Atorvastatin reduces IOP in ocular hypertension \textit{in vivo} and suppresses ECM in trabecular meshwork perhaps via FGD4

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Abstract. To explore the role of atorvastatin in regulating intraocular pressure (IOP) in glaucoma \textit{in vivo}, and to investigate its related molecular pathway \textit{in vitro}, an ocular hypertension model was generated by intravitreal injection of an adenoviral vector encoding transforming growth factor (TGF)-\(\beta\)2 in the right eye of BALB/cJ mice, while the left was treated with an empty control adenovirus. To determine its anti-intraocular hypertension role, these induced hyper-IOP mice were gavaged with atorvastatin (20 mg/kg/day). Furthermore, extracellular matrix (ECM) factors were examined in the primary human trabecular meshwork (HTM) cells followed atorvastatin (0~200 µM) treatment \textit{in vitro}. Whole genome microarray was employed to identify potential therapeutic target molecules associated with ECM regulation. Unilateral murine ocular hypertension was induced, via intravitreal injection of the adenoviral vector carrying the human TGF-\(\beta\)2 gene (Ad.hTGF-\(\beta\)2226/228), raising IOP from 12±1.6 to 32.3±0.7 mmHg (n=6, \(P<0.05\)) at day 15, which plateaued from day 15 to 30. Atorvastatin administration from day 15 to 30 decreased IOP from 32.3±0.7 to 15.4±1.1 mmHg (n=6, \(P<0.05\)) at day 30. Additionally, atorvastatin administration changed the morphology of cultured HTM cells from an elongated and adherent morphology into rounded, less elongated and less adherent cells, accompanied with suppressed expression of ECM. Gene Ontology and Genome analysis revealed that FGD4 (FYVE, RhoGEF and PH domain containing 4) might be a key factor contributing to these changes. Our data demonstrated that atorvastatin reduced TGF-\(\beta\)2-induced ocular hypertension \textit{in vivo}, perhaps via modifying cellular structure and decreasing ECM, using the FGD4 signaling pathway, as demonstrated in HTM cells. Our findings provide some useful information for the management of glaucoma, with statin therapy revealing a potential novel therapeutic pathway for glaucoma treatment.

Introduction

Primary open angle glaucoma (POAG) leads to irreversible blindness (1), which is characterized by the gradual apoptosis of retinal ganglion cells, resulting in visual field defects (2). Intraocular pressure (IOP) elevation is the major risk factor for the progression of POAG (3). However, the underlying pathogenesis of POAG remains inconclusive. Considering that ~75% of aqueous humor outflow occurs through the trabecular meshwork (TM) pathway (4), increased IOP-associated POAG is considered to be mainly due to elevated resistance to the TM outflow (5).

Most of the increased resistance to aqueous humor outflow is due to an increase in extracellular matrix (ECM) deposition (6-8). ECM contains elastin, collagens and fibronectin (FN). Considering the involvement of the ECM in increasing the resistance of the TM to aqueous humor outflow, pathological dysregulation of the ECM is strongly implicated in POAG, in part mediated via the matricellular proteins, which are important regulatory factors-extracellular proteins, including tenascin-C, secreted protein and rich in cysteine (SPARC) and connective tissue growth factor (CTGF), and communicate with each other and regulate their surrounding ECM (9,10). CTGF and SPARC may promote tissue fibrosis and abnormal tissue remodelling and disrupt ECM homeostasis, which eventually contributes to the pathogenesis of glaucoma (11,12).

In addition, aging, actomyosin contractile systems (e.g. ciliary muscle contractions) and inflammation also contribute to the TM resistance to outflow (13,14).

FYVE, RhoGEF and PH domain containing 4 (FGD4) is a guanine nucleotide exchange factor specific for activation of cell division control protein 42 homolog (CDC42) (GTP bound) (15). The FGD4/CDC42 pathway is involved in the regulation of cell morphology, cytoskeleton organization, intercellular adhesion and ECM synthesis of non-ocular cells (16-18). Thus, the FGD4/CDC42 pathway may also contribute to the regulation of cell morphology, cytoskeletal organization, cell adhesion and ECM synthesis in human trabecular meshwork (HTM) cells.
There are several therapeutic agents available for reducing IOP in glaucoma (19). However, few of them are designed specifically for controlling the pathogenesis of POAG, e.g., targeting ECM accumulation in the TM. Moreover, the currently available anti-glaucoma agents can only decrease IOP, but not prevent the progression of, nor cure POAG (20,21).

Statins reduce serum cholesterol via selectively inhibiting HMG-CoA reductase for the biosynthesis of cholesterol (22-24). Statins are well known for their 'pleiotropic effects', e.g., anti-fibrotic, anti-inflammatory and immunomodulatory (25-29). Interestingly, statins also reduce the progression of POAG in specific populations (30-32), suggesting that statins minimize the risk of the development of glaucoma, as well as delay the progression of visual field defect (32). Because statins suppress ECM synthesis in non-ocular tissues, we hypothesized that statins can also decrease ECM dysregulation in human trabecular meshwork (HTM), constituting a potential anti-glaucoma mechanism (33,34). We demonstrated previously that atorvastatin improved aqueous humor outflow in a porcine whole-eye perfusion model ex vivo (35), but it is unclear whether atorvastatin is able to reduce IOP in POAG in vivo.

Transforming growth factor (TGF)-β2, expressed in TM cells, contributes to ECM generation (36). An adenoviral TGF-β2-induced ocular hypertension murine model was found to display a high glaucoma risk via decreasing aqueous outflow facility (36,37). Such an animal model is expected to be a useful model to investigate the pathogenesis of glaucoma (38).

Our present study aimed to investigate whether atorvastatin was able to reduce TGF-β2-induced intraocular hypertension in vivo, as well as to investigate the possible molecular expression in the dysregulation of the ECM in primary HTM cells following atorvastatin administration in vitro.

Materials and methods

Animals. All animals used in this research followed the ARVO statement for the Use of Animals (https://www.arvo.org/About/policies/statement-for-the-use-of-animals-in-ophthalmic-and-visual-research/). BALB/cJ mice (8 weeks; 3 females and 3 males per group, 4 groups and 24 mice in this study; 22-25 g), purchased from the Lingchang Animal Facility (Shanghai, China), were offered food and water ad libitum on a 12 h light/12 h dark cycle at 20°C room temperature. At the end of the experiment the animals were euthanized by cervical dislocation. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee of the Huashan Hospital, Fudan University (Protocol #: JS-194, Approval Date: 19 February 2019).

Induction of ocular hypertension in vivo. The ocular hypertension model was induced by injecting an adenoviral vector of human TGF-β2 (Ad.hTGF-β2226/228) in 2 µl (6x10^7 pfu) intravitreally in the right eye, as described previously (36). The left eye was given carrier Ad with an empty vector (6x10^7 pfu in 2 µl), as a control. There was also a blank, no-injection control group. The Ad.hTGF-β2226/228-injected animals were further divided into groups with and without atorvastatin administration (20 mg/kg/day, gavage), starting at day 15. Thus, there were totally four groups of experimental mice: Ad.hTGF-β2226/228-induced (n=6); Ad.hTGF-β2226/228-induced with atorvastatin from day 15 (n=6); Ad. empty-injected (n=6); and non-injection (n=6). All IOP measurements were implemented using a Tonolab rebound tonometer (Icare), as described (39).

Trabecular meshwork cell culture. All experiments were carried out according to the guidelines of the Human Ethics Committee of Huashan Hospital, Fudan University (protocol #: 2020-611, approval date: 31 March 2020) (40). Primary HTM cells were isolated and characterized as previously (41). There were 4 donors from Huashan Hospital, a Red Cross Hospital (Shanghai, China), aged 30, 35, 48 and 50 years, including 2 females and 2 males, respectively. Formal written consent was obtained from each of the donors in this study. All the donors had no previous diagnosis or history of eye disease. A more detailed description of the procedures for generating primary HTM cells were as follows. Human TM tissues were explanted from the corneal edge and were further minced in culture solution. The minced tissues were further cultured for 4 h at 37°C under 5% CO₂ until the cells were adherent to the bottom of the culture flask. The culture flasks were washed with fresh culture medium to remove dead unadhered cells. The culture medium consisted of DMEM containing 15% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.). HTM cells were sub-cultured when the cells reached 80% confluence. The HTM cells were passaged at a ratio of 1:3 to maintain the same conditions. The third passage of HTM cells was identified and validated as HTM cells immunohistochemically for the specific proteins FN, vimentin and laminin (LN). The cultured HTM cells were stimulated with 250 and 500 nM dexamethasone (DEX) (Sigma-Aldrich; Merck KGaA) with fresh medium every 2 days until the 6th day. The level of myocilin (MYOC), which correlates with the trabecular meshwork glucocorticoid response, was assessed with western blot analysis. These HTM cells were passaged for 5 cultures in this study.

Atorvastatin treatment. The HTM cells were serum-deprived for 12 h until they reached 90% confluence. These cells were subsequently treated with atorvastatin (Sigma-Aldrich; Merck KGaA) at different dosages (0, 50, 100, 150 and 200 µM), and for different times (12, 24, 36 and 48 h). The morphological changes in the HTM cells were captured under phase contrast microscopy (ECLIPSE Ni-U; Nikon). The effect of atorvastatin on the viability of HTM cells was determined using a CCK-8 assay kit (Dojindo Molecular Technologies, Inc.) following atorvastatin treatment (0, 50, 100, 150, 200 µM), as previously described (35).

Real-time PCR. Whole cell RNA from HTM cells was extracted, using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Prime-Script RT reagent kit (RR036A; Takara Biotechnology Co.) was used for cDNA synthesis from 1 µg RNA. Real-time PCR was conducted using a qPCR kit (RR820A; Takara Biotechnology Co.), and the primers used are shown in Table I. The procedures have been described in detail previously (35).
**Western blot analysis.** Western blot analysis was performed, as described previously (35,42). The concentration of each protein sample was determined using the Bradford protein-detection method (Bio-Rad, Laboratories, Inc.) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide), then transferred onto a polyvinylidene fluoride (PVDF) membrane. The blots were incubated with the primary antibodies: rabbit anti-collagen I antibody (14695-1-AP, 1:1,000 dilution; Proteintech), rabbit anti-fibronectin antibody (15613-1-AP, 1:1,000 dilution; Proteintech), rabbit anti-CTGF antibody (ab6992, 1:1,000 dilution; Abcam), rabbit anti-SPARC antibody (15274-1-AP, 1:1,000 dilution, Proteintech), GAPDH (60004-1-lg, 1:500 dilution, Proteintech) in blocking solution overnight at 4˚C. Then the membranes were incubated with goat anti-rabbit secondary antibody (SE134, 1:1,000 dilution, Solarbio) at room temperature for 1 h, followed by washing with TBST for 3 times. Then, typically enhanced chemiluminescent (ECL) kit was used according to the manufacturer's instructions (POO18FS, Beyotime). A particular band of each sample was visualized to each GAPdh band (version 1.48v; National Institutes of Health). The protein density of every band was analyzed with Image J software and normalized as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to compare results among two or more groups, followed by least significant difference (LSD) post hoc analysis; P<0.05 was deemed to be a statistically significant difference.

**GO and KEGG analysis.** The differentially expressed genes were analyzed by Gene Oncology (GO) enrichment, and were implemented, using the top GO R packages based on the Fisher's exact test (43). Clusters of Orthologous Groups and KEGG databases were used to classify and analyze differentially expressed genes (44), and P<0.05 was used as the significance enrichment standard.

**Statistical analysis.** Data were analyzed using SPSS17.0 version 12.0 software (SPSS, Inc.). All data are represented as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to compare results among two or more groups, followed by least significant difference (LSD) post hoc analysis; P<0.05 was deemed to be a statistically significant difference.

**Results**

Atorvastatin reduces intraocular pressure in a mouse model of ocular hypertension. IOP was increased 2-fold following intravitreal injection of Ad.hTGF-β2/28 (12±1.6 vs. 24±1.7 mmHg, P<0.01) at day 5 (Fig. 1). A significantly elevated IOP of 32.3±0.7 mmHg was observed by day 15 following vector intravitreal injection, which plateaued until day 30, which was ~1.6-fold higher than the non-injection eyes (12.8±0.4 mmHg) or the vehicle (Ad. Empty) eyes (12.6±0.8 mmHg) (P<0.001). By day 20 (5 days after the commencement of the gavage of atorvastatin), IOP was significantly decreased in the hypertensive IOP group treated with atorvastatin compared to the non-atorvastatin-treated group (21.5±0.7 vs. 32.5±0.7 mmHg, P<0.01). By day 30, the IOP of the atorvastatin treatment subgroup of the Ad.hTGF-β2/28-induced hypertensive IOP group continued to become significantly lower than that of the non-atorvastatin-treated group (15.4±1.1 vs. 32.3±0.7 mmHg, respectively).
Atorvastatin toxicity and HTM cell morphology. The isolated primary HTM cells grew out from the HTM tissues (Fig. 2A) and expressed FN, vimentin and LN (Fig. 2B), and MYOC was increased after stimulation with DEX in a dose-dependent manner (Fig. 2C). There was no significant difference in viability of the HTM cells treated with and without atorvastatin (50-100 μM), except with atorvastatin at a dosage of 200 μM for 48 h (Fig. 3A). Thus, the time and the dose of atorvastatin were selected to be no more than 24 h nor over 200 μM in the subsequent experiments (Fig. 3A). There were morphological changes in the HTM cells following 24 h of statin treatment. The shape of the HTM cells was altered from an elongated and adherent cell morphology into rounded and detached cells with reduced elongation in a dose-dependent manner following statin treatment (Fig. 3B).

Verification of the expression of FGD protein isoforms in HTM and the inhibitory effect of atorvastatin on FGD4 gene expression in trabecular meshwork cells. Our study found that the FGD4 gene was the most highly expressed isoform of FGD in HTM cells, and FGD1 was the second most highly expressed among the FGD genes (Fig. 7A). The expression of the FGD4 gene was decreased with increasing atorvastatin concentration (Fig. 7B). There was no significant change in FGD1 gene expression following atorvastatin treatment (Fig. 7C).

Discussion

In the present study, we observed that atorvastatin reduced intraocular pressure (IOP) of ocular hypertension in vivo and in vitro, consistent with the observed morphologic change of isolated HTM cells. Atorvastatin also suppressed two key matricellular proteins [secreted protein and rich in cysteine (SPARC) and connective tissue growth factor (CTGF)] and decreased extracellular matrix (ECM) synthesis [collagen I and fibronectin (FN)] in human trabecular meshwork (HTM) cells. In addition, our data support the hypothesis that FYVE, RhoGEF and PH domain containing 4 (FGD4)/cell division control protein 42 homolog (CDC42) might be the pivotal factors to trigger these changes, which could be a novel potential therapeutic target for glaucoma pathogenesis and treatment. All the findings suggest that atorvastatin may be able to be a useful agent in the management of glaucoma.

Ocular hypertension induced by Ad.hTGF-β226/228 can be reduced following atorvastatin treatment, suggesting that statins are effective in the management of ocular hypertension via the transforming growth factor (TGF)-β2 pathway, which is in line with others (37,38), showing hypertensive IOP is induced via the TGF-β2 route in vivo. However, the precise underlying mechanism of atorvastatin in reducing IOP is still unclear and will be clarified in the future, because elevated IOP is the most pivotal risk factor contributing to the progression of glaucoma (1,2).

Furthermore, there was no obvious cytotoxicity observed in the HTM cells in response to atorvastatin treatment, even
at a rather high concentration, suggesting that the dosage of atorvastatin used in the present study is safe and reliable.

Intercellular space between HTM cells was increased and HTM cell size was reduced following atorvastatin treatment in a dose-response manner, suggesting that there is a potential role for statins in reducing IOP, which may also be associated with a decrease in actin stress fibers and focal adhesion (45). The increased intercellular space and smaller size of HTM cells could improve permeability of the TM and reduce resistance of the TM to outflow. The permeability of the TM is acutely modulated by the combined action of contractile and volume-regulatory properties (46). Taken together, this could explain why atorvastatin reduced IOP via decreasing cell-cell adhesion and increasing intercellular space.

It has been reported that statins suppress ECM synthesis in various non-ocular tissues (33,34). Furthermore, there is a correlation between IOP and synthesis of ECM of the TM, where reducing ECM contributes to a decrease in outflow resistance (47), which is in line with our finding that atorvastatin decreased collagen I and FN expression in HTM cells. Thus, these data suggest that atorvastatin acts as a novel anti-glaucoma medicine, targeting the pathogenesis of primary open angle glaucoma (POAG). Moreover, we also found that atorvastatin inhibited matricellular protein expression (SPARC and CTGF), which enhance fibrosis and increase ECM deposition (10), providing a possible explanation for the involvement of a reduction in IOP at the molecular level.
FGD4 is closely related to the interaction among ECM receptors, adhesion junctions, and the regulation of the actin cytoskeleton, as demonstrated following GO and KEGG gene enrichment analysis. We found that FGD4 was significantly inhibited in response to atorvastatin treatment, suggesting the importance of FGD4 during the development of POAG. FGD4 activates CDC42 via GTP. CDC42 is one of the three members of the Rho protein guanosine triphosphatase (GTPase) family (48), which is closely related to the pharmacological effects of statins (49). The FGD4/CDC42 signaling pathway influences cytoskeleton remodelling and ECM generation (50,51), and thus might also play a pivotal role in glaucoma pathogenesis.
Considering data from whole genome microarray, we believe that atorvastatin reduces IOP via the FGD4/CDC42 signaling pathway, which suppresses ECM expression and cytoskeleton remodelling, which will be verified in a future study.

In conclusion, the present study demonstrated that atorvastatin reduced IOP, accompanied by an anti-ECM synthesis effect, improved cellular spacing, and decreased focal adhesion proteins. Statins may be good candidates as a novel treatment for glaucoma. In future research, the precise underlying mechanisms of the FGD4/CDC42 signaling pathway involvement in the anti-glaucoma effect of atorvastatin will be investigated.
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Figure 6. (A) Expression and enrichment analysis of different genes in HTM cells treated with atorvastatin. (B) Expression levels of 'extracellular matrix synthesis pathway', 'cell-cell adhesion pathway', 'cytoskeletal pathway' related genes in HTM cells treated with atorvastatin. Among these genes, the FGD4 gene was downregulated the most in a drug concentration-dependent manner. HTM, human trabecular meshwork; FGD4, FYVE, RhoGEF and PH domain containing 4.

Figure 7. (A) FGD4 was the most highly expressed isoform in HTM cells. (B) The expression level of FGD4 was decreased in response to the increased dosages of atorvastatin. *P<0.05 vs. the untreated control. (C) No significant difference in FGD1 expression was observed among the different atorvastatin concentrations. HTM, human trabecular meshwork; FGD4, FYVE, RhoGEF and PH domain containing 4.
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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XYS and YYZ completed all experimental components and contributed to the interpretation of the results. YYC, WTL, LC, JLZ and YZ performed the statistical analysis and contributed to the interpretation of the results. YYC and WTL participated in the revision of this paper. YYZ contributed to the experimental design. XYS and YYZ wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work (including all data) are appropriately investigated and resolved.

Ethics approval and consent to participate

The animal study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Animal Care and Use Committee of the Huashan Hospital, Fudan University (Protocol #: JS-194, Approval Date: 19 February 2019). The primary HTM cell experiments were carried out according to the guidelines of the Human Ethics Committee of Huashan Hospital, Fudan University (protocol #: 2020-611, approval date: 31 March 2020). Formal written consent was obtained from each of the donors in this study.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare no competing interests. This research is original and has not been published elsewhere.

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