Experimental Eye Research 136 (2015) 29–33

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Elevated intraocular pressure induces Rho GTPase mediated contractile signaling in the trabecular meshwork

Padmanabhan P. Pattabiraman [a, 1], Toshihiro Inoue [a, 1, 2], P. Vasantha Rao [a, b, *]

[a] Department of Ophthalmology, Duke University School of Medicine, Durham, NC, USA
[b] Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC, USA

ARTICLE INFO

Article history:
Received 4 February 2015
Received in revised form
30 April 2015
Accepted in revised form 4 May 2015
Available online 5 May 2015

Keywords:
Trabecular meshwork
Rho GTPase
Mechanotransduction
Intraocular pressure
Outflow resistance

ABSTRACT

Rho GTPase regulated contractile signaling in the trabecular meshwork (TM) has been shown to modulate aqueous humor (AH) outflow and intraocular pressure (IOP). To explore whether elevated IOP, a major risk factor for primary open angle glaucoma (POAG) influences Rho GTPase signaling in the TM, we recorded AH outflow in enucleated contralateral porcine eyes perfused for 4–5 h at either 15 mm or 50 mm Hg pressure. After perfusion, TM tissue extracted from perfused eyes was evaluated for the activation status of Rho GTPase, myosin light chain (MLC), myosin phosphatase target substrate 1 (MYPT1), myristoylated alanine-rich C-kinase substrate (MARCKS) and paxillin. Eyes perfused at 50 mm Hg exhibited a significant decrease in AH outflow facility compared with those perfused at 15 mm Hg. Additionally, TM tissue from eyes perfused at 50 mm Hg revealed significantly increased levels of activated RhoA and phosphorylated MLC, MYPT1, MARCKS and paxillin compared to TM tissue derived from eyes perfused at 15 mm Hg. Taken together, these observations indicate that elevated IOP-induced activation of Rho GTPase-dependent contractile signaling in the TM is associated with increased resistance to AH outflow through the trabecular pathway, and demonstrate the sensitivity of Rho GTPase signaling to mechanical force in the AH outflow pathway.

Glaucoma is the second leading cause of blindness globally, and if untreated, can lead to irreversible blindness due to optic nerve degeneration and loss of retinal ganglion cells (Kwon et al., 2009). Primary open-angle glaucoma (POAG), the most prevalent form of glaucoma in the United States, is associated with elevated intraocular pressure (IOP), which is considered a definitive risk factor for POAG (Kwon et al., 2009; Quigley, 1993; Weinreb and Khaw, 2004). Importantly, lowering IOP has been shown to delay vision loss in glaucoma patients, and has remained only treatment available for all types of glaucoma (Kwon et al., 2009; Lee and Goldberg, 2011; Weinreb and Khaw, 2004). However, the pathobiology of elevated IOP and glaucoma has remained elusive. Therefore, a thorough understanding of molecular basis of both physiological and pathological IOP is expected to offer novel insights into the development of efficacious new IOP lowering medication.

It is commonly believed that elevated IOP derives primarily from the increased resistance to AH outflow through the conventional or trabecular pathway consisting of the trabecular meshwork (TM), Schlemm's canal (SC), and juxtacanalicular connective tissue (JCT) (Gabelt and Kaufman, 2005; Lee and Goldberg, 2011). Although the molecular basis for increased resistance to AH outflow is not completely clear, it is believed that accumulation of extracellular matrix, tissue stiffness, compromised phagocytosis and cell death are some of the factors associated with increased resistance to AH outflow and elevated IOP (Stamer and Acott, 2012; Stamer et al., 2015). Various growth factors (e.g. TGF-β, connective tissue growth factor, Wnt, Nitric oxide, lysophosphatidic acid, sphingosine-1-phosphate and endothelin-1), cytokines, extracellular matrix (ECM) proteins and ECM degrading enzymes have also been shown to modulate AH outflow through the TM by influencing actomyosin organization, cell adhesive interactions and contractile properties of the TM (Stamer and Acott, 2012). Importantly, Rho GTPase and its downstream effector, Rho kinase, have been demonstrated to play a critical role in the signaling mechanisms triggered by physiological factors and to thereby regulate TM

* Corresponding author. Department of Ophthalmology, Duke University School of Medicine, Durham, NC 27710, USA.
E-mail address: p.rao@dm.duke.edu (P.V. Rao).
[1] Equal contribution.
[2] Current address: Department of Ophthalmology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan.

http://dx.doi.org/10.1016/j.exer.2015.05.001
0014-4835/

contractile properties, ECM synthesis and AH outflow through the TM (Inoue and Tanihara, 2013; Pattabiraman et al., 2014; Pattabiraman and Rao, 2010; Pattabiraman et al., 2015; Rao et al., 2001; Zhang et al., 2008). Additionally, mechanical stretch, matrix rigidity, fluid flow force and tissue fibrosis have also been shown to regulate Rho GTPase signaling activity in vascular and other smooth muscle tissues and in endothelial cells suggesting involvement of Rho GTPase in mechanotransduction (Boopathi et al., 2014; Chiquet et al., 2009; Higashida et al., 2013; Huang et al., 2012; Mammoto et al., 2008; Mattias et al., 2014; Provenzano and Keely, 2011; Tan et al., 2013; Zhao et al., 2007; Zhou et al., 2013). Conversely, elevated IOP has been reported to influence the expression profile of various genes in TM as a homeostatic response to control fluctuations in AH outflow and IOP (Borras, 2003). Since dysregulated Rho GTPase signaling in the trabecular AH outflow pathway has been demonstrated to increase resistance to AH outflow under experimental conditions (Junglas et al., 2012; Pattabiraman et al., 2015; Zhang et al., 2008), and Rho kinase inhibitors are in advanced clinical trials as agents for lowering IOP in human patients (Inoue and Tanihara, 2013; Williams et al., 2011), in this study, we sought to address whether elevated IOP influences the activity of Rho GTPase and its downstream effector proteins involved in contractile and cell adhesive responses in TM in the context of AH outflow resistance.

To this end, experiments were conducted on freshly enucleated cadaver porcine eyes (contralateral pairs) perfused with Dulbecco's Phosphate Buffered Saline, pH7.4 containing 5.5 mM D-glucose at 32 °C, using a Grant perfusion system as we described previously (Epstein et al., 1999; Rao et al., 2001). Eyes were initially perfused at 15 mm Hg for 1 h to record baseline AH outflow, following which one eye from each pair continued to be perfused at 15 mm Hg, while the contralateral eye was perfused at 50 mm Hg for 4 h. Aqueous outflow was recorded at hourly intervals and after completion of perfusion, aqueous outflow rate (μl/min) and facility (μl/min/mm Hg) data were analyzed by a paired two-tailed Student's t-test or ANOVA (analysis of variance) to determine significance of any observed changes. Fig. 1A which illustrates percent change in aqueous outflow rate from baseline, shows a significant increase in aqueous outflow ($p < 0.05$; $n = 7$) rate in eyes perfused at 50 mm Hg compared to control eyes perfused at 15 mm Hg. On the other hand, aqueous outflow facility in eyes perfused at 50 mm Hg showed a significant decrease compared to eyes perfused at 15 mm Hg (Fig. 1B). This decrease in aqueous outflow facility in eyes perfused at 50 mm Hg was evident from the first hour and persisted till the 4th hour post baseline perfusion, indicating increased resistance to aqueous outflow in response to elevated perfusion pressure. This observation was found to be consistent with several previous reports in which an inverse association has been confirmed between IOP and AH outflow facility (Battista et al., 2008; Becker and Constant, 1956; Brubaker, 1975; Dai et al., 2009; Hashimoto and Epstein, 1980). These data also indicate that under conditions of acutely increased perfusion pressure, the AH outflow tissues appear to adapt to facilitate increased AH drainage through the conventional pathway within the first hour, based on the observed increase in the aqueous outflow rate in the eyes perfused at 50 mm Hg.

Additionally, when perfusion pressure was lowered to 15 mm Hg in eyes initially perfused at high pressure (50 mm Hg for 4 h), the outflow facility began to increase significantly within three hours, indicating that perfusion pressure exerts a reversible response on outflow facility in the short term perfusion model (data not shown). In Fig. 1A and B, porcine eyes perfused at 15 mm Hg also show a steady increase in aqueous outflow rate and facility with increased perfusion time, a response attributed to the washout effect (Epstein et al., 1999). Although, the molecular mechanism involved in washout effect is not thoroughly understood, enucleated eyes from certain species exhibit increased outflow facility with time during perfusion with normal perfusion medium (Battista et al., 2008; Epstein et al., 1999). Since contralateral paired eyes were used to evaluate the effects of 15 and 50 mm Hg intraocular pressure on outflow facility, the washout response observed in controls eyes was subtracted from the values of outflow facility measured in eyes perfused at 50 mm Hg.

The eyes perfused for five hours at 15 and 50 mm Hg were fixed for histological analysis of changes as we described earlier (Rao et al., 2001), then examined for the changes in the integrity of aqueous outflow pathway tissues including TM and aqueous plexi (equivalent to SC in humans), using transmission electron microscopy (Fig. 1C). These analyses revealed a much reduced lumen space in aqueous plexi (AP) of eyes perfused at 50 mm Hg compared to those perfused at 15 mm Hg. Similarly, there was an obvious disorganization and compression of TM in eyes perfused at 50 mm Hg relative to eyes perfused at 15 mm Hg (Fig. 1C). Consistent with previously reported observations (Battista et al., 2008), these histological changes observed in the TM and AP of porcine eyes subjected to high pressure perfusion demonstrate the collapsible nature of AP and disorganization of TM under high pressure perfusion and correlated well with increased resistance to aqueous humor outflow.

The Rho GTPase/Rho kinase signaling pathway has been shown to modulate AH outflow by regulating the contractile properties of TM and SC cells in both, enucleated eyes and in live animals of different species, including humans (Inoue and Tanihara, 2013; Pattabiraman et al., 2015; Rao et al., 2001). Moreover, Rho GTPase regulates the formation of focal adhesions which are well-recognized mechanosensors for transduction of mechanical forces into biological signals (mechanotransduction) involving integrins, ECM, focal adhesion adaptor proteins, actomyosin cytoskeleton and tyrosine kinases (Goldmann, 2014; Provenzano and Keely, 2011; Schwarz et al., 2006; Shemesh et al., 2005). Therefore, to explore the plausible influence of elevated IOP on Rho GTPase activation, contractile activity and focal adhesion formation in TM tissue, we extracted TM tissue from enucleated porcine eyes perfused either at 15 or 50 mm Hg for 5 h as described earlier. For these analyses, each sample was comprised of pooled TM tissue from 4 eyes, and 3 pooled samples were analyzed per experimental condition – i.e., the basal (15 mm Hg) and the high (50 mm Hg) pressure conditions. Such pooled samples were collected separately for the Rho GTPase activation assay (Set 1) and phosphorylation status of MLC, MARCKS, MYPT1 and paxillin (Set 2). For the set 1 samples, TM tissue was snap frozen in liquid nitrogen immediately upon extraction from the perfused eye, and stored at −80 °C till further analysis. For assessment of Rho activation status, TM samples were processed for RhoA-GTP pulldown assay using a RhoA activation assay kit (Cytoskeleton Inc, Denver, CO) as we described earlier (Zhang et al., 2008). Assays were done in triplicates with appropriate positive and negative controls as per the manufacturer's instructions. Tissue lysates containing 500 μg of protein were processed in the Rho-GTP pull-down assay to quantify the activated Rho GTPase (Rho-GTP form), using rhotekin-Rho binding domain (RBD) beads, followed by immunoblot analysis using an anti-RhoA monoclonal antibody (Cytoskeleton Inc), as we described earlier (Zhang et al., 2008). Fig. 2A shows a representative immunoblot of activated Rho GTPase from both basal (15 mm Hg) and high pressure (50 mm Hg) TM specimens along with total RhoA GTPase which was probed as a loading control using anti-RhoA polyclonal antibody (sc179; Santa Cruz Biotechnology). Quantitative analysis of the levels of activated RhoA in TM tissue from high pressure perfused eyes showed a significant increase ($p < 0.5$, $n = 3$; based on student's t-test) compared with TM tissue from eyes perfused at