RhoA GTPase-Induced Ocular Hypertension in a Rodent Model Is Associated with Increased Fibrogenic Activity in the Trabecular Meshwork

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Ocular hypertension arising from increased resistance to aqueous humor (AH) outflow through the trabecular meshwork is a primary risk factor for open-angle glaucoma, a leading cause of blindness. Ongoing efforts have found little about the molecular and cellular bases of increased resistance to AH outflow through the trabecular meshwork in ocular hypertension patients. To test the hypothesis that dysregulated Rho GTPase signaling and a resulting fibrotic activity within the trabecular meshwork may result in ocular hypertension, we investigated the effects of expressing a constitutively active RhoA GTPase (RhoAV14) in the AH outflow pathway in Sprague-Dawley rats by using lentiviral vector-based gene delivery. Rats expressing RhoAV14 in the iridocorneal angle exhibited a significantly elevated intraocular pressure. Elevated intraocular pressure in the RhoAV14-expressing rats was associated with fibrotic trabecular meshwork and increased levels of F-actin, phosphorylated myosin light chain, α-smooth muscle actin, collagen-1A, and total collagen in the trabecular AH outflow pathway. Most of these changes were ameliorated by topical application of Rho kinase inhibitor. Human autopsy eyes from patients with glaucoma exhibited significant increases in levels of collagen-1A and total collagen in the trabecular AH outflow pathway. Collectively, these observations indicate that increased fibrogenic activity because of dysregulated RhoA GTPase activity in the trabecular AH outflow pathway increases intraocular pressure in a Rho kinase-dependent manner. (Am J Pathol 2015, 185: 496–512; http://dx.doi.org/10.1016/j.ajpath.2014.10.023)
changes such as tissue stiffness because of altered ECM deposition and turnover in the JCT and basement membrane of the inner wall of the SC could lead to increased resistance to AH outflow and elevated IOP. However, little is known about the origin and activation of matrix-producing cells and their role in ECM deposition and turnover in resistance to AH outflow in both normal and glaucomatous eyes. We and others have identified the ability of various physiologic agents, including bioactive lipids (lysophosphatidic acid and sphingosine-1-phosphate), endothelin-1, transforming growth factor (TGF)-β2, connective tissue growth factor (CTGF), and autotaxin, the levels of some of which are known to be elevated in the AH of glaucoma patients, to regulate AH outflow resistance via activation of the Rho/Rho kinase signaling pathway. Most significantly, and in contrast to the effects of Rho kinase and Rho GTPase inhibitors, activation of RhoA signaling in organ-cultured porcine eyes resulted in decreased AH outflow facility, leading us to explore the cellular and molecular bases for the increased resistance to AH outflow elicited by activation of Rho GTPase. Moreover, our recent studies found that TM cells expressing a constitutively active RhoA GTPase (RhoAV14) or treatment of human TM cells with TGF-β2, CTGF, or lysophosphatidic acid led to increased production of not only ECM (collagen-1A, tenascin-C, fibronectin, and laminin) proteins, cytokines, and α-smooth muscle actin (α-SMA) but importantly also induced the expression of fibroblast-specific protein-1 (also known as S100A4), α-SMA, and regulators of epithelial/endothelial to mesenchymal transition, including serum response factor, myocardin-related transcription factor, slug and twist in a Rho kinase-dependent manner to indicate the propensity of TM cells for transdifferentiating into myofibroblast-like α-SMA and collagen-1A–expressing contractile cells. Furthermore, glaucoma patients are reported to have elevated levels of Rho GTPase in the optic nerve head, suggesting plausible involvement of the Rho GTPase signaling pathway in glaucoma pathobiology. Significantly, dysregulation of Rho/Rho kinase signaling is confirmed to be involved in pathophysiology of various diseases, including cardiac, neurologic, kidney, pulmonary, and fibrotic diseases and cancer.

The activated, GTP-bound form of Rho GTPase works in concert with its downstream effector Rho kinase, which is a serine-threonine kinase, to regulate various cellular processes, including cellular contraction, migration, polarity, adhesion, and transcription by controlling actin cytoskeletal dynamics, myosin II activity, and cell adhesive interactions. Further, it is now well recognized that the Rho GTPase/Rho kinase signaling pathway not only controls AH outflow through the TM in perfused eyes but also affects IOP in different animal models and humans. Importantly, these consistent observations have prompted several ongoing phase 2/3 human clinical trials to investigate the use of Rho kinase inhibitors for lowering IOP and treatment of glaucoma. However, the cellular and molecular bases for ocular hypertensive activity of Rho kinase inhibitors is not thoroughly understood. Therefore, in this study we have explored the in vivo effects of constitutively activated RhoA GTPase on IOP in rats and on the fibrogenic response in the trabecular AH outflow pathway. In addition, we evaluated the profiles of collagen-1A and total collagen expression in the AH outflow pathway of human glaucoma specimens along with age-matched nonglaucoma human eyes.

Materials and Methods

Lentivirus Vector Production and Purification

Human RhoA G14V mutant (RhoAV14) was amplified by PCR from a pcDNA 3.1 plasmid (obtained from Dr. Patrick Casey, Duke University, Durham, NC), cloned into a HIV-based bicistronic lentiviral vector TSIN-green fluorescent protein (GFP). The vector allowed simultaneous expression of RhoAV14 and GFP and was purified by using the endotoxin free maxiprep kit (Qiagen Sciences, Germantown, MD). Vasicular stomatitis virus glycoprotein G–pseudotyped HIV vectors were generated with packaging plasmids that expressed constitutively active RhoAV14-GFP as we described earlier. A lentiviral vector expressing GFP alone was prepared and purified for use as a control. Briefly, RhoAV14-expressing transfer construct along with packaging construct (CMV6R8.91) and the envelope plasmid [pMD.G (vasicular stomatitis virus glycoprotein G)] mixed in the ratio of 3:3:1 was used to transfect human embryonic kidney 293T cells by using calcium phosphate transfection. The transfection protocol included treatment of cells with the plasmid DNA of interest, 0.01 mol/L Tris pH 8.0, 2.5 mol/L calcium chloride, and 2× HEPES-buffered saline for a period of 16 hours. Transfection mix was subsequently replaced with fresh cell culture media, and 48 hours later vector was harvested by filtering cell culture conditioned media through a 0.45-μm low protein-binding filter (Millex-HV; Millipore, Cork, Ireland). Supernatant was then concentrated with two rounds of ultracentrifugation, with the second round using a sucrose gradient, and the viral pellet was resuspended in phosphate-buffered saline (PBS). Human embryonic kidney 293T cells were transduced with the vector, and the potency was determined with flow cytometric analysis by using the reporter gene GFP in human embryonic kidney 293T cells.

Animals

Sprague-Dawley female rats (176 to 200 g of weight and aged >60 days) were purchased from Charles River Laboratories (Wilmington, MA) and housed under a standard 12-hour light and dark cycle with food and water provided ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Duke University Medical Center and conducted in accordance with the Declaration of Helsinki. To test the effects of
lentivirus-based RhoAV14 expression on IOP, rats were divided into three groups: with group I (n = 26) and group II (n = 27) serving as controls and receiving either an injection of 10 μL of PBS or GFP-expressing vector [10 μL, titer of 1 × 10^7 transduction units (TU/mL)], respectively. The rats in group III (n = 27) received the RhoAV14-GFP—expressing vector (10 μL, titer of 1 × 10^7 TU/mL). Injections were administered only to the right eye of each rat, whereas the left eye served as the untreated control. The experiments were performed in three independent sets, with Set-1 having 8 rats in each group, Set-2 having 12 rats in each group, and Set-3 having 6 rats in the saline- and 7 in the GFP and RhoAV14-injected groups. Set-3 was used to evaluate the effects of Rho kinase inhibitor on IOP in the RhoAV14-expressing rats.

**Intracameral Injections**

Rats were anesthetized with i.p. injection of ketamine/xylazine at 60 mg per 8 mg/kg body weight. Before administering intracameral injections, the cornea was treated with a drop of topical anesthetic (0.05% proparacaine hydrochloride ophthalmic solution; Akorn Inc., Lake Forest, IL). Intracameral lentiviral vector delivery was performed under an operating microscope, using a 33-gauge needle Hamilton syringe (Hamilton, Reno, NV). Ten microliter of viral vector prep that expressed either GFP or RhoAV14/GFP was injected into the right eye of rats with the needle inserted through the superior cornea at the limbus with the bevel up, to gently reach the center of the anterior chamber. During this process of needle insertion, approximately 10 μL of AH was released to avoid generating high IOP immediately after intracameral injection. When the needle was inside the anterior chamber, the sample was delivered with a gentle push for >30 seconds, and the fluid entry was monitored by direct visualization through the operating microscope. The needle was left in place for at least 2 minutes and was withdrawn gradually to minimize AH leakage. Rats were returned to their cages after they were fully awake.

**IOP Detection**

At the beginning of the study, basal IOPs were recorded in rats by using a Tonolab (iCare Laboratory, Espoo, Finland) after mild sedation with ketamine/xylazine. After injection with saline or lentiviral vectors, IOPs were recorded in both the eyes of sedated rats in a blinded fashion, once every 3 to 4 weeks for the next 5 months (155 days). For IOP measurements, rats were positioned with the visual axis horizontal to the probe, and five consecutive readings (units that correlate to IOP) were obtained. For the Rho kinase inhibitor study, IOP was measured between 1 and 1.5 hours after topical application of Y27632 [Rho kinase inhibitor; (R)-(++)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide dihydrochloride; Tocris Bioscience, Minneapolis, MN]. Day-time IOP recordings were recorded between 10 AM and 1 PM, and night-time recordings were acquired between 10 PM and 1 AM.

Y27632 was reconstituted in PBS (pH 7.4). For evaluating the effects of topical application of this inhibitor, two 4-μL drops of Y27632 at a concentration of 10 mmol/L were applied to eyes (right) that had been intracamerally injected with saline, GFP, or RhoAV14/GFP-expressing viral vector particles, approximately for 5 months after the injection. The left eye (uninjected) of each rat received vehicle control (PBS). Topical administration of Rho kinase inhibitor to the central cornea was performed at 30-second intervals with the eyelids retracted to prevent blinking. Drug application was done every day in the morning between 10 AM and 12 PM for 15 days consecutively.

**Tissue Processing**

Rats were sacrificed on completion of the IOP studies by anesthetization with ketamine/xylazine and transcardially followed by perfusion with PBS and fixation with 4% paraformaldehyde in PBS. The eyes were enucleated, and tiny incisions were made to the cornea to enable entry of the fixative (2.5% glutaraldehyde and 2% formaldehyde for electron microscopy studies) into the anterior chamber. For paraffin embedding, enucleated eyes were formalin-fixed (3.7% neutral-buffered formalin), processed on an automatic tissue processor, followed by paraffin embedding and tissue sectioning (5-μm sections) with the use of core services offered by the Pathology Department, Duke University. For cryosectioning, enucleated eyes were fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4°C for 18 hours and transferred sequentially into 5% and 30% sucrose in the same buffer, each at 4°C overnight. Eye anterior segment cryosections (5 μm) were obtained with a cryostat (Microm HM 550) and mounted on gelatin-coated slides as described earlier by us.45

**Electron Microscopy and Immunogold Labeling**

The glutaraldehyde and formaldehyde-fixed eyes were bisected at the equator, and the anterior segments were cut into four quadrants. Semithin sections of eye anterior segments (0.5 μm) were stained with toluidine blue and were visualized by light microscopy. For transmission electron microscopy (TEM), tissue specimens were fixed with 1.0% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer and were stained with 1% uranyl acetate. Finally, 70-nm-thick microtome sections were stained with KMnO₄ and Sato stain and were photographed with TEM (JEM-1400; JEOL USA, Peabody, MA) as we described previously.45

For immunologic electron microscopy, tissue samples were fixed with 2% to 4% paraformaldehyde in 0.1 mol/L phosphate buffer, dehydrated through a graded series of ethyl alcohols, and embedded in Unicryl (Vector Laboratories, Burlingame, CA). Thin sections (70- to 90-nm thick) were mounted on Formvar/carbon-coated nickel grids. After drying, the grids were floated on drops of 50 mmol/L
glycine for 15 minutes to quench the aldehydes. After rinsing with PBS, the grids were then placed into the blocking buffer for a block/permeabilization step of 30 to 45 minutes. The grids were then placed in the primary antibody (collagen-1A) overnight at 4°C, rinsed with the incubation buffer, and floated on drops of the secondary antibody with attached 6-μm gold particles for 2 hours at room temperature as we described earlier. After rinsing with a rinse buffer (incubation buffer without Tween 20), the grids were placed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer for 5 minutes. Finally, the tissue specimens were imaged with JEM-1400 TEM (JEOL USA).

**Human Specimens**

Formalin-fixed, paraffin-embedded human eyes from patients with or without glaucoma were prepared as part of the routine, complete autopsies at Duke University Medical Center. Electronic and paper medical records of autopsy specimens were used to identify patient age, sex, race, ocular diseases, systemic diseases, ocular medications, and systemic medications as described earlier by us. Sample use for research was done after approval from the Duke University Medical Center Institutional Review Board. Ten eyes from 10 patients with a history of glaucoma or typical histologic evidence of POAG and 10 eyes from 10 age-matched nonglaucoma (control) patients were analyzed. The demographic details of human specimens used in this study are provided in Supplemental Table S1. IOP readings and visual field scores were not available for any of these patients.

**Immunohistochemistry**

For both rat and human paraffin-embedded sections, a standard tissue immunohistochemistry protocol was followed as we described earlier. Briefly, 5-μm-thick tissue sections were deparaffinized and rehydrated with three changes of xylene, followed by three changes of absolute ethyl alcohol, and washed with three changes of water. To unmask the antigen epitopes, heat-induced antigen retrieval was performed with 0.1 mol/L sodium citrate buffer pH 6.0 (Vector Laboratories) for 20 minutes at 100°C. The slides were then rinsed in water and washed two times with Tris-buffered saline (10 mmol/L Tris and 100 mmol/L NaCl; Tris-buffered saline). Nonspecific staining was blocked with Sniper Background Reducer (Biocare Medical, Concord, CA). The tissue sections were incubated with primary antibody overnight at 4°C in a humidified chamber. All primary antibodies were diluted in 1% bovine albumin (fatty acid free and low endotoxin) that contained Tris-buffered saline and were used at a 1:50 dilution. Polyclonal anti-rabbit collagen-1A (Abcam, Cambridge, MA) and α-SMA (Sigma-Aldrich, St. Louis, MO) antibodies were used. After incubation, the slides were washed with Tris-buffered saline three times and stained uniformly. To stain normal and glaucomatous human specimens simultaneously, we split our subject population into two groups, with each group containing a mixture of both normal and glaucoma specimens. All eyes were assigned a numeric code so investigators were masked to sample identity. All tissue specimens were stained in duplicate on different days, and a negative control (without primary antibody) was run for each assay. Detection of mouse primary antibody binding was done with Polylink-2 AP Plus Mouse NR Detection system and Permanent Red Kit for Immunohistochemistry (GBI Labs, Mukilteo, WA). Detection of rabbit primary antibody binding was done with Mach 4 Universal AP Polymer Kit (Biocare Medicals, Concord, CA) in combination with Vulcan Fast Red chromogen (Biocare Medicals) according to the manufacturer’s protocol. The sections were counterstained with hematoxylin and were rinsed with deionized water. The tissue sections were protected with a coverslip with the use of Permount mounting medium (Fisher Scientific, Waltham, MA). Slides were viewed and graded with a Zeiss Axioplan 2 microscope (Carl Zeiss GmbH, Jena, Germany), and images were captured with an Olympus Vanox microscope (Olympus America, Center Valley, PA).

Interpretation of immunohistochemistry results was performed as described in our previous work with minor modifications. For the rat and human specimens, two (P.P.P. and P.V.R.) and three reviewers (P.P.P., A.P., and P.V.R.), respectively, independently scored immunostaining intensity of AH outflow pathway tissues. The slides were masked such that the reviewers were unaware of which samples were from saline-, GFP-, or RhoAV14-injected eyes in the rat study or which specimens were derived from control versus glaucoma patients in the human study. In the case of human samples, to minimize scoring bias because of recognition of eyes with glaucomatous damage to the optic nerve retina, the observers focused their attention on the anterior chamber angle. The reviewers scored the uveoscleral (US)-TM, corneoscleral (CS)-TM, and JCT and SC on both the nasal and temporal side of each globe for the human eyes as we described earlier. In the case of rat eyes, the scoring was done on the TM region (without distinguishing them into CS or US and JCT) and SC region because the JCT region was not distinguishable from TM in many samples. Staining intensity was scored from 0 (no staining) to 5 (most intense staining) as described in our earlier work. Any of the regions that were not scored by at least two of the three reviewers were omitted from analysis; this was usually because of a reviewer being concerned about possible artifactual presence or absence of staining throughout the section or sectioning anomalies such as folds or tears. The score was excluded from analysis if there was more than a two-point discrepancy between grading by the reviewers. After all reviewers had scored the slides, the code was broken. The scores from the nasal and temporal sides of the outflow tract in each eye were pooled, and values for the duplicate slides were averaged to accommodate for the small variability inherent in immunohistochemical staining.


Immunofluorescence Staining

For F-actin staining, preblocked rat eye tissue cryosections were labeled with tetra-rhodamine isothiocyanate—conjugated phalloidin (500 ng/mL; Sigma-Aldrich) as we described earlier.21 For phosphorylated myosin light chain (pMLC), air-dried rat eye tissue cryosections were blocked and immunostained with polyclonal antibody to pMLC (Cell Signaling Technologies, Danvers, MA), at a dilution of 1:50. After incubation with primary antibody, slides were washed and incubated in the dark for 2 hours at room temperature, with Alexa Fluor 568-conjugated secondary antibody (Invitrogen, Carlsbad, CA), as we described earlier.30 Slides were mounted with Vector mount and nail polish and were photographed with a Nikon Eclipse 90i confocal laser scanning microscope (Nikon, Melville, NY).

Sirius Red stain, a strong anionic dye, was used for collagen detection.55 Tissue sections (rats or human) were deparaffinized and hydrated via a descending ethanol series (100%, 95%, 80%, and 70%), followed by two washes in distilled water. The specimens were then immersed in Sirius Red solution (0.1% in saturated picric acid) for 60 minutes for rat sections and 90 minutes for human sections. The sections were rinsed with running tap water for exactly 3 minutes and subsequently with acidified water, followed by dehydration and mounted with Permount mounting medium (Fisher Scientific). Stained sections were graded with a Zeiss Axioplan 2 microscope (Carl Zeiss GmbH), and images were captured with an Olympus Vanox microscope (Olympus America).

Cell Cultures

Human TM (HTM) cells were cultured from TM tissue isolated from leftover donor corneal rings after they had been used for corneal transplantation at the Duke Ophthalmology Clinical Service as described previously.21 HTM cells were cultured in Dulbecco’s modified Eagle’s medium that contained 10% fetal bovine serum and 100 U/500 mL penicillin, 100 µg/500 mL streptomycin, and 4 mmol/L glutamine. All cell culture experiments were conducted with confluent cultures (derived from passages between four and six times) that were serum starved for at least 24 hours before analysis of changes in cell morphology. As expected, RhoAV14-expressing cells showed a change in morphology on the basis of phase-contrast images and were infected with lentiviral vectors that expressed either GFP or RhoAV14/GFP (107 TU/mL) for 10-cm plate. After 36 hours, virus media were replaced with normal Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and observed for the GFP expression. GFP expression was seen within 4 to 6 days. At this point, cells were serum starved for 48 hours, and they were fixed for immunostaining, or cell lysates were prepared for immunoblotting.

Immunoblot Analysis

The cell lysates derived from HTM cells that expressed RhoAV14/GFP or GFP alone were immunoblotted for total RhoA, GFP, pMLC, phosphorylated-myosin phosphatase targeting subunit (pMYPT1), α-SMA, and β-actin with the use of the respective polyclonal (RhoA from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; GFP from BD Biosciences, San Jose, CA; pMLC from Cell Signaling Technologies; and pMYPT1 from Millipore, Billerica, MA) or monoclonal (α-SMA and β-actin from Sigma-Aldrich) primary antibodies in conjunction with relevant secondary antibodies as described earlier.21,30 Immunoblots were developed by enhanced chemiluminescence and were scanned densitometrically with a Gel Doc scanner (FOTODYNE Inc., Hartland, WI) with TL100 software. Densitometry analyses were performed with ImageJ software version 1.46 (NIH, Bethesda, MD).

Statistical Analysis

Analysis of variance was performed to test IOP changes between saline-, GFP-, and RhoAV14-injected groups. If the test proved significant, independent groups were assessed for significance by using the nonparametric Wilcoxon rank sum test. The nonparametric Wilcoxon rank sum test of differences in medians was performed to compare final scores for both rat and human samples by region and stain. The cell culture-based experiments were analyzed with Student’s t-test, with P < 0.05 being statistically significant.

Results

RhoAV14 Induces Changes in Actin Cytoskeletal Organization and Contractile Properties in the HTM Cells

Before testing the effects of RhoAV14 expression on IOP in live rats, we assessed the effectiveness of the lentiviral vector that we generated for expressing RhoAV14 and GFP in cultured HTM cells. We monitored GFP expression, changes in actin cytoskeletal organization, and phosphorylation status of MYPT1 and MLC which regulate cellular contraction induced via the activated Rho/Rho kinase signaling pathway. Figure 1A depicts the gene map of the lentiviral transfer vector. Virus titers were in the range of 3.6 ± 1.2 × 10^7 TU/mL and 3.0 ± 0.58 × 10^7 TU/mL for GFP and RhoAV14 GFP, respectively (means ± SD of three independent preparations). The titers were adjusted to 1 × 10^7 TU/mL and used to infect HTM cells in cultures. GFP expression was noted in HTM cells 4 days after transduction (Figure 1B). Cells were then serum starved for 48 hours before analysis of changes in cell morphology. As expected, RhoAV14-expressing cells exhibited either a rounded or contractile (or stiffened) morphology on the basis of phase-contrast images and compared with the GFP-expressing control cells (not shown). In addition, the RhoAV14-expressing HTM cells showed a
**Figure 1** RhoAV14-induced effects on cell morphology, F-actin, and contractile activity in cultured HTM cells. A: Map of bicistronic transfer construct depicting the RhoAV14-IRES-GFP insert and the CMV promoter that allows simultaneous expression of RhoAV14 and GFP from the same mRNA transcript. **B:** Four days after viral vector treatment, HTM cells expressing either RhoAV14/GFP or GFP alone were serum starved for 48 hours. HTM cells expressing RhoAV14/GFP exhibit a stiffened and contractile (rounding) cell morphology compared with the flattened and elongated morphology seen in GFP-expressing control cells. RhoAV14-expressing cells also display increased actin stress fibers (stained with rhodamine-phalloidin) compared with the GFP-expressing control cells. Representative images are shown to depict these changes. C: Under the conditions described above, immunoblot analyses of HTM cells reveals similar levels of GFP in both GFP-expressing and RhoAV14/GFP-expressing cells, confirming the expression of recombinant proteins. In addition, RhoAV14-expressing HTM cells show a significant increase in the levels of total RhoA, pMYPT1, pMLC, and α-SMA compared with GFP-expressing control HTM cells. β-actin served as a loading control. D: Histograms show quantitative changes that are based on densitometric analysis of immunoblots for RhoA, pMYPT1, pMLC, and α-SMA normalized against β-actin between the GFP control and RhoAV14-expressing cells. Values are expressed as means ± SD. n = 3 independent specimens. *P < 0.05. Scale bar = 50 μm. amP, ampicillin resistance; CMV, cytomegalovirus; eGFP, enhanced green fluorescent protein; HTM, human trabecular meshwork; IRES, internal ribosome entry site; pMLC, phosphorylated myosin light chain; pMYPT1, phosphorylated-myosin phosphatase targeting subunit; polyA, polyadenylic acid; RhoAV14, constitutively active RhoA GTPase; α-SMA, α-smooth muscle actin; SV40, simian virus 40.

Robust increase in actin stress fibers as evidenced by rhodamine-phalloidin fluorescence (Figure 1B), and they also exhibited significant increases in the levels of total RhoA, α-SMA, pMYPT1, and MLC relative to the GFP-expressing control cells (Figure 1, C and D). GFP expression was also confirmed by immunoblot analysis in both the controls (treated with GFP-expressing vector) and RhoAV14-expressing cells (treated with the RhoAV14 and GFP-expressing vector). These cell-based observations confirm the suitability of lentiviral vector to drive expression of the two transgenes in HTM cells and the activated state of the expressed recombinant RhoAV14 GTPase.

Expression of RhoAV14 in the Iridocorneal Angle of Rats Leads to OHT

To determine the role of Rho GTPase activity in regulation of IOP in live rats, we evaluated the effects of expressing RhoAV14 in the trabecular AH outflow pathway of rats on IOP. For this study, adult Sprague-Dawley female rats were divided into three groups with group I and group II serving as controls that received either a 10-μL saline injection or 10 μL of 1 × 10^7 TU/mL GFP-expressing lentiviral vector, respectively. The rats in group III received 10 μL of RhoAV14/GFP-expressing vector at a titer of 1 × 10^7 TU/mL. Only one eye (right) of each rat from all three groups received intracameral injections, and the contralateral eyes were untreated. A total of 20 rats from each group (Set-1, n = 8; Set-2, n = 12) received a single intracameral injection of saline or vector that expressed GFP or RhoAV14/GFP. Basal IOPs were measured in all rats a few days before the intracameral injections, and IOP recording under sedation was continued after injection, once every 3 to 4 weeks for >22 weeks, using a Tonolab (iCare Laboratory). In the Set-1 and Set-2 rats that expressed RhoAV14/GFP, we recorded a significant increase in IOP at 68 and 94 days after injection, respectively, compared with the GFP-expressing and saline-injected control rats (Figure 2). IOP values in the Set-1 RhoAV14-expressing rat eyes were found to be 12.07 ± 0.7 mm Hg (means ± SEM; n = 8) and significantly higher (P < 0.01) than in the saline-injected (10.1 ± 0.8 mm Hg) and GFP-expressing control (9.9 ± 0.6 mm Hg) rats. Similarly, IOP values in the Set-2 RhoAV14-expressing rat eyes were found to be 14.2 ± 0.6 mm Hg (means ± SEM; n = 12) and significantly higher (P < 0.01) than in the saline-injected (11.8 ± 0.73 mm Hg) and GFP-expressing (11.3 ± 0.9 mm Hg) rats. Increased IOP in the RhoAV14-expressing eyes was sustained until the end of the study (155 days after injection). The mean percentage ± SEM increase in IOP in the RhoAV14-expressing eyes was 21.7% ± 0.46% (Set 1) and 21.3% ± 2.18% (Set 2) compared with the saline-injected and GFP-expressing control eyes (Supplemental Table S2). The noninjected contralateral eyes did not show any significant difference in IOP among the three groups in both Set-1 and Set-2 rats (data not shown). In the Set-1 rats, IOPs were also measured at night on days 116 and 136 after injections. As is well known, 56 IOP values were higher at night in all three groups (Figure 2). Moreover, the nightly values of IOP in the RhoAV14-expressing rat eyes were significantly higher (33.4% ± 0.36%; n = 8) than in the respective saline and GFP control rat eyes (Supplemental Table S2).

RhoAV14 Expression in the Trabecular AH Outflow Pathway of Rats Induces Contractile Response

At the end of the IOP evaluation (155 days) studies described in the previous section, rats were sacrificed, and...
the enucleated eyes from all three groups (RhoAV14/GFP, GFP, and saline) were processed to analyze the distribution profile of GFP, which served as a reporter gene that confirmed expression of the candidate recombinant protein (RhoAV14 GTPase) in lentiviral vector-injected rat eyes. Sagittal plane thin cryosections of rat eye anterior segments through the iridocorneal angle mounted onto glass slides were directly visualized under a fluorescence microscope to assess GFP expression and distribution in the AH outflow pathway. Analysis of rat eyes injected (intracameral) with RhoAV14/GFP- or GFP-expressing viral vectors confirmed the expression of GFP in the iridocorneal angle, including ciliary body and the trabecular AH outflow pathway (TM and SC) (Figure 3A). As expected, no GFP was found in the eyes of saline-injected rats. A minimum of four individual specimens from each group were examined to confirm expression of recombinant proteins in the AH outflow pathway on the basis of GFP expression, and all specimens from both the RhoAV14/GFP and GFP groups were confirmed to express GFP. Expression of GFP is much stronger in the iridocorneal angle than in other ocular tissues (eg, cornea) (Figure 3A), and the lens tissue did not exhibit detectable GFP expression (data not shown). In addition, lentiviral vector-based expression of either RhoAV14 or GFP in the iridocorneal angle of rat eye did not cause any noticeable effect on the anterior chamber angle opening. Light microscope-based histologic examination of the eye specimens from all three groups showed an open anterior chamber angle morphology, confirming that the changes noted in IOP of RhoAV14-expressing rats are not related to structural integrity of the eye anterior chamber angle (Figure 3B).

To confirm the functionality of the recombinant RhoAV14 expressed in vector-injected rat eyes, we examined changes in F-actin distribution (Figure 3C) and pMLC (Figure 3D) in tissues of the AH drainage pathway. These experiments were performed by confocal microscopy-based monitoring of rhodamine–phalloidin-based (F-actin) fluorescence and pMLC immunofluorescence, respectively, using tissue cryosections derived from all three groups (RhoAV14/GFP, GFP, and saline). The fluorescence staining intensity for F-actin was relatively intense in the TM and SC of RhoAV14-expressing specimens compared with GFP-expressing (Figure 3C) and saline (data not shown) controls. F-actin staining associated with the ciliary muscle and iris in RhoAV14-expressing specimens was not different from that found in the GFP (Figure 3C) and saline (data not shown) controls. Consistent with the increase in F-actin staining intensity, pMLC immunofluorescence was also found to be much higher in the AH outflow pathway region, especially in the TM and SC tissues in RhoAV14-expressing specimens, than in the GFP (Figure 3D) and saline (data not shown) controls. Appropriate negative controls for the nonspecific tissue and secondary antibody fluorescence were included in these comparisons (data not shown).

Before proceeding further, we attempted to determine whether inflammation or immunologic reactions contributed

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**Figure 2** The expression of RhoAV14 in the iridocorneal angle of live rats induces elevated IOP. Two sets of Sprague-Dawley rats were injected intracameraly with 10 μL of saline solution (group I) or 1 × 10^7 TU/mL of either GFP- (group II) or RhoAV14 (group III)-expressing vector particles (Set-1 and Set-2). The injections were administered to the right eye, with the contralateral left eye serving as the untreated control. The day of injection was considered as day 1. IOPs were measured a few days before (<7 days) the intracameral injections to record basal values and were continued after injections for up to day 155, with IOP measurements being performed once every 3 to 4 weeks. Day-time IOPs were measured (Set-1 and Set-2) between 10 am and 1 pm, with night-time IOPs (Set-1) being measured between 10 pm and 1 am. IOPs were compared at the indicated time points between the saline-treated and the GFP- and RhoAV14-expressing groups. Analysis of variance was performed to test IOP changes between saline-, GFP-, and RhoAV14-injected groups. If the test proved significant, independent groups were assessed for significance by using the nonparametric Wilcoxon rank sum test. A and B: Histograms show the day-time IOP values in the right eye (injected) of rats from Set-1 and Set-2, respectively, during the pre- and postinjection periods. The RhoAV14-expressing eyes display a significant increase in IOP compared with controls (saline-injected or GFP-expressing groups) at days 68 and 94 after injection in the Set-1 and Set-2 rats, respectively. The triangle on the x axis indicates the day of intracameral injections. C: Histograms of the night-time IOP values in the right eye of injected rats at the indicated postinjection periods. Values are expressed as means ± SEM; n = 8 per Set-1 group; n = 12 per Set-2 group, *P < 0.05. GFP, green fluorescent protein; IOP, intraocular pressure; RhoAV14, constitutively active RhoA GTPase; TU, transduction unit.
to the viral vector-mediated effects within the anterior chamber angle of the eye. We examined the expression and distribution profiles of markers for macrophage (F4/80) and monocytes (Iba1) by immunofluorescence staining of tissue cryosections. These analyses did not reveal any positive staining for either of the inflammatory markers in the iridocorneal angle, indicating that lentiviral vector injections in the eye anterior chamber did not trigger inflammation or immunologic reaction (data not shown).

RhoAV14 Expression in the AH Outflow Pathway of Rats Induces Fibrotic Changes

To determine whether elevated IOP in the RhoAV14-expressing rats is associated with the structural alterations in the AH outflow pathway, we examined the histology and ultrastructure of the AH outflow pathway by light microscopy and TEM. Analysis of semithin sections by light microscopy did not show structural difference in the opening of the eye anterior chamber angle in RhoAV14-expressing rats compared with that in GFP-expressing specimens (Figure 4). Because we did not find differences in IOP or anterior chamber angle histology between the GFP-expressing control rats and saline-injected rats, further analysis of ultrastructural changes in the trabecular AH outflow pathway by TEM focused only on comparison of changes between RhoAV14/GFP-expressing and GFP-expressing (control) specimens. TEM of the eye anterior chamber angle specimens revealed noticeable compaction of the TM and JCT in >60% of the RhoAV14/GFP-expressing eyes compared with the GFP-expressing eyes (Figure 4). Unlike in GFP-expressing control specimens, in which the TM beams appear to be loose with noticeable spaces between the beams, the TM beams/JCT area appeared compact and dense in the RhoAV14-expressing specimens (Figure 4). This fibrotic/ultrastructurally dense-looking TM in RhoAV14 eyes was associated with optically dense extracellular material relative to the GFP controls in which there seemed to be more optically empty space between the beams (Figure 4B). Importantly, in support of this conclusion, because it relates to possible changes in the ECM within the trabecular AH outflow pathway of RhoAV14-expressing specimens, we performed immunogold labeling for collagen-1A. The results indicated that collagen-1A was distributed mostly within the TM and JCT regions, with relatively higher/denser accumulation of gold particle deposition observed in the RhoAV14-expressing specimens compared with the GFP-expressing controls (Figure 4C). The images are representative of a total of four to six independent specimens analyzed in the study.

Figure 3  Expression of GFP and RhoAV14/GFP in the iridocorneal angle of live rats and contractile changes in the AH outflow pathway. A: Anterior chamber tissue cryosections derived from rat eyes injected intracamerally with GFP- or RhoAV14/GFP-expressing viral vector particles show GFP-specific green fluorescence in the conventional AH pathway, confirming the expression of recombinant proteins with lentivirus-based gene delivery approach. GFP expression was distributed in the AH outflow pathway tract mainly in the TM and around the walls of SC. GFP expression was also seen in the regions around the CB. The saline-injected rat eyes, as expected, did not exhibit GFP-specific green fluorescence. B: Light microscope-based histologic examination of the rat eyes injected with viral particles that express either RhoAV14/GFP or GFP alone or injected with saline alone show indistinguishable and normal morphology of open AH drainage angle in all three groups, confirming no notable adverse influence of vector on the opening of AH drainage angle. C and D: The AH outflow pathway tissues (cryosections), including TM and SC, show increased levels of F-actin and pMLC on the basis of rhodamine-phalloidin fluorescence staining and anti-pMLC immunofluorescence staining, respectively, in the RhoAV14-expressing rats (arrows) compared with the GFP-expressing control rats. Lower panels show magnified area of the AH outflow pathway in which the changes in F-actin and pMLC are easily evident. Scale bars: 50 μm (A–D). AH, aqueous humor; CB, ciliary body; GFP, green fluorescent protein; pMLC, phosphorylated myosin light chain; RhoAV14, constitutively active RhoA GTPase; SC, Schlemm canal, TM, trabecular meshwork.
chamber angle of RhoAV14-expressing eyes exhibited a relatively strong positive immunostaining for α-SMA (Figure 5A) and collagen-1A (Figure 5B) and deposition of total collagen as detected by Sirius Red staining (Figure 5C) compared with eyes expressing GFP alone (Figure 5, A–C). The ciliary body, which stained positive for α-SMA, collagen-1A, and total collagen in the GFP- and the RhoAV14-expressing specimens, exhibited higher staining intensity for each of the three proteins in the RhoAV14-expressing specimens. In addition to the visual comparison of staining intensity shown in Figure 5, we used a grading system comprised of masked result review by two independent reviewers (n = 4 samples from Set-2, with each sample immunostained and analyzed in triplicate). Nonparametric comparison of the calculated scores for staining intensity confirmed that specimens from RhoAV14-expressing eyes exhibited higher staining intensity than the GFP-expressing control eye specimens for α-SMA (Figure 5D). However, this difference was found to be statistically significant only in the SC region (42% increase) of RhoAV14-expressing specimens compared with the corresponding region from GFP-expressing controls (Figure 5D). Although the staining intensity of α-SMA in the TM/JCT combined region from RhoAV14-expressing eyes was increased by 22% compared with the corresponding region in GFP-expressing specimens, this difference was not significant (Figure 5D). However, the immunostaining intensity of collagen-1A and Sirius Red staining of total collagen showed a significant increase by approximately 50% in the TM and SC regions in RhoAV14-expressing specimens (P < 0.03 and P < 0.02, respectively; n = 4) compared with the GFP-expressing specimens (Figure 5D and Supplemental Table S3).

Y27632 Lowers IOP by Suppressing the Fibrogenic Response in the TM of RhoAV14-Expressing Rats

To determine whether the effects of RhoAV14 on IOP are mediated via Rho kinase, we tested the topical effects of Y27632, a well-characterized small molecular weight Rho kinase inhibitor on IOP in the RhoAV14-expressing rats.39 For this experiment (Set-3), intracameral injections of either saline (n = 6), vector particles expressing GFP (n = 7), or RhoAV14/GFP (n = 7) were administered to the anterior chamber of the right eye in adult live rats, with the untreated contralateral left eye serving as untreated control. IOP was subsequently monitored for nearly 22 weeks as described earlier (Figure 2). After this, Y27632 was applied topically (4 μL of 10 mmol/L in PBS; twice with 30-second delay) once per day to the right eye, whereas the contralateral (left) eye received PBS (4 μL, twice) for a total of 15 days. IOPs were recorded under mild anesthesia, between 1 and 1.5 hours after administration of Rho kinase inhibitor, on days 1, 3, 8, 11, and 15, as described earlier. Consistent with the data obtained from rats from Set-1 and Set-2, the RhoAV14-expressing rats in Set-3 exhibited significant elevation (P < 0.001; n = 7) in IOP, starting from day 54.

RhoAV14 Expression in the Trabecular AH Outflow Pathway of Rats Induces Accumulation of Collagen-1A, Total Collagen, and α-SMA

On the basis of the histologic observations described in the previous section which suggest a possible fibrotic response in the RhoAV14-expressing rat AH outflow pathway, we evaluated for changes in the levels of collagen-1A, total collagen, and α-SMA by immunohistochemical analysis and Sirius Red staining with the use of paraffin-embedded tissue sections from RhoAV14-expressing (n = 4 from Set 2) and GFP-expressing (n = 4 from Set-2) specimens. The anterior-
compared with the saline \((n = 6; \text{data not shown})\) and GFP \((n = 7)\) expressing rats (Figure 6). This trend was sustained until day 155 consistent with the observations from Set-1 and Set-2. The percentage increase in IOP in the RhoAV14-expressing rats was approximately 23.4% at day 155 relative to the GFP and saline control rats. After treatment with Y27632, no difference was detected in IOP between the RhoAV14-expressing and GFP-expressing and saline-treated (data not shown) control rats. Topical administration of Y27632 was found to cause a significant (20%) decrease in the IOP in RhoAV14-expressing rat eyes (Figure 6 and Supplemental Table S4). GFP-expressing control rat eyes exhibited a slight (4%) but insignificant decrease in IOP in response to topically administered

**Figure 5** Evidence for RhoAV14-induced fibrogenic activity in the AH outflow pathway of live rats. **A–C:** Representative images of immunohistochemical localization of α-SMA and collagen-1A and Sirius Red stain of total collagen, respectively, in the AH outflow pathway of RhoAV14-expressing and GFP-expressing rat specimens (paraffin-embedded). On the basis of relative intensity of staining pattern within the AH outflow pathway, the RhoAV14-expressing specimens show increased levels of α-SMA, collagen-1A, and total collagen in the TM, JCT, and SC compared with the GFP-expressing eyes. **D:** Quantification of the staining intensity on the basis of grading done by two individuals in a masked manner shows an increase of α-SMA staining intensity in both TM \((21.7\%)\) and SC \((42\%)\); however, it was found to be significant only in the SC of RhoAV14-expressing specimens compared with the GFP controls \((P < 0.03)\). Collagen-1A shows significantly increased staining intensity in both TM \((P < 0.03)\) and SC \((P < 0.03)\) of RhoAV14-expressing specimens compared with the GFP-expressing controls. Likewise, the total collagen staining based on Sirius Red stain shows a significant increase in the TM \((P < 0.02)\) and SC \((P < 0.03)\) of RhoAV14-expressing specimens compared with the GFP-expressing controls. The box and whisker plots represent median and the interquartile range in the distribution, \(n = 4\) RhoAV14-expressing specimens (D). *\(P < 0.05\). Scale bars: 20 μm (A–C). AH, aqueous humor; GFP, green fluorescent protein; JCT, juxtacanalicular tissue; RhoAV14, constitutively active RhoA GTPase; SC, Schlemm canal; α-SMA, α-smooth muscle actin; TM, trabecular meshwork.
RhoAV14-expressing rats treated with the Rho kinase inhibitor exhibit a decrease in IOP compared with IOP of the same respective groups treated with Y27632. Rho kinase inhibitor also decreases IOP in the GFP-expressing controls compared with IOP of the same groups treated with PBS. Y27632, at 10 μmol/L, was applied topically once daily for 15 days. Eyes from rats injected with virus were treated with Rho kinase inhibitor (10 μmol/L Y27632; two 4-μL drops; once daily for 15 days). Eyes from RhoAV14-expressing rats treated with the Rho kinase inhibitor exhibited a significant decrease in IOP compared with IOP in the same rats before the treatment with Y27632. Rho kinase inhibitor also decreases IOP in the GFP-expressing controls compared with IOP of the same respective groups before the Rho kinase inhibitor; however, the difference is not significant between these two groups. Values are expressed as means ± SEM. *P < 0.05. GFP, green fluorescent protein; IOP, intraocular pressure; RhoAV14, constitutively active RhoA GTPase; TU, transduction unit; Y27632, Rho kinase inhibitor [(R)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride].

Figure 6  Rho kinase inhibitor decreases IOP induced by RhoAV14 in live rats. Sprague-Dawley rats (Set-3) injected intracamerally with GFP-expressing viral vector or RhoAV14/GFP-expressing viral vector (1 × 10⁷ TU/mL in 10 μL) show a significant increase in day-time IOP in the RhoAV14-expressing rats, starting from day 54 after injection, with the trend continuing until day 155. After this period (155 days), the right eyes from rats injected with virus were treated with Rho kinase inhibitor (10 mmol/L Y27632; two 4-μL drops; once daily for 15 days). Eyes from RhoAV14-expressing rats treated with the Rho kinase inhibitor exhibit a significant decrease in IOP compared with IOP in the same rats before the treatment with Y27632. Rho kinase inhibitor also decreases IOP in the GFP-expressing controls compared with IOP of the same respective groups before the Rho kinase inhibitor; however, the difference is not significant between these two groups. Values are expressed as means ± SEM. n = 7 rats in each group. *P < 0.05. GFP, green fluorescent protein; IOP, intraocular pressure; RhoAV14, constitutively active RhoA GTPase; TU, transduction unit; Y27632, Rho kinase inhibitor [(R)-(−)trans-4-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride].

Y27632. The contralateral eyes treated with PBS showed a slight decrease in IOP, but this decrease was not found to be significant compared with basal values in either RhoAV14 or GFP-expressing or in saline-treated rat eyes (data not shown). We excluded IOP values from analysis acquired on day 1 after treatment with Rho kinase inhibitor (Figure 6) because of the erratic nature of the Tonopen recordings.

On completion of the IOP studies, ultrastructural and immunohistochemical analyses were performed to evaluate the effects of Y27632 on the fibrogenic response evoked by RhoAV14 in the trabecular AH outflow pathway. We used the GFP- and RhoAV14-expressing specimens from Set-2 to compare with the Y27632-treated specimens (Set-3; n = 3). Examination of the histologic structure of the anterior chamber angle of GFP- and RhoAV14-expressing specimens in the presence and absence of Rho kinase inhibitor did not reveal any noticeable differences (Figure 7A). TEM analyses of specimens from RhoAV14-expressing eyes treated with topical Y27632 (Figure 7B) revealed a marked absence of the extracellular material deposition noted in the TM and JCT regions of untreated RhoAV14-expressing eyes. This effect of Y27632 was also evidenced by the large and wide optically empty spaces in the TM and JCT regions (Figure 7B) of RhoAV14-expressing specimens treated with Y27632, as opposed to RhoAV14-expressing eyes. Immunologic electron microscopy for collagen-1A showed decreased collagen-1A–dense particles in the AH outflow pathway of RhoAV14-expressing eyes treated with Y27632 (Figure 7C) compared with untreated specimens (Figure 7C). Saline-treated and GFP-expressing specimens that received Y27632 drops exhibited comparable levels of collagenous material in the TM and JCT regions of the outflow pathway with no clear differences relative to those noted between the GFP- and RhoAV14-expressing specimens (Set-2; data not shown).

Immunohistochemical analyses of α-SMA and collagen-1A and Sirius Red stain analysis for total collagen were also changes (Figure 7B). Immunologic electron microscopy for collagen-1A showed decreased collagen-1A–dense particles in the AH outflow pathway of RhoAV14-expressing eyes treated with Y27632 (Figure 7C) compared with untreated specimens (Figure 7C). Saline-treated and GFP-expressing specimens that received Y27632 drops exhibited comparable levels of collagenous material in the TM and JCT regions of the outflow pathway with no clear differences relative to those noted between the GFP- and RhoAV14-expressing specimens (Set-2; data not shown).
performed to compare the effects of the Rho kinase inhibitor on RhoAV14-induced changes in the AH outflow pathway (Figure 8). Immunostained and Sirius Red-stained specimens were graded in a masked manner, and the average values of grading scores were evaluated for statistically significant differences. RhoAV14-expressing rat eyes treated with Y27632 exhibited a significant decrease in immunostaining for \( \alpha \)-SMA \((P < 0.03; \ n = 3) \) (Figure 8, A and D) and collagen-1A \((P < 0.03; \ n = 3) \) (Figure 8, B and D) and in Sirius Red staining for total collagen \((P < 0.03; \ n = 3) \) (Figure 8, C and D) in the TM/JCT areas, relative to untreated RhoAV14-expressing rat eyes. Collagen-1A expression \((P < 0.02; \ n = 3) \) and total collagen distribution \((P < 0.02; \ n = 3) \) were also decreased significantly around the endothelial walls of SC (Figure 8, B–D), whereas \( \alpha \)-SMA expression was unaltered \((P < 0.08; \ n = 3) \) in the Y27632-treated specimens compared with the RhoA-expressing specimens (Figure 8A). The percentage change in score calculated for Rho kinase inhibitor-treated RhoAV14-expressing specimens were all negative, indicating a decrease in the staining intensity relative to the corresponding untreated, RhoAV14-expressing eyes (Supplemental Table S5).

**Human Glaucoma Eye Specimens Exhibit Increased Accumulation of Collagen-1A and Collagen in the Trabecular AH Outflow Pathway**

To determine the relevance of the findings from the rat model for ECM-associated changes in the trabecular AH outflow pathway of human glaucoma and age-matched control eyes, we evaluated the distribution and accumulation of collagen-1A

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**Figure 8** Rho kinase inhibitor suppresses the RhoAV14-induced expression of \( \alpha \)-SMA, collagen-1A, and total collagen in the AH outflow pathway of rats. A–C: Representative images of immunohistochemical localization of \( \alpha \)-SMA and collagen-1A and of total collagen on the basis of Sirius Red stain in the AH outflow pathway of RhoAV14-expressing eyes treated or untreated with Y27632, respectively. Daily, topical application of Y27632 for 15 days leads to a marked decrease in the staining intensity of \( \alpha \)-SMA, collagen-1A, and total collagen both in the TM and SC of the RhoAV14-expressing eyes compared with untreated RhoAV14-expressing specimens. D: Histograms depict changes in the intensity of staining of \( \alpha \)-SMA, collagen-1A, and total collagen in the AH outflow pathway of RhoAV14-expressing rat specimens treated or untreated with Y27632. Staining intensity was graded in a masked manner by two individuals. Treatment with Y27632 decreases the staining intensity of \( \alpha \)-SMA, collagen-1A, and total collagen in the TM compared with the staining recorded in untreated RhoAV14-expressing specimens. Similarly, the staining of collagen-1A and total collagen in the SC is significantly decreased in the AH outflow pathway of RhoAV14-expressing specimens treated with Y27632 compared with the untreated RhoAV14-expressing specimens. Box and whisker plots represent median and the interquartile range in the distribution. \( n = 4 \). \( *P < 0.05 \). Scale bars: 20 \( \mu \)m (A–C). AH, aqueous humor; RhoAV14, constitutively active RhoA GTPase; SC, Schlemm canal; \( \alpha \)-SMA, \( \alpha \)-smooth muscle actin; TM, trabecular meshwork; Y27632, Rho kinase inhibitor \([(R)-(+)\)-trans-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide dihydrochloride].

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and total collagen by immunostaining analysis and Sirius Red staining, respectively, of paraffin-embedded autopsy eyes from glaucoma \( (n = 10) \) and age-matched nonglaucoma \( (n = 10) \) patients. The demography details, including age, sex, race, and medication of these patients, are described in Supplemental Table S1 and confirm there were no significant differences in any of these factors between the glaucoma and nonglaucoma patients used in this study. The relative staining intensity of collagen-1A and total collagen was graded by three individuals in a masked fashion, and average scores were compared statistically. The nonglaucoma eyes displayed detectable positive staining for both collagen-1A (Figure 9A) and total collagen (Figure 10A) in the TM and JCT regions.

Relatively weak staining was noted in the SC region compared with the TM and JCT regions for both proteins, particularly for collagen-1A (Figure 9A, A and B). The relative staining intensity scores for collagen-1A and total collagen derived from the different regions of AH outflow pathway are shown in Figures 9B and 10B, respectively. The glaucoma eye specimens showed a significant increase in collagen-1A staining in the TM, including both the CS-TM \( (P < 0.01; n = 10) \) and US-TM \( (P < 0.003; n = 10) \) (Figure 9A) regions compared with the nonglaucomatous specimens. Interestingly, the staining pattern of collagen-1A in the JCT and SC regions of glaucoma specimens did not show much difference compared with the nonglaucoma specimens (Figure 9, A and B). The total collagen distribution evaluated by Sirius Red staining presented as a strong signal in the AH outflow pathway of both

Figure 9 Increased collagen-1A accumulation in the AH outflow pathway of human glaucoma specimens. \( A \): The images depict the distribution pattern of collagen-1A in the AH outflow pathway of age-matched nonglaucoma control subjects and glaucoma subjects. The glaucoma specimens show intense immunostaining of collagen-1A in both the CS-TM and US-TM, which is distributed at moderate levels throughout the JCT and SC. \( B \): Grading of staining intensity of collagen-1A in the AH outflow pathway by three individuals in a masked manner reveals a significant increase in both the CS-TM and US-TM \( (P < 0.01 \text{ and } 0.003, \text{ respectively}) \) in the glaucoma specimens compared with the age-matched nonglaucoma specimens. The difference in staining intensity of collagen-1A in the JCT and SC regions of glaucoma and control specimens is insignificant. \( n = 10 \) (B). \( * P < 0.05. \) Scale bars: 100 \( \mu m \) (A). AC, anterior chamber; AH, aqueous humor; CB, ciliary body; CS_TM, corneoscleral trabecular meshwork; JCT, juxtacanalicular tissue; SC, Schlemm canal; US_TM, uveoscleral trabecular meshwork.

Figure 10 Increased total collagen staining in the AH outflow pathway of human glaucoma specimens. \( A \): Images show the distribution profile of total collagen in the AH outflow pathway of age-matched nonglaucoma and glaucoma specimens on the basis of Sirius Red staining. Compared with age-matched control specimens, the glaucoma human specimens show intense staining for total collagen in the areas of CS-TM, US-TM, JCT, and SC. \( B \): Grading values derived on the basis of the intensity of Sirius Red stain by three individuals in a masked manner reveals a significant increase in total collagen in the regions of CS-TM, US-TM, JCT, and SC of glaucoma specimens compared with the age-matched controls. \( n = 10. \) \( * P < 0.05; \) Scale bars: 100 \( \mu m \) (A). AC, anterior chamber; AH, aqueous humor; CB, ciliary body; CS_TM, corneoscleral trabecular meshwork; JCT, juxtacanalicular tissue; SC, Schlemm canal; US_TM, uveoscleral trabecular meshwork.
Discussion

Our objective was to determine whether dysregulated Rho GTPase signaling in the AH outflow pathway affects homeostasis of IOP, and if in fact it does influence IOP, to understand the molecular basis for such alterations. To address this objective on a mechanistic level with the use of an experimental animal model, we investigated the effects of expressing a constitutively active RhoA GTPase mutant (RhoAV14) on IOP, using a lentiviral vector to target RhoAV14 expression to the AH outflow pathway of live rats. Here, we demonstrated not only that sustained activation of Rho GTPase signaling in the conventional AH outflow pathway induces OHT in live rats but importantly that the elevated IOP appears to be associated with augmented fibrogenic activity in the cells of the AH outflow pathway in a Rho kinase-dependent manner. Moreover, we record evidence for an increased accumulation of collagen-1A and total collagen in the trabecular AH outflow pathway of human glaucoma specimens. These observations collectively infer that increased fibrogenic activity of the cells of the conventional AH outflow pathway induced by the dysregulated cellular mechanisms, especially the Rho/Rho kinase signaling pathway which controls the contractile and biomechanical properties of cells and tissues, impairs AH outflow through the TM, resulting in OHT. Significantly, inhibition of Rho kinase activity ameliorates the fibrotic responses of TM and lowers IOP, further supporting the promising therapeutic potential of Rho kinase inhibitors in lowering IOP in glaucoma patients.

OHT, a primary risk factor for POAG is well recognized to be the result of increased resistance to AH outflow through the conventional pathway. Among various possible mechanisms involved in increased resistance to AH outflow, it is becoming increasingly evident that the accumulation of ECM and associated matrix stiffness, altered contractile and material properties of the TM, is considered to be some of the significant factors that contribute to elevated IOP. Most significantly, POAG patients are known to have elevated levels of TGF-β2, CTGF, endothelin-1, and autotaxin activity in the AH. Moreover, these different factors were found to influence the contractile properties of TM tissue, ECM synthesis, and actin cytoskeletal reorganization by activating Rho GTPase/Rho kinase signaling and other signaling pathways.

However, in a broader sense the molecular mechanisms involved in increased resistance to AH outflow are not well understood. In addition, despite overwhelming interest from the pharmaceutical industry to explore the therapeutic use of Rho kinase inhibitors in lowering IOP in glaucoma, we still do not have a complete understanding of the cellular basis by which Rho kinase inhibitors increase AH outflow through the TM and how these inhibitors may reduce resistance to AH outflow.

RhoA and IOP

Toward a better understanding of the significance of Rho/Rho kinase signaling in regulating the resistance to AH outflow and on the basis of our recent observations in cultured TM cells, in which we gained mechanistic evidence for the role of Rho GTPase signaling in ECM synthesis and organization and the expression of α-SMA, here, we investigated the in vivo effects of a constitutively active form of Rho GTPase on IOP in a rat model. For a mechanistic understanding and to induce sustained Rho GTPase activity, we expressed RhoAV14 under the control of a cytomegalovirus promoter in the AH outflow pathway of rat eye, using a lentivirus vector-based gene delivery and intracameral administration of viral vector particles.

Expression of RhoAV14, which is confirmed by detecting GFP tracer protein fluorescence in the AH outflow pathway, increased IOP significantly and consistently in three independent sets of rats in the absence of detectable inflammation. As expected, RhoAV14 expression is not restricted to the conventional AH outflow pathway and was detected in the ciliary muscle and iris as well. Although the lentivirus-based gene expression approach maintained a sustained expression of RhoAV14 in the AH outflow pathway for >15 weeks and IOP continued to be elevated significantly compared with the GFP controls, IOP did not increase progressively after the initial significant increase noted by 7 to 9 weeks after injections. Moreover, with this gene delivery approach, there was only 20% to 30% elevation in IOP during day and night compared with the GFP controls. The possible reason for this modest elevation in IOP with RhoAV14 expression could be relevant to the inherent inefficient approach available to induce the recombinant gene expression in the conventional pathway and insufficient viral vector concentration used in this study. Ideally, 1 × 10⁸ TU/mL of viral vector particles would have been appropriate, but we could not generate this high titer and used a titer of 1 × 10⁷ TU/mL instead. However, although IOP was elevated moderately but significantly in the RhoAV14-expressing rats, it was sustained until the rats were sacrificed at 22 weeks after injection. As is known, the use of lentiviral vector is associated with a considerable delay (ranging between 7 and 9 weeks) relative to adenoviral gene delivery, in the context of detecting the initial significant differences in IOP in RhoAV14-expressing rats. In addition, it is necessary to note here that we recorded IOP only once every 3 to 4 weeks. Therefore, there could be a 1- to 2-week
discrepancy in our estimation of the exact time of onset of elevated IOP in the RhoAV14-expressing rats.

RhoA and Fibrogenic Activity in the AH Outflow Pathway

Although an initial glimpse into the histologic integrity of the AH outflow pathway in the RhoAV14-expressing rats by light microscopy showed no abnormality in the opening of the anterior chamber angle, we noted the appearance of a relatively more compact and firm AH outflow pathway in a high number (>60%) of these rats by TEM, compared with the GFP-expressing controls (approximately 15%). The TM beams in particular were found to display a fibrotic or stiffened morphology in the RhoAV14 AH pathway relative to the GFP controls. Importantly, this histologic phenotype in the RhoAV14-expressing specimens was associated with increased accumulation of collagen-1A in the conventional outflow pathway as detected by immunolocal electron microscopy. After these observations and the use of immunohistochemical analysis of paraffin-embedded tissue specimens, we recorded significantly elevated levels of α-SMA, collagen-1A, and total collagen in the AH outflow pathway of RhoAV14-expressing rats compared with the GFP controls. These observations in the AH outflow pathway are not only consistent with the changes that we reported in the RhoAV14-expressing cultured human TM cells but also confirm that elevated IOP associates closely with increased levels of α-SMA, collagen-1A, and total collagen in the AH outflow pathway. Moreover, these changes most likely are involved in altered material properties (stiffer or fibrotic appearance) of AH outflow pathway noted in the RhoAV14-expressing specimens. It is highly likely that the increase in levels of α-SMA, collagen-1A, and total collagen observed in the AH outflow pathway of RhoAV14-expressing rat specimens is induced by the actin/serum response factor/myocardin-related transcription factor transcriptional axis in response to sustained activation of Rho GTPase activity as we have found in cultured TM cells. This possibility has not been confirmed in the present study, however, because of limitations with availability of TM tissue from rats. Similarly, confirmation of whether the increased levels of α-SMA, collagen-1A, and total collagen in the AH outflow pathway of RhoAV14-expressing rat specimens derive from myofibroblast activation and endothelial-to-mesenchymal transition of TM cells will require additional studies. However, increased RhoA activity was demonstrated to induce fibrotic response in various tissues and to play a significant role in TGF-β- and CTGF-induced epithelial-mesenchymal transition/endothelial-to-mesenchymal transition and ECM synthesis in different cell types. Moreover, our previous study has reported that Rho GTPase activation in TM cells induces expression of TGF-β2 and CTGF, which are recognized profibrogenic factors. Whether the optic nerve head in RhoAV14-expressing rats shows fibrogenic changes similar to those observed in the AH outflow pathway was not determined in this study.

Rho Kinase Inhibitor and Ocular Hypotensive Activity

Taken together, it is plausible that increased IOP in the RhoAV14-expressing rats could be associated with increased resistance to AH outflow through the conventional pathway because of alterations in collagen synthesis and deposition and the resulting effect on material properties of AH pathway tissues such as the TM and SC. Therefore, it is possible that the noted delay in onset of significant elevation in IOP in the RhoAV14-expressing rats observed in this study could also be related to a gradual progression of fibrosis or fibrogenic activity in the conventional AH pathway induced by Rho GTPase activation. Because we did not measure AH outflow facility in this study, we cannot rule out possible involvement of the nonconventional or unveojocular pathway in the observed elevated IOP in RhoAV14-expressing rats. Likewise, the question of whether AH production by the ciliary epithelium is influenced by Rho GTPase activation remains open because we documented expression of recombiant RhoAV14 in the ciliary muscle.

Consistent with Rho GTPase-induced OHT in rats (Figure 2), topical application of Rho kinase inhibitor (Y27632) decreased elevated IOP in the RhoAV14-expressing rats. Treatment of RhoAV14-expressing rats with topical Rho kinase inhibitor was found to be associated not only with normal histology in the AH outflow pathway but also comparable levels of α-SMA, collagen-1A, and total collagen in the AH outflow pathway relative to GFP-expressing controls. These observations reveal that the Rho kinase inhibitor suppresses RhoAV14-induced fibrogenic activity in the AH outflow pathway. This set of results is also consistent with our previous observations that used human TM cells in which both Rho kinase inhibitor (Y27632) and pirfenidone, a known antifibrotic agent, decreased expression of α-SMA, collagen-1A, and total collagen in the presence of TGF-β2. Moreover, Rho kinase inhibitors and statins that mimic Rho kinase inhibitor responses were found to suppress fibrosis in various organs and tissues. Therefore, it is most likely that the known ocular hypotensive effects of the Rho kinase inhibitors are related partly to their antifibrogenic activity in addition to their possible influence on cellular relaxation and paracellular permeability through the inner wall of SC. Likewise, Rho kinase inhibitors are also known to suppress the fibrogenic activity of TGF-β, lysophosphatidic acid, endothelin-1, and CTGF in different cell types. Each of these factors was also confirmed to increase resistance to AH outflow through the TM.

Elevated Levels of Collagen in the AH Outflow Pathway and Glaucoma

Finally, in support of our observations documented in the live rat, in which we found a good correlation between elevated IOP and fibrogenic activity induced by RhoAV14, our analyses of human autopsy glaucoma eyes provided evidence for significantly increased collagen-1A and total collagen in the TM,
JCT, and SC areas in glaucomatous eyes compared with the age-matched nonglaucomatous controls, implying a plausible association between augmented fibrogenic activity in the AH outflow pathway and POAG. To our surprise and contrary to the general thought,8,10,18 the accumulation of collagen-1A and total collagen was found to be much higher in the TM (both CS-TM and US-TM regions) compared with the JCT and SC. Because we found a good match between the glaucoma specimens and the nonglaucoma control specimens with respect to age, sex, and race, we do not suspect that any of these factors contributed to the increased accumulation of collagen in the AH outflow pathway. These observations provide a significant insight into the potential role of accumulation of ECM in the glaucoma specimens and its presumed involvement in increased resistance to AH outflow and OHT.18

Conclusion

This study provides experimental evidence for increased fibrogenic activity because of dysregulated RhoA GTPase activity in the trabecular AH outflow pathway leading to increased IOP. Inhibition of Rho kinase prevents the fibrogenic response in a live OHT rodent model, further supporting the importance of targeting the Rho/Rho kinase signaling pathway for lowering IOP in glaucoma patients.

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Supplemental Data

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References


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