 4°C for 48 hours. Protease inhibitors were added, and the samples were stored in -80°C until being subjected to immunoblotting (see the following text). RNA quality control testing was performed with commercial analytic instrumentation (2100 Bioanalyzer; Agilent Technologies, Inc., Santa Clara, CA) based on the UCLA data quality guide (<http://core.genetics.ucla.edu/public/EDCGuidelines>). Linear amplification, cRNA synthesis, and hybridization to bovine oligonucleotide arrays (GeneChip Bovine

Genome Array; Affymetrix, Santa Clara, CA) were performed at the Mount Sinai microarray core facility. Signal intensities for approximately 23,000 probe sets (each probe set consists of 11 perfect matches and 11 mismatches) were obtained from each array. Data quality was verified by inspection of various plots of the probe signal intensities such as histograms, box plots, and RNA degradation plots as well as different quality metrics (Affymetrix) such as the percentage of probe sets that are called "present" by an expression summary algorithm (MAS5.0 algorithm; Affymetrix),¹⁰ the average background, the scale factor, and the glyceraldehyde-3-phosphate dehydrogenase and β -actin 3':5' ratios.

Probe intensities of the whole data set were processed using a commercial package (GeneChip Robust Multi-Array [gcrma]¹¹ Bioconductor Software).¹² The gcrma package adjusts for background intensities in array data (Affymetrix), which include optical noise and non-specific binding, and converts background-adjusted probe intensities to expression measures after normalization. The summarized data are thus normalized signal intensities that are logarithm base 2 transformed. These summarized data were subjected to statistical analysis (see the following text).

Quantitative Real-Time-PCR Gene Expression Quantification

Quantitative real-time PCR (QRT-PCR) was performed to confirm a number of the genes identified to be differentially expressed in the MA procedure. RNA from paired samples from the same eyes used for MA were reverse transcribed with random hexamers to cDNA using a reverse transcription kit (Quantitect; Qiagen), in accordance with the manufacturer's instructions. cDNA was prepared for RT-PCR using a commercial kit (QuantiTect SYBR Green QRT-PCR Kit; Qiagen) and specific primer sets (Table 1). Primer specificity was confirmed by verification of generation of a single PCR product of the expected size after 30 rounds of amplification on a thermal cycler (Biometer T1; Biometra GmbH, Goettingen, Germany) using cDNA transcribed from other cow eye tissues. RT-PCT analysis was performed (SYBR Green labeling) at the Mount Sinai QRT-PCR core facility.

Immunoblotting

Proteins selected for immunoblotting analysis were, at the time, the only proteins corresponding to genes with changing expression for which an antibody with documented bovine specificity was commercially available.

Protein samples were diluted in Laemmli sample buffer, separated in a 4–20% SDS-PAGE gel (Bio-Rad, Hercules, CA), and transferred to polyvinylidene difluoride membranes. The membranes were blocked with a commercial blocking solution (Pierce Superblock; Thermo Fisher Scientific, Rockford, IL) and incubated with a mouse monoclonal goat polyclonal antichondromodulin antibody (Mab41681, 1:1000, overnight at 4°C; R&D Systems, Minneapolis, MN) or a rabbit polyclonal anti-GPNMB antibody (H-121, 1:1000, overnight at 4°C; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with a horseradish peroxidase-conjugated anti-mouse or rabbit IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 2 hours at room temperature. A rabbit anti- β -actin-specific antibody (1:5000, overnight at 4°C; Abcam, Cambridge, MA) was used to verify

TABLE 1. Primers Used in QRT-PCR

Gene ID	Gene Name	Forward Primer	Reverse Primer
CHM-1	Chondromodulin 1	cccagactggatcatgaagg	ttatagggccatgggttggtat
KCNMA 1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	tgccagttaaagtgcacaaca	ttggcccattctattcatcc
TPM2	Tropomyosin 2	cgaaaaccgagctatgaagg	ctcatatttgcggtccgaat
TYRP1	Tyrosinase-related protein 1	tttgccctccagttaccaac	ctggcagcaatcagacaaga
VEGFA	Vascular endothelial growth factor	tttccaatctctctctctgatcg	ccttatttcaaaggaatgtgtgg
ADRB2	Adrenergic receptor beta 2	tcctcttgcctggaacttg	cgaagggtccgagagactca

that equal amounts of protein have been loaded in each lane. Antibody binding was visualized using an enhanced chemiluminescent substrate (ECL Kit; Pierce, Rockford, IL) and a commercial imager (Kodak Image Station 440CF; Kodak, Boston, MA) and quantified using image analysis software (ImageJ developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>) after normalizing for actin concentration.

Statistical and Bioinformatics Analysis

TM samples from cow eyes were compared as pairs. For each gene, the comparison between TM from the steroid-treated and that from the untreated eye is based on a moderated *t*-statistic, defined to be

$$\frac{\bar{x} - \bar{y}}{s + s_0}$$

where $\bar{x}, \bar{y}$ are the average log 2 signal intensities from the *gcrma* package for the two groups, respectively, and s is the SE of the $\bar{x} - \bar{y}$, equal to $\sqrt{s_x^2/n_x + s_y^2/n_y}$. s_0 takes the 10th percentile of s among 23,000 probe sets, which is used to make sure the two-sample *t*-statistic does not become extremely large due to just an extremely small value of s . Significant genes were selected by the following criteria: the moderated *t*-statistic is not <3 in absolute value, the maximal signal intensity is not <4 , and the log 2-fold change ($\bar{x} - \bar{y}$) is not <0.5 in absolute value.

Probe set identifiers (Affymetrix Chip [Affy] IDs) that were identified to be differentially expressed were manually curated to determine whether they correspond to specific genes or whether they represent only expressed sequence tags but cannot be assigned to any specific genes.

Genes thus selected were subjected to bioinformatics analysis using commercial analysis software (Ingenuity Pathway; Ingenuity Systems, Redwood City, CA) to determine functional gene networks and canonical groups whose expression is differentially affected between the TM of eyes with steroid-induced IOP elevation and that of contralateral eyes. Since the knowledge database (Ingenuity Systems) does not contain bovine data, pathway analysis was performed using mouse, rat, and human data.

RESULTS

IOP in Steroid-Treated Cow Eyes

Mean IOP at baseline was 14.8 ± 0.7 mm Hg (mean $\pm$ SD) for control eyes and 14.9 ± 0.8 mm Hg for experimental eyes ($P > 0.05$, *t*-test). IOP increased substantially during the course of the experiments in all eyes treated with corticosteroid when compared with both baseline reading as well as that of contralateral eyes ($P < 0.0001$, ANOVA) (Fig. 1). All animals had at least a 5 mm Hg IOP difference between contralateral eyes at the time of euthanasia (which represents a 31% increase in IOP from the nonsteroid-treated eye). This IOP difference was maintained for at least 5 days in all animals before euthanasia.

Gene Expression Changes in the TM

Paired comparison of gene expression in the TM of steroid-treated eyes identified 278 downregulated and 341 upregulated probe set identifiers (Affy IDs) when compared with the TM of placebo-treated eyes. These identifiers (Affy IDs) corresponded to 187 and 258 genes, respectively. The first 20 (with the largest fold change) of each of the up- and downregulated genes and their log 2-fold changes are shown in Table 2. Human orthologs of 197 of the genes with changing expression ($\sim 44\%$ of the genes identified) are known to have potential glucocorticoid-responsive elements up to 20 kB upstream of the initiation codon.

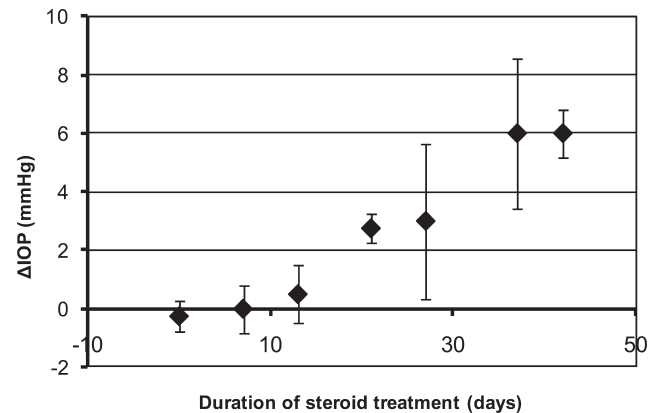


FIGURE 1. IOP history of cow eyes used in MA. Δ IOP represents the difference between steroid-treated and -untreated eyes. Error bars represent SDs.

QRT-PCR

To confirm microarray results a number of up- and downregulated genes, belonging to different gene networks, were selected and their expression differences were confirmed by QRT-PCR (Fig. 2).

Immunoblotting

To determine whether changes in gene expression translate into protein expression changes, one of the upregulated (*Chondromodulin* [*ChM*]) and one of the downregulated (*GPNMB*) genes were selected. Differences in the amount of the respective proteins detected by immunoblotting were normalized for the amounts of β -actin. Protein quantity increased ($P < 0.003$, *t*-test) and decreased ($P < 0.04$, *t*-test), respectively, in TM of eyes treated with corticosteroid compared with contralateral control eyes, in accordance with gene expression changes observed using MA (Fig. 3).

Bioinformatic Analysis of Microarray Data

Of the genes affected, 47 encoded for proteins with known localization to the extracellular space, 116 with known localization to the cell membrane, 126 with known localization to the cytoplasm, and 50 with known nuclear localization. Genes affected encoded for a variety of classes of molecules (Table 3).

Further bioinformatic analysis (Ingenuity Pathway) revealed that of the genes with differential expression, 390 could be mapped to 24 functional networks. The most heavily populated network included 28 genes (80% of the genes included in the network) and is related to cellular compromise, gene expression, cellular assembly, and organization. Twenty-two of the 24 networks identified overlapped by one or more molecules, linking approximately 770 genes, 388 ($\sim 50\%$) of which expression appeared to change. Two of the top 10 networks with molecules that have been previously linked to steroid-induced glaucoma as well as novel ones are shown in Figures 4 and 5.

When organized in canonical pathways, integrin signaling ($P = 6.78\text{E-}07$), germ cell-Sertoli cell junction signaling ($P = 4.73\text{E-}06$), and integrin-linked signaling ($P = 6.13\text{E-}06$) were the most affected, with approximately between 8 and 9% of the molecules in each of these pathways changing expression (18 of 205, 15 of 168, and 16 of 191 molecules, respectively). Molecules with changing expression in the integrin signaling pathway are listed in Table 4.

DISCUSSION

Glucocorticosteroid-induced IOP elevation is a well-described side effect of steroid therapy in some individuals. Corticoste-

TABLE 2. Top Up- and Downregulated Genes in the TM of Eyes with Steroid-Induced IOP Elevation Compared with Their Contralateral Controls

Gene ID	Gene Name	Log 2-Fold Change
Upregulated Genes		
<i>KCNMB1</i>	Potassium large conductance calcium-activated channel, subfamily M, beta member 1	4.12
<i>ITGA8</i>	Integrin, alpha 8	3.35
<i>DES</i>	Desmin	3.29
<i>PLN</i>	Phospholamban	2.82
<i>ACTA2</i>	Actin, alpha 2, smooth muscle, aorta	2.75
<i>RBM24</i>	RNA binding motif protein 24	2.58
<i>PTPRR</i>	Protein tyrosine phosphatase, receptor type, R	2.37
<i>COL24A1</i>	Collagen, type XXIV, alpha 1	2.32
<i>CNN1</i>	Calponin 1, basic, smooth muscle	2.18
<i>AGT</i>	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	2.16
<i>SMTN</i>	Smoothelin	2.06
<i>RASL12</i>	RAS-like, family 12	2.04
<i>TGFB111</i>	Transforming growth factor beta 1 induced transcript 1	1.99
<i>CD55</i>	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	1.95
<i>CKB</i>	Creatine kinase, brain	1.91
<i>MRV11</i>	Murine retrovirus integration site 1 homolog	1.89
<i>PCP4L1</i>	Purkinje cell protein like 1	1.88
<i>HSPB8</i>	Heat shock 22-kDa protein 8	1.80
<i>TAGLN</i>	Transgelin	1.73
<i>GPR162</i>	G-protein-coupled receptor 162	1.72
Downregulated Genes		
<i>RGS1</i>	Regulator of G-protein signaling 1	-2.78
<i>GPR37</i> /// <i>LOC790388</i>	G-protein-coupled receptor 37 (endothelin receptor type B-like) /// similar to probable G-protein-coupled receptor 37 precursor (endothelin B receptor-like protein 1) (ETBR-LP-1) (Parkin-associated endothelin receptor-like receptor (PAELR))	-2.23
<i>SLC45A2</i>	Solute carrier family 45, member 2	-2.13
<i>CA2</i>	Carbonic anhydrase II	-1.91
<i>TRPA1</i>	Transient receptor potential cation channel, subfamily A, member 1	-1.88
<i>ATP6V0D2</i>	ATPase, H ⁺ transporting, lysosomal 38 kDa, V0 subunit d2	-1.82
<i>IGHG1</i>	Immunoglobulin heavy constant gamma 1	-1.81
<i>TMEM22</i>	Transmembrane protein 22	-1.80
<i>MREG</i>	Melanoregulin	-1.77
<i>NELL2</i>	NEL-like 2 (chicken)	-1.69
<i>SLC6A1</i>	Solute carrier family 6 (neurotransmitter transporter, GABA), member 1	-1.66
<i>F2RL1</i>	Coagulation factor II receptor-like 1	-1.65
<i>CEACAM8</i>	Carcinoembryonic antigen-related cell adhesion molecule 8	-1.62
<i>LOC514078</i>	Similar to Myosin-Vb	-1.60
<i>ALDH1A3</i> /// <i>LOC534200</i>	Aldehyde dehydrogenase 1 family, member A3///similar to aldehyde dehydrogenase family 1, subfamily A3	-1.56
<i>GLT25D2</i>	Glycosyltransferase 25 domain containing 2	-1.56
<i>PAH</i>	Phenylalanine hydroxylase	-1.54
<i>MGC148992</i>	Similar to RGC-32	-1.50
<i>LOC514078</i>	Similar to Myosin-Vb	-1.48
<i>HOPX</i>	HOP homeobox	-1.47

roids seem to affect IOP in a dose- and duration-dependent manner and can exert such an effect when administered by a variety of routes.¹³ If the duration of corticosteroid therapy is lengthy it can lead to glaucomatous optic neuropathy.⁴ The prevalence of steroid-induced glaucoma has been increasing over the past few years because potent, long-lasting steroids are increasingly used to treat many posterior pole conditions.^{14,15} It has been suggested that a specific common relationship exists between this ocular response to corticosteroids and the factors in the TM that cause primary open-angle glaucoma.¹⁶ Steroid-induced glaucoma is currently treated medically by either decreasing aqueous inflow (β -blockers, carbonic-anhydrase inhibitors, α 2-agonists) or increasing uveoscleral (nontrabecular) outflow (prostaglandin analogs and α 2-agonists). However, neither of these approaches addresses the underlying pathology at the level of the TM, because the underlying mechanisms for development of steroid-induced reduction in outflow facility remain largely unknown. Therefore, understanding the cellular and molecular processes that

lead to corticosteroid-induced ocular hypertension is important because it may illuminate the mechanisms for primary open-angle glaucoma and lead to new therapies to lower IOP. The present work is a first attempt to understand the early molecular events that lead to steroid-induced IOP elevation.

Many investigators have studied the effects of glucocorticoids on the TM (reviewed in Wordinger and Clark¹⁷ and Jones 3rd and Rhee¹⁸). The work has used cultured TM cells,^{19–23} organ cultured eyes,^{24–30} and some in vivo models.^{31–33} These investigations have targeted morphologic changes, as well as gene expression, extracellular matrix (ECM), cytoskeleton, and cell adhesion molecules in the TM (extensively reviewed in Wordinger and Clark¹⁷ and Borrás^{34,35}). However, most of these studies are performed on cell or organ cultures that are not necessarily representative of the in vivo condition. For example, myocilin—one of the molecules associated with the development of glaucoma when mutated—is expressed in high amounts in trabecular cells in vivo,³⁶ but is barely expressed in vitro unless cultures are exposed to corticosteroids.³⁷ The few

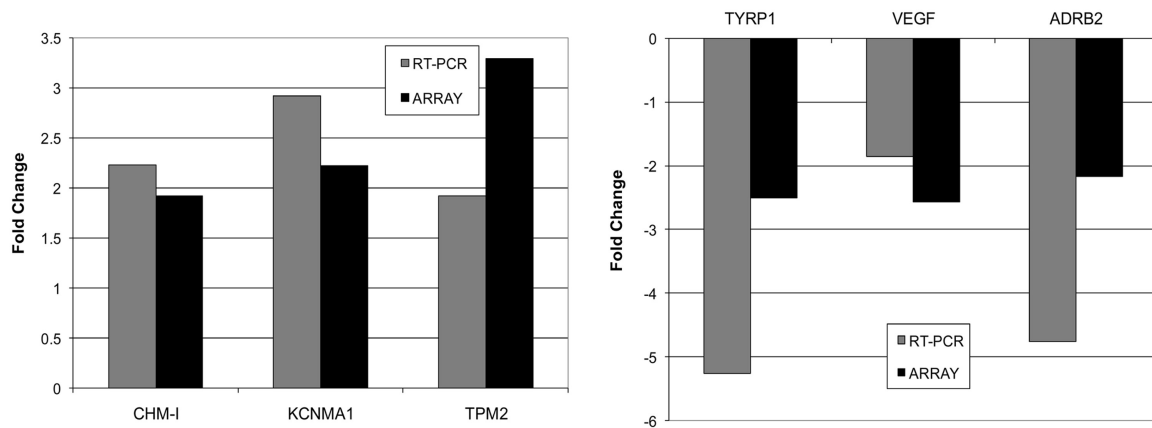


FIGURE 2. Comparison of fold-change of genes with changing expression by MA and by QRT-PCR. Three genes each from both up- and downregulated genes detected by MA were confirmed by QRT-PCR. CHM-I, chondromodulin 1; KCNMA1, potassium large conductance calcium-activated channel, subfamily M, alpha member 1; TPM2, tropomyosin 2; TYRP1, tyrosinase-related protein 1; VEGF, vascular endothelial growth factor; ADRB2, adrenergic receptor beta 2.

in vivo studies performed thus far have initially focused on anatomic alterations³⁸ and on candidate genes and their protein products.³⁹ A few global gene expression investigations have compared gene expression in organ-cultured TM between normal and glaucomatous eyes.^{40,41} The present work represents the first time that global gene expression in the TM has been studied, shortly after the induction of elevated IOP in vivo. Although direct (gene by gene) comparison with previous studies mentioned earlier is not appropriate because of differences in the species, differences in the doses and type of steroids, and differences in the experimental setup (in vitro versus in vivo), the role of certain cell processes, pathways, or components is further corroborated (e.g., the role of ECM modulation).

In contrast to humans and monkeys, where the elevation of IOP induced by corticosteroids appears to occur in only approximately 30%⁴² and 50%,⁴³ respectively, 100% of cows tested⁵ and other ruminants⁴⁴ develop IOP elevation. Thus, these animals are ideal for studying the molecular mechanisms that lead to steroid-induced IOP elevation. The animal model that was used in our present work shares significant similarities with the human eye, both anatomically and in terms of the physiology of the aqueous humor formation. Bovine aqueous humor, as in humans, has a higher concentration of chloride than that of plasma,⁴⁵ and the isolated bovine ciliary epithelium transports chloride and is inhibited by carbonic anhydrase inhibitors.⁴⁶ On the outflow side, TM in the bovine eye is anatomically similar to the human TM (although a more formed pectinate ligament is present) and also shares other important homologies.^{47–49} The

bovine aqueous plexus is the equivalent of the human Schlemm's canal.⁵⁰ At the same time it should be pointed out that there are also differences between the bovine and human TM function. In perfused anterior segments, human eyes do not show the phenomenon of "washout,"⁵¹ whereas bovine eyes do.⁵² It has been proposed that the human eyes have a more extensive network of elastic fibers in the cribriform plexus, preventing separation of inner wall from the juxta-canalicular tissue during perfusion.⁵³ This anatomic difference may account for the large amounts of extracellular material accumulating in this region in the cow eyes after relatively short term exposure to steroids.⁶

We have previously reported the use of cows for studying the microscopic and ultramicroscopic changes at the level of the TM after steroid-induced IOP elevation.⁶ In those experiments, as in the present study, we used animals that were subjected to at least 6 weeks of steroid treatment. Although investigating an earlier time point could detect a gene set enriched in genes leading to IOP elevation, it was elected to use the same time point as that in our earlier study. This time point was chosen not only to make the results comparable but also to ensure that IOP elevation would be present in all treated eyes, thus minimizing the variability inherent in experiments that do not use genetically inbred strains of animals (IOP elevation is not entirely synchronous, although it occurs in all animals tested). At the same time we did not want to have IOP elevated for prolonged periods of time to minimize secondary changes in gene expression. As expected, IOP elevations were achieved in all experimental eyes after approximately 4 weeks

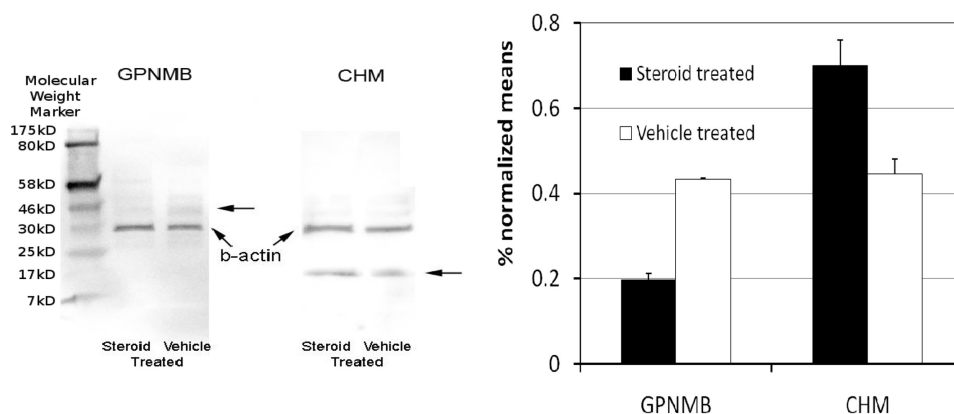


FIGURE 3. Representative immunoblot of bovine TM from an animal treated in one eye with topical prednisolone acetate three times a day for 45 days. The contralateral eye received vehicle only. Immunoblots were incubated with antibodies against GPNMB and chondromodulin (ChM). Arrows point to protein-specific bands. Lane 1 is MW marker. Error bars on quantification bar graph represent SEM.

TABLE 3. Numbers of Genes Encoding for Various Categories of Proteins with Changing Expression in the TM of the Bovine Steroid-Induced IOP Model

Class of Encoded Proteins	Number Affected	Examples
Cytokines	3	Family with sequence similarity 3, member C
Enzymes	69	Glycoprotein (transmembrane) nmb
G-protein-coupled receptors	10	Adrenergic, beta-2-, receptor
Growth factors	6	Vascular endothelial growth factor A
Ion channels	10	Potassium large conductance calcium-activated channel, subfamily M, beta member 1
Kinases	17	Mitogen-activated protein kinase 14
Ligand-dependent nuclear receptors	1	Nuclear receptor subfamily 2, group F, member 2
Peptidases	10	Complement component 1, s subcomponent
Phosphatases	7	Protein tyrosine phosphatase, receptor type, R
Transcription regulators	22	TEA domain family member 3
Transmembrane receptors	7	Retinoic acid receptor responder (tazarotene induced) 2
Transporters	30	ATPase, H ⁺ transporting, lysosomal 38 kDa, V0 subunit d2
Others	186	Smoothelin

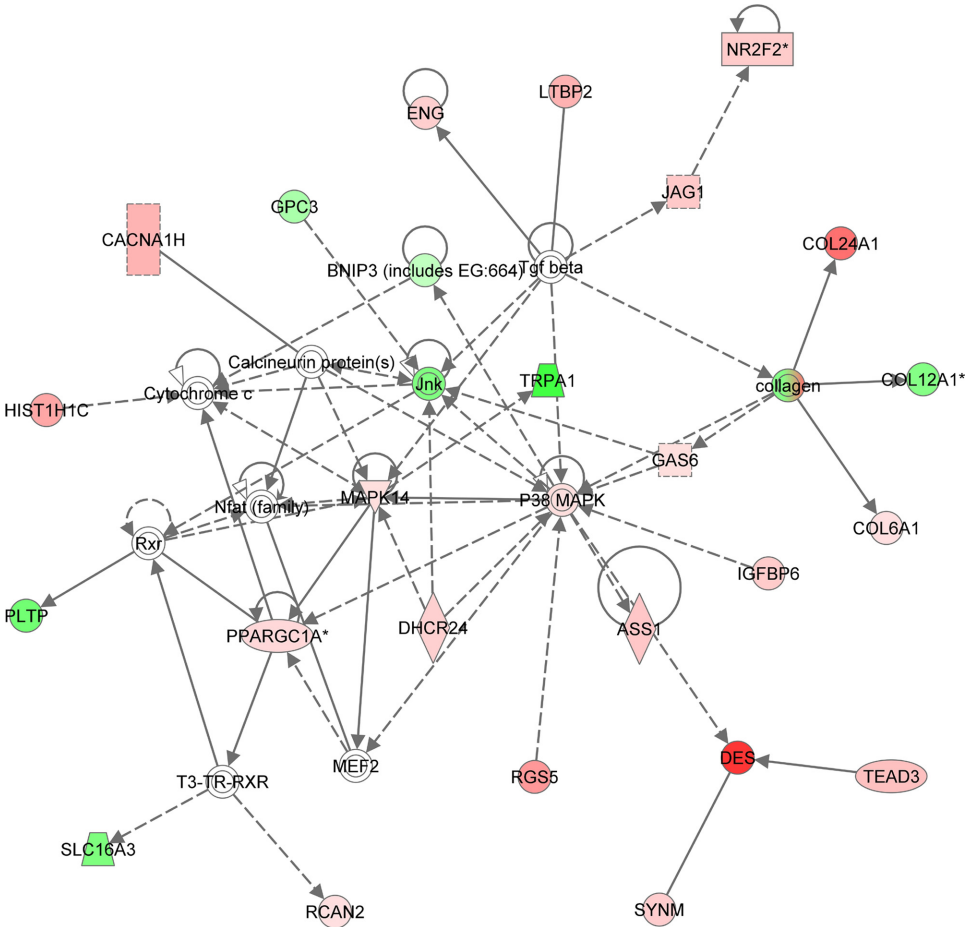
of treatment. IOP elevation was comparable in this set of animals to that in our previous report.⁶

We have used oligonucleotide microarrays to study global gene expression in the TM. Oligonucleotide arrays have the advantage of greater specificity because they can be tailored to minimize chances of cross-hybridization.^{5,4} Other major advantages of this approach include a uniform probe length and the ability to discern splice variants. The availability of genetic information for the cow has allowed us to use species-specific arrays, thus decreasing the ambiguity in gene calls. Using this approach we were able to identify a significant number of genes with changing expres-

sion as a result of steroid treatment and subsequent IOP elevation. The genes with changing expression can be in either one of the following four categories:

1. Genes that are involved in IOP elevation
2. Genes that are directly affected by steroid treatment but are unrelated to IOP elevation
3. Genes with expression that is changing because of IOP elevation
4. Genes with expression that is indirectly changing as a response to steroid-induced changes in the tissue but that are unrelated to IOP elevation

FIGURE 4. One of the top networks of genes with altered expression in the TM of unilaterally steroid-treated cows identified by network analysis (Ingenuity Pathway); 28 of 35 genes (80%) in this network appear to be changing in expression. Downregulated genes are shown in green, upregulated genes in red. Intensity of color indicates fold change (i.e., deeper colors represent greater change). Note that Jnk is downregulated, whereas MAPK14 is upregulated.



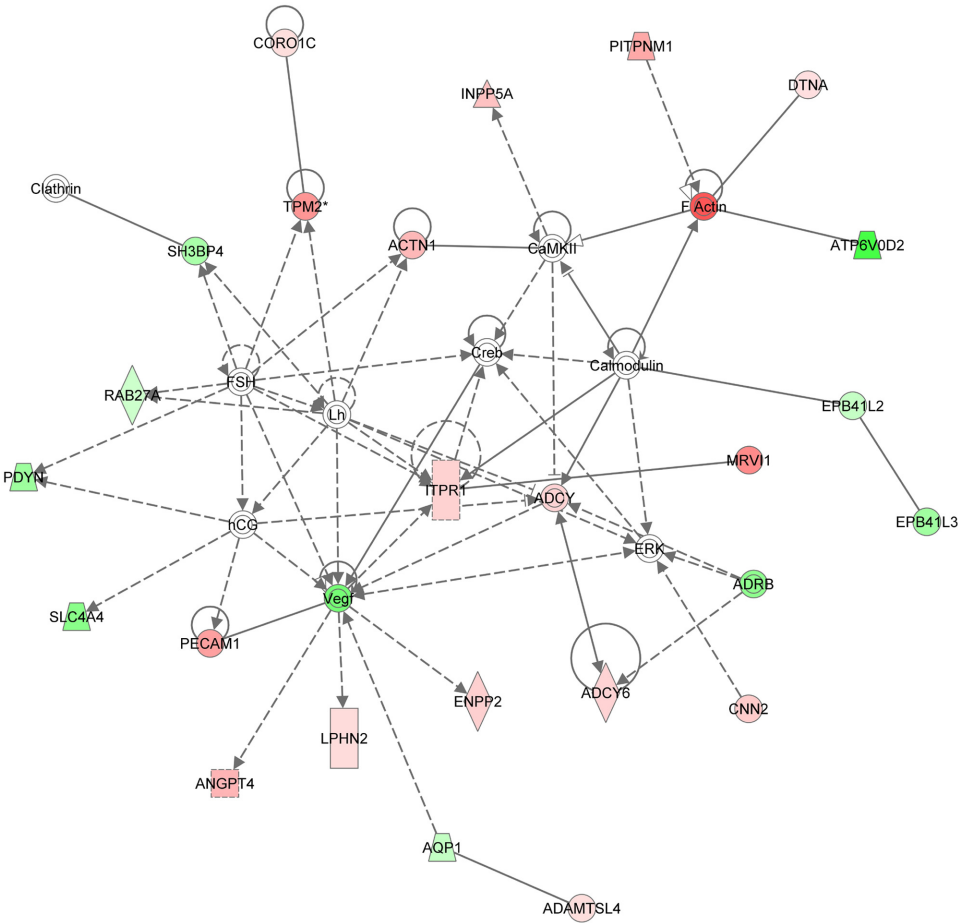


FIGURE 5. One of the top networks of genes with altered expression in the TM of unilaterally steroid-treated cows identified by network analysis (Ingenuity Pathway); 27 of 35 genes (~77%) in this network appear to be changing in expression. Down-regulated genes are shown in green, up-regulated genes in red. Intensity of color indicates fold change (i.e., deeper colors represent greater change). Note that VEGF is down-regulated, whereas adenylate cyclase (ADCY) is upregulated.

Thus gene expression changes can be either the direct effect of steroid therapy on TM cells (some causing IOP elevation) or occur secondarily as a result of activation/or suppression of other genes. The design of the present experiment does not allow us to determine which category the identified genes

TABLE 4. Integrin Pathway Genes with Changing Expression in the TM of Eyes with Steroid-Induced IOP Elevation Compared with Their Contralateral Controls

Gene Symbol	Gene Name	Log 2-Fold Change
ACTA2	Actin, alpha 2, smooth muscle, aorta	2.75
ACTN1	Actinin, alpha 1	1.16
CAPN6	Calpain 6	1.39
DIRAS3	DIRAS family, GTP-binding RAS-like 3	-1.16
ILK	Integrin-linked kinase	0.80
ITGA8	Integrin alpha 8	3.35
MYLK	Myosin light chain kinase	1.45
PIK3C2B	Phosphoinositide-3-kinase, class 2, beta polypeptide	0.77
PPP1CC	Protein phosphatase 1, catalytic subunit, gamma isozyme	-0.59
RAP2B	RAP2B, member of RAS oncogene family	0.53
RHOC	ras homolog gene family, member C	0.59
RRAS	Related RAS viral (r-ras) oncogene homolog	1.36
TLN1	Talin 1	0.80
TSPAN4	Tetraspanin 4	0.77
TSPAN5	Tetraspanin 5	-0.51
TSPAN6	Tetraspanin 6	-0.82
TTN	—	-0.77
VCL	Vinculin	0.85

fall into, but does narrow the field of possible genes involved. Based on human genomic data, approximately half of the genes identified are potentially directly regulated by steroids. Indeed, some of the genes identified using the present approach have been previously implicated by others in the pathogenesis of steroid-induced IOP elevation and in open-angle glaucoma. For example, 7 (17.5%) of the 40 genes proposed to constitute the molecular signature of human glaucomatous TM³⁵ were also detected to be changing by the present analysis (these are aB-crystallin, cadherin, insulin-like growth factor-binding proteins, metallothioneins, thrombomodulin, transgelin, and tropomyosin). Using microarrays to identify genes, of course, poses some limitations that are inherent to this methodology.^{55,56} Genes with low-fold change are less likely to be detected than genes with dramatically different gene expression. In addition, a portion of the genes identified are false positives and some genes with truly changing expression fail to be identified. Thus, for example, myocilin whose expression is known to change in steroid-induced IOP elevation in perfused human⁵⁷ organ cultures failed to reach threshold values and was called “nonchanging.” Although this is rather surprising, it could also represent a true finding. Myocilin polymorphisms have not been associated with steroid-induced glaucoma in humans or primates⁴³ and myocilin induction by steroids has to our knowledge been reported only in cultured bovine TM cells⁴⁸ (but not in perfused bovine anterior segments). To overcome some of the inherent limitations of microarrays, traditional strategies such as confirmation of gene changes using QRT-PCR can be used. It is generally accepted that QRT-PCR is more reliable than MA when it comes to

individual genes.⁵⁸ However, QRT-PCR analysis can be performed on only a limited number of genes. We have used QRT-PCR to confirm a limited number of interesting genes. Some of these genes have been previously implicated in IOP elevation, whereas the role of others is yet unclear. In particular we would point out the changes in expression of *TYRP1*. Lack of *TYRP1* has been implicated in glaucoma development in DBA/2 mice.⁵⁹

Using a bioinformatics analysis approach, we have identified a number of gene networks that are affected in steroid-induced IOP elevation in the cow TM. Some of these networks (Fig. 4) are predictable based on what we already know about glaucoma. *TGF β* is known to be involved in the pathogenesis of the disease.⁶⁰ Thus, even if the selection criteria for identifying genes with changing gene expression failed to detect change in *TGF β* expression, its known role in the development of IOP elevation confirms the importance of the associated molecules within this network. Moreover, given the substantial number of genes in each of the networks identified one can safely assume that the whole network activity has been altered. One of the genes identified in this manner is *MAPK14* (mitogen-activated protein kinase 14, p38 MAPK) (see Fig. 4). Its position in the network allows it to regulate a number of other genes, some of which have been implicated in glaucoma by the present work and that of others.^{60–66} *MAPK14* is normally activated by various environmental stresses and proinflammatory cytokines and plays a critical role in the production of some cytokines (e.g., IL-6). In the bovine steroid-induced IOP elevation model *MAPK14* expression in the TM was upregulated 1.4-fold in the steroid-treated eye. It has been reported⁶⁷ that in open-angle glaucoma *MAPK14* is relatively unresponsive to IL-1 signaling, indicating a constitutive activation. Interestingly other MAPKs (e.g., *MAPK10*) seem to be downregulated in the bovine steroid-induced IOP elevation model.

Other networks, like the one depicted in Figure 5, suggest that additional genes may be involved. Some of these genes such as *VEGF* can be potentially interesting. Although it is unclear what its role in the TM is, one can speculate that since *VEGF* can affect vascular permeability, it may have a similar role in affecting TM permeability. Acute elevation of IOP in perfused TM leads to *VEGF* upregulation, although perfusion for longer times has been associated with downregulation of the gene.³⁵ Similarly, changes in expression of the adrenergic receptor beta 2 (*ADRB2*), may be relevant because its main ligand (epinephrine) is known to affect outflow facility⁶⁸ through beta adrenergic receptors,⁶⁹ probably by increasing paracellular flow.⁷⁰ It is of course because of the design of these experiments that it is difficult to distinguish which of the steroid effects are related to IOP elevation and which are concurrent but unrelated effects of steroids on the TM. However, these networks provide a framework for confirming that individual genes are involved in a process and exploring how related genes are also affected. Moreover, it potentially allows identification of key molecules that can potentially affect the behavior of the whole network.

We have previously identified collagen VI as one of the components of plaques accumulating in steroid-induced glaucoma.⁶ Given the presence of plaques in the extracellular space at the same time point in the same model, we further focused on changes in expression of genes encoding structural proteins of the ECM. Although collagen VI upregulation is detected by MA, collagen XXIV appears to have the greatest fold change among the collagen genes (approximately sevenfold upregulation). COL24A1 (Collagen XXIV A1) encodes one of the lesser studied fibrillar collagens. Its function is currently unclear, although based on the temporal pattern of its expression it has been suggested that it may participate in regulating type I collagen fibrillogenesis at specific anatomic locations

during fetal development.⁷¹ It has been previously detected in the bone, the cornea, and the retina. In addition (and most interestingly) it has multiple glycine-isoleucine pairs that are the target of proteolysis by MMP-1 (upregulation of which we have shown to be effective in preventing as well as reversing IOP elevation in the steroid-induced ovine model⁷²). In addition, collagen XII was downregulated. Collagen XII is a member of the FACIT (fibril-associated collagens with interrupted triple helices) collagen family. It is a homotrimer found in association with type I collagen, an association that is thought to modify the interactions between collagen I fibrils and the surrounding matrix. Other genes encoding for structural proteins that have been identified include laminin γ 1, gelsolin, hyaluronan, and proteoglycan linking protein 1, latent transforming growth factor binding protein 2, and cadherin. It thus appears that even at this relatively early point after the induction of IOP elevation by steroids, significant change in the ECM of the TM occurs. This change is coupled with changes in the integrin pathway that allow cells to communicate with and control the composition of the ECM (see Table 4).

Changes in gene expression do not by themselves affect cellular physiology unless they are followed by changes in protein production. Confirmation of the changes in protein amounts of some of the genes differentially expressed has traditionally been part of many studies of differential gene expression and has been done by immunoblotting. The very limited amount of protein that can often be extracted from small fragments of tissue make the task of confirming differential protein expression one at a time virtually impossible. In this study we have elected to confirm changes in the amounts of two proteins with commercially available antibodies that are documented to cross-react with the bovine protein. One of the proteins, GPNMB, is interesting because its absence is known to be critical for the development of glaucoma in DBA/2 mice similarly to *TYRP1* protein as discussed earlier.⁵⁹ As expected in the steroid-induced cow model GPNMB is also downregulated.

In summary we have identified a number of genes with changing gene expression in the TM of the bovine steroid-induced IOP elevation model. A number of these genes are involved in the pathogenesis of this condition. It is hoped that understanding the interrelations of these genes and the sequence of molecular events that lead to IOP elevation will enable us to devise novel therapeutic strategies to treat steroid-induced glaucoma as well as other open-angle glaucomas. This understanding will be further enhanced from studying earlier points in the process of IOP elevation that we are currently pursuing.

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