Statins reduce TGF-beta2-modulation of the extracellular matrix in cultured astrocytes of the human optic nerve head

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ABSTRACT

Statins are cholesterol lowering drugs and have shown beneficial effects on glaucoma. With regard to the mechanism of statin action on glaucoma, we investigated the effects of statins on transforming growth factor-beta 2 (TGF-beta2)-induced expression of extracellular matrix (ECM) proteins in human astrocytes of the optic nerve head (ONH) lamina cribrosa (LC). By using primary human ONH astrocytes, we found that both simvastatin and lovastatin inhibited TGF-beta2-mediated expression of ECM proteins such as connective tissue growth factor, collagen I, fibronectin, and plasminogen activator inhibitor-1. Suppression of ECM related proteins is due to inhibition of Smad2/3 activation as statins inhibit TGF-beta2 signaling. In ONH astrocytes, TGF-beta2 does not induce MAPK activation. In this study we found an anti-fibrotic effect of statins in human astrocytes of the ONH and identified TGF-beta2 as a mediator of statin action, which may support a beneficial role for statins in blocking glaucomatous axonal damage induced by ECM remodeling.

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1. Introduction

Glaucoma is a progressive optic neuropathy that may lead to blindness without appropriate and timely treatments. One of the pathogenic characteristics of glaucoma is a remodeling of the extracellular matrix (ECM) at the lamina cribrosa (LC) of the optic nerve head (ONH) (Radius and Anderson, 1981; Quigley and Anderson, 1977, Quigley et al., 1981). The LC of the ONH is a multi-layered sieve-like supporting structure from which the axons of retinal ganglion cells exit the eye. During glaucomatous optic disc damage, the LC of the ONH experiences considerable deformation, becoming thinner and posteriorly displaced, with these changes aggravating the axonal damage caused by the remodeling of the ECM in the LC (Hernandez et al., 1990; Pena et al., 1996). As the main glial cells in the LC of the ONH, astrocytes play critical roles in maintaining neuronal health. Astrocytes may also mediate ECM restructuring during glaucoma development (Hernandez et al., 1988, 2000; Neumann et al., 2008; Yu et al., 2007; Zode et al., 2011).

Transforming growth factor-beta (TGF-beta) modulates diverse cellular responses, including differentiation, proliferation, and chemotaxis. TGF-beta signaling can be mediated by the canonical Smad pathway through Smad2/3 or a non-Smad pathway. TGF-beta2 signaling is initiated by the binding of TGF-beta2 to its receptors. Activation of type I receptor induces phosphorylation of downstream effectors. In the canonical Smad pathway, phosphorylated Smad2/3 forms a heterodimer with Smad4 and translocates to the nucleus, resulting in target gene expression. The non-Smad pathway is mediated by phosphorylation of Akt, ERK, p38, or JNK. TGF-beta2, the predominant TGF-beta isofrom in the eye, has been suggested as one of the molecular modulators of the astrocyte rearrangement of the ECM in the glaucomatous ONH (Pena et al., 1999; Zode et al., 2011). The TGF-beta2 level is significantly increased in the ONH of glaucoma patients, with the source of the TGF-beta determined to be reactive astrocytes in the ONH (Pena et al., 1999; Zode et al., 2011). Consistent with the in vivo data, TGF-beta2 treatment of cultured astrocytes increases the expression of ECM molecules such as collagen I, fibronectin, and plasminogen activator inhibitor (PAI)-1 (Fuchshofer et al., 2005; Zode et al., 2011). Hence, attenuation of
ECM production via a TGF-β2 intervention would theoretically protect neurons against glaucomatous axonal damage.

Statins are HMG-CoA reductase inhibitors that lower lipid levels by blocking the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol. Due to their cholesterol-lowering properties, statins such as simvastatin and lovastatin have been widely prescribed for cardiovascular disease. The effects of statins on astrocytes have additionally been studied in various brain lesions (Kurata et al., 2012; Li et al., 2009; Wang et al., 2006; Yongjun et al., 2013) and statins have shown neuroprotective effects in studies of other brain pathologies. For example, systemic simvastatin treatment rescues retinal ganglion cells from optic nerve injury, possibly by suppressing astroglial NF-κB activation (Morishita et al., 2014). Simvastatin reduces the activation of astrocytes and microglia cells after traumatic brain injury (Li et al., 2009). Statins also have beneficial effects on glaucoma with patients taking statins showing a reduced risk of open-angle glaucoma development and normal tension glaucoma development (Leung et al., 2010; Stein et al., 2012). One potential mechanism of action of statins in glaucoma could be an effect on ONH circulation but these and other mechanisms remain to be fully investigated. Because statins have neuroprotective effects in other brain regions, statins might also protect retinal ganglion cells from glaucomatous damage.

With regard to the mechanism underlying the neuroprotective effect of statins in ONH astrocytes, we hypothesized that it could involve regulation of the TGF-β2 pathway. As mentioned above, TGF-β2-mediated ECM remodeling is a pathological phenomenon during glaucoma development. Statins have been reported to attenuate TGF-β signaling, with simvastatin attenuating TGF-β1-mediated alpha-smooth muscle actin (α-SMA), fibronectin, and connective tissue growth factor (CTGF) expression by suppressing Smad2/3 phosphorylation in human alveolar epithelial cells (Yang et al., 2013). Lovastatin decreases TGF-β-mediated CTGF transcription and α-SMA expression in human Tenon fibroblasts (Meyer-Ter-Vehn et al., 2008). Therefore, we investigated in our current study whether statins modulate TGF-β2 signaling and, consequently, TGF-β2-mediated ECM protein expression in cultured astrocytes from the LC of the human ONH.

2. Material and methods

2.1. Cell culture

Ten pairs of human eyes were harvested within 10 h of death from Asian donors (28–58 years of age) with no history of ocular disease. The study protocol was approved by the institutional review board of Asan Medical Center. This study followed the tenets of the Declaration of Helsinki, and informed consent was obtained from appropriate family members. ONH was isolated from the neighboring tissues using a microscope and sagittally dissected, and the LC was identified. LC discs were prepared by dissecting the pre- and post-laminar tissues. Each disc was then cut into multiple explants and placed in 25-cm² tissue culture flasks (Falcon, Lincoln Park, NJ) at 37 °C in a 5% CO₂ incubator. Each explant was cultured in 0.5 ml Dulbecco’s modified Eagle’s medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS; Invitrogen-Gibco-Life Science Technology, Karlsruhe, Germany), 5 ng/mL human basic fibroblast growth factor (bFGF; Sigma-Aldrich, Darmstadt, Germany), 5 ng/mL human platelet-derived growth factor-A chain (PDGF-AA; Sigma-Aldrich), 50 U/mL penicillin, and 50 g/mL streptomycin (Invitrogen-Gibco-Life Science Technology). The growth medium was changed twice per week. Human LC explants were cultured as described by Hernandez et al. (1988). Once the initial outgrowth was detected, the medium was increased to 2 mL. Three weeks later, mixed cells were passaged using 0.25% trypsin-EDTA (Invitrogen-Gibco-Life Science Technology). The medium was then changed to serum-free astrocyte growth medium (AGM; Cambrex Bio Science, Verviers, Belgium) for 24 h to isolate the ONH astrocytes and then to 5% FBS-containing AGM (Yu et al., 2007).

2.2. Immunofluorescent staining of human ONH astrocytes

Astrocytes were seeded in 24-well culture plates containing 12-mm glass coverslips in growth medium. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% triton X-100. After two washes with phosphate-buffered saline (PBS), the cells were incubated overnight at 4 °C with primary antibodies (1:100) in PBS with 2% bovine serum albumin (BSA). After washing twice for 5-min washes in PBS, the cells were incubated for 1 h at room temperature with secondary antibody (Cy-3) (1:200) in PBS. Astrocytes were confirmed through positive staining for the glial fibrillary acidic protein (GFAP; Sigma-Aldrich), neural cell adhesion molecule (NCAM; Abcam, Cambridge, UK), Pax2 (Abcam), S100 (Abcam) and negative staining for A2B5 (Abcam). Cells were incubated with 1 µg/mL 4’, 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen-Gibco-Life Science Technology) to stain the nuclei. The coverslips were mounted on Fluorsave reagent (Calbiochem, San Diego, CA) and fluorescent images were taken using a confocal laser scanning system (TSC-SP2; Leica, Heidelberg, Germany) and Zeiss ZEN imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.3. Cell viability

Cytotoxicity was assayed using the stable tetrazolium salt WST-1 Cell Counting Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s protocol. Astrocytes were seeded into a 96-well plate and treated with statins for 24 h. Then, cells were incubated with 10 µL/well WST-1 reagent (Roche Applied Science) for 2 h. After incubation, any formazan dye that formed was quantitated using an automated microplate reader (Vmax; Molecular Devices, Palo Alto, CA). The measured absorbance is directly correlated with the number of viable cells.

2.4. Western blot analysis

Whole cell lysates for western blotting were prepared from cultured ONH astrocytes in protein extraction solution. After centrifugation for 15 min at 13,000 rpm at 4 °C, the supernatant was transferred to new tubes. Protein concentrations were determined using the Bradford protein-detection method (Bio-Rad, Munich, Germany). Each sample was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes, which were then blocked in bovine serum albumin (BSA) for 1 h. The blots were incubated with antibodies against collagen I (COLI; Abcam), fibronectin (Abcam), CTGF (Abcam), pSmad2/3 (Cell Signaling Technology, Danvers, MA), pAkt (Cell Signaling Technology), Akt (Cell Signaling Technology), pERK (Cell Signaling Technology), ERK (Cell Signaling Technology), and GAPDH (Cell Signaling Technology) in blocking solution overnight at 4 °C. After the membranes were washed with TBST, they were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The membranes were developed using the Enhanced Chemiluminescence (ECL) detection system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The band densities were quantified using FUJIFILM Science Laboratories Image Gauge Ver. 4.0 (Fuji Photo Film Co., Ltd, Japan).
2.5. Localization of pSmad2/3 and Smad4

Astrocytes were seeded in 24-well culture plates containing 12-mm glass coverslips in growth medium. The cells were pretreated with statins for 1 h and then treated with TGF-β2 for another hour. They were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. After two washes with phosphate-buffered saline (PBS), the cells were incubated overnight at 4 °C with pSmad2/3 antibody (1:100; Cell Signaling Technology) and Smad4 (1:100; Merck KGaA, Darmstadt, Germany) in PBS with 2% BSA. After two 5-min washes in PBS, the cells were incubated for 1 h at room temperature with secondary antibody (Cy-3) (1:200 in PBS). Cell nuclei were counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen-Gibco-Life Science Technology) at room temperature for 5 min. Confocal images were obtained using a confocal laser scanning system (TSC-SP2; Leica, Heidelberg, Germany) and Zeiss ZEN imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.6. Statistical analysis

All values are expressed as the mean ± standard deviation. Unpaired t-test and one way ANOVA test were used for statistical comparison. All statistical analyses were performed using an ANOVA test with SPSS (version 15.0; SPSS Inc., Chicago, IL). P < 0.05 was considered significant.

3. Results

3.1. Characterization of astrocytes from the human laminar cribrosa (LC) of the optic nerve head (ONH)

Primary astrocytes from the human ONH were subjected to immunostaining to ensure purity of the culture (Fig. 1). More than 95% of the cells were positive for GFAP which is constitutively immunostaining to ensure purity of the culture (Fig. 1). More than 95% of the cells were positive for GFAP which is constitutively expressed in astrocytes (Yang and Hernandez, 2003). GFAP staining was also distinguished astrocytes from LC cells, another major cell type in ONH which are GFAP-negative. Most of the primary cells were positive for other astrocyte markers including NCAM, Pax2 and S100 (Kobayashi et al., 1997; Yang and Hernandez, 2003). These cells were negative for A2B5 which is expressed in oligodendrocytes to study the effects of statin.

3.2. Evaluation of ECM protein expression in ONH astrocytes

In vivo, the TGF-β2 level is significantly elevated in ONHs from glaucomatous eyes compared with normal age-matched controls (Pena et al., 1999). In vitro, TGF-β2 substantially increases the levels of ECM proteins in astrocytes and LC cells of the human ONH (Fuchshofer et al., 2005; Zode et al., 2011). Therefore, we speculated that TGF-β2 might play a critical role in glaucoma development by inducing ECM remodeling, one of the pathological characteristics of glaucoma. To confirm the effect of TGF-β2 on the expression of ECM-related proteins, we treated primary ONH astrocytes with recombinant TGF-β2 at concentrations of 1, 5 and 10 ng/mL for 24 h. Cell lysates were then subjected to western blotting to determine the levels of proteins related to ECM maintenance (Fig. 2). We tested collagen I and fibronectin, which are major components of ECM, and PAI-1, which regulates the degradation of ECM molecules. In addition, we examined CTGF, which is a downstream effector of TGF-β2 and mediates ECM synthesis by TGF-β2 (Pena et al., 1999). Recombinant TGF-β2 induced the expression of the ECM proteins collagen I and fibronectin by 2 to 2.5-fold in ONH astrocytes. PAI-1 and CTGF expression were also induced by TGF-β2 in the ONH astrocytes. Protein levels were induced by TGF-β2 in a dose-dependent manner and saturated at 5 ng/mL in the ONH astrocytes. Thus, TGF-β2 induces the expression of ECM-related proteins in human ONH astrocytes in vitro.

3.3. Effects of statins on astrocyte viability

Statins induce cell death in cancer cells, airway smooth muscle cells, and fibroblasts (Ghavami et al., 2010; Tu et al., 2011). To test their effects on astrocyte viability, human astrocyte cultures were treated with different concentration of statins for 24 h and cell viability was tested using a WST-1 assay (Fig. 3). Cell viabilities (vs. a DMSO control) were measured at 93% for simvastatin and 96% for lovastatin with 0.1 μg/mL statins. With 0.5 μg/mL statins, cell viabilities were 93% for simvastatin and 92% for lovastatin. Cell viabilities were 84% for simvastatin and 88% for lovastatin with 1 μg/mL statins and 83% for simvastatin and 85% for lovastatin with 5 μg/mL statins. In our current study, 5 μg/mL concentration was used for experiments. Cytotoxicity by lovastatin was not significant to 5 μg/mL concentration whereas simvastatin starts to show some effect on cell viability from 1 μg/mL.

3.4. Statins inhibit TGF-β2-induced ECM protein expression

We next examined the effects of statins on the TGF-β2 pathway in ONH astrocytes. Previous reports have suggested that statins negatively regulate the TGF-β pathway, with simvastatin shown to attenuate the TGF-β1-mediated phosphorylation of Smad2/3, CTGF expression, and MMP-2 and MMP-9 secretion in human alveolar epithelial cells (Yang et al., 2013). In addition, lovastatin was reported to decrease TGF-β-mediated CTGF transcription and α-SMA expression in human Tenon fibroblasts (Tu et al., 2011). However, statins have also shown positive effects on the TGF-β pathway, with simvastatin and atorvastatin found to increase TGF-β1 secretion, TR-II expression, Smad2/3 phosphorylation, and ECM regulatory protein expression in vascular smooth muscle cells (Rodriguez-Vita et al., 2008). The effects of statins on the TGF-β pathway in astrocytes remain to be studied. To address whether statins positively or negatively regulate the TGF-β2 signaling pathway in astrocytes in our current study, we measured TGF-β2-mediated ECM protein levels in ONH astrocytes, with and without statins (simvastatin, Fig. 4A; lovastatin, Fig. 4B). The cells were treated with statins for 1 h followed by TGF-β2 for 24 h 1 μg/mL statins did not inhibit TGF-β2-mediated collagen I and fibronectin expression in preliminary experiments (data not shown). Since cell viability at 1 μg/mL and 5 μg/mL statins was in the similar range (84% for simvastatin and 88% for lovastatin with 1 μg/mL statins and 83% for simvastatin and 85% for lovastatin with 5 μg/mL statins), we used 5 μg/mL statins. At a 5 μg/mL concentration, both simvastatin and lovastatin inhibited the TGF-β2-mediated expression of ECM proteins levels. The P values for simvastatin with TGF-β versus TGF-β alone were 0.0022 for collagen I, 0.047 for fibronectin, 0.012 for CTGF and 0.049 for PAI-1. The P values for lovastatin with TGF-β versus TGF-β alone were 0.00034 for collagen I, 0.028 for fibronectin, 0.0174 for CTGF and 0.016 for PAI-1. Collagen I, CTGF, and PAI-1 were found in our analysis to be downregulated to the level comparable to simvastatin alone. Similarly, we observed that lovastatin inhibited TGF-β2-mediated induction of collagen I, CTGF, and PAI-1. Fibronectin expression was only partially inhibited by simvastatin (30% reduction) and lovastatin (20% reduction) but this reduction was significant. These data suggested that both simvastatin and lovastatin negatively regulate TGF-β2-mediated ECM protein expression in human ONH astrocytes.
Fig. 1. Characterization of human primary astrocytes. Primary astrocyte culture form human ONH was immunostained for astrocytes markers, glial fibrillary acidic protein (GFAP), neural cell adhesion molecule (NCAM), Pax2, and S100 to confirm their identification as astrocytes. More than 95% cells were positive for GFAP, NCAM, Pax2, and S100. Cells were negative for A2B5, an oligodendrocyte marker. Nuclei were visualized with DAPI (blue).

Fig. 2. Effects of TGF-β2 on the expression of collagen I, fibronectin, CTGF, and PAI-1 in human ONH astrocytes. Astrocytes were serum starved overnight in serum-free medium and then treated with 1, 5, and 10 ng/mL recombinant TGF-β2 for 24 h or with DMSO as a negative control. (A) The expression levels of collagen I, fibronectin, CTGF, and PAI-1 were determined by western blotting. Representative images from the results of four different donor eyes were shown. (B) Protein levels were normalized to GAPDH for loading control. No treatment was set control at 100% and relative protein levels to control were expressed. Data are expressed as mean ± standard deviation of results from four different donor eyes (n = 4).
3.5. Statins attenuate TGF-β2-induced nuclear localization of Smad2/3 and Smad4 but have no effect on the phosphorylation of non-smad pathway molecules

TGF-β2 binding to its receptors initiates several downstream pathways, such as the canonical Smad pathway, non-canonical MAPK pathway, and Rho pathway. TGF-β2 also induces activation of Smad2/3 pathway in human ONH astrocytes and LC cells but has no effect on the phosphorylation status of non-Smad pathway molecules such as p38, ERK, and JNK (Zode et al., 2011). We examined the phosphorylation of Smad2/3 by TGF-β2. ONH astrocytes were serum starved overnight and then treated with TGF-β2 for 15 min, 30 min, 1 h, 2 h and 6 h. Cell lysates were then immunoblotted with phospho-specific antibodies (Fig. 5A). The phosphorylation of Smad2 was found to be induced by TGF-β2 from 15 min and this activity persisted for up to 6 h. Phosphorylation of Smad3 was not significantly induced by TGF-β2 (Fig. 5A). We also examined the phosphorylation of Akt, ERK, p38 and JNK and these MAPK pathways were not induced by TGF-β2 in human ONH astrocytes (Fig. 5B).

Fig. 3. Effects of statins on the viability of human ONH astrocytes. Astrocytes were serum starved overnight in serum-free medium and then treated with 0.1, 0.5, 1, and 5 μg/mL statins for 24 h. Cytotoxic effects of simvastatin and lovastatin were examined in statin- and DMSO-treated astrocytes using a WST-1 assay. Data are expressed as mean ± standard deviation of results from five different donors (n = 5).

Fig. 4. Effects of statins on TGF-β2-induced expression of collagen I, fibronectin, CTGF, and PAI-1 (ECM) in human ONH astrocytes. Astrocytes were first serum starved overnight in serum-free medium. Astrocytes were then pretreated with simvastatin (5 μg/mL) (A) or lovastatin (5 μg/mL) (B) for 1 h followed by TGF-β2 (5 ng/mL) for 24 h or TGF-β2 alone (5 ng/mL) for 24 h. The expression levels of ECM proteins were determined by western blotting. Protein levels were normalized to GAPDH for loading control. No treatment was set control at 100% and relative protein levels to control were expressed. Data are expressed as mean ± standard deviation of results from four different donors (n = 4). Representative blot image of four different donors were shown.
We next investigated the effects of statins on the TGF-β2-mediated Smad pathway in ONH astrocytes. The cells were treated with TGF-β2 for 1 h in the presence or absence of statins and Smad2/3 phosphorylation was determined using a phospho-specific antibody (Fig. 6A). Phospho-Smad2 was found to be induced by TGF-β2 and pre-treatment with simvastatin or lovastatin for 1 h reduced Smad2 phosphorylation to the level comparable to control (Fig. 6A). These data suggested that statins suppress the TGF-β2-mediated phosphorylation of Smad2 in ONH astrocytes.

We also investigated Smad2/3 nuclear localization to further investigate Smad2/3 activation. Upon activation by TGF-β2, Smad2/3 forms a complex with Smad4 and translocates to the nucleus where it regulates TGF-β2-responsive gene expression. ONH astrocytes were treated with TGF-β2 for 1 h because Smad2/3 translocation occurs within a short period after TGF-β2 treatment (Zode et al., 2011), as compared to TGF-β2-responsive gene expression which was detected after 24 h. The nuclear accumulation of Smad2/3 and Smad4 was visualized by staining with a phospho-Smad2/3 antibody and a Smad4 antibody, respectively (Fig. 6B). In the untreated astrocytes, the phospho-Smad2/3 signal was detectable throughout the cytosol and nucleus. Neither simvastatin nor lovastatin alone had any effect on Smad2/3 distribution whereas we observed strong nuclear staining of phospho-Smad2/3 in TGF-β2-treated astrocytes, indicating translocalization of the active Smad2/3 protein (Fig. 6B). When astrocytes were treated with statins and TGF-β2 together, the strong nuclear staining of phospho-Smad2/3 was found to have dispersed with signal detectable throughout the cytosol and nucleus, indicating inhibition of Smad2/3 nuclear accumulation by statins. Similar to the phospho-Smad2/3 staining profile, Smad4 signal was concentrated in the nucleus following TGF-β2 treatment. However, astrocytes treated with both statins and TGF-β2 showed diffused Smad4 staining. These data suggested that the statins suppress TGF-β2-mediated Smad2/3 nuclear accumulation. Based on these observations, we conclude that statins interfere with TGF-β2 signaling upstream of Smad2/3 activation.

4. Discussion

Statins are drugs with cholesterol-lowering properties that are typically used to treat cardiovascular diseases. In addition to their role in modulating lipid metabolism, statins also have non-lipid effects, such as antifibrotic (Schaafsma et al., 2011) and neuroprotective (Schmeer et al., 2006) effects, allowing their possible use to treat other diseases. Statins exert beneficial effects on glaucoma,
Fig. 6. Effects of statins on TGF-β2-induced phosphorylation of Smad2 and nuclear localization of phospho-Smad2/3 and Smad4 in human ONH astrocytes. (A) Human ONH astrocytes cultures were serum starved overnight and then treated with TGF-β2 (5 ng/mL) alone for 1 h, statin (5 μg/mL) alone for 2 h or 1 h statin treatment followed by 1 h TGF-β2 (5 ng/mL). The phosphorylated Smad2 levels were normalized to total Smad2 and vehicle only sample was set control at 100% and relative protein levels to control were expressed. Data are expressed as mean ± standard deviation of results from four different donors. Representative data of four different donors and quantitative mean densitometry results are shown (n = 4). (B) Human ONH astrocytes cultures were grown on 24-well culture plates containing 12-mm glass coverslips in growth medium. Cells were treated with lovastatin (5 μg/mL) or simvastatin (5 μg/mL) for 1 h and then treated with TGF-β2 (5 ng/mL) for additional 1 h or TGF-β2 (5 ng/mL) alone for 1 h. Cells were then fixed and stained with phospho-Smad2/3-specific antibody and Smad4-specific antibody. Cell nuclei were counterstained with DAPI. Representative data of four different donors were shown (n = 4).
with patients taking statins showing reduced risk of open-angle glaucoma development (Stein et al., 2012). In addition, statins have been found to slow disease progression in open-angle glaucoma suspects (De Castro et al., 2007). However, the mechanism of action of statins in glaucoma has not been fully explored.

In the present study, we observed that statins inhibit the TGF-β2-mediated expression of ECM-related proteins in human astrocytes of the ONH (Fig. 4). This inhibition occurs at the point of Smad activation by TGF-β2 as the statins inhibit the phosphorylation of Smad2 and nuclear accumulation of Smad2/3, canonical TGF-β2 signaling effectors (Fig. 6A and B). The question then arose as to the levels at which statins interfere with the TGF-β2 pathway. Statins have been shown to regulate TGF-β receptor expression (Rodríguez-Vita et al., 2008) and also the expression of TGF-β itself and its downstream effector Smad2 (Chen et al., 2013). In our present experiments, Smad2/3 expression was found to be unaffected by statin treatment (Fig. 5). We speculated that Rhoa was most likely candidate factor through which statins would function in regulating TGF-β. Statins inhibit Rhoa activation as a result of the inhibition of its isoprenylation (Pokrovskaya et al., 2014). Rhoa signaling is required for TGF-β1-mediated PAI-1 expression and TGF-β1-mediated smooth muscle cell differentiation as dominant-negative Rhoa blocks the nuclear translocation of Smad2 and Smad3 by inhibiting their phosphorylation (Chen et al., 2006; Samarakoon et al., 2008). Simvastatin inhibits TGF-β-induced ECM proteins expression through the inhibition of Rhoa activation in keloid fibroblasts (Mun et al., 2013) and the inhibition of Rhoa pathway by the ROCK (a major downstream effector of Rhoa) inhibitor suppresses TGF-β2-mediated collagen I expression (Itoh et al., 2007). As we observed in our present analysis that statin pretreatment for 1 h followed by 1 h TGF-β treatment is sufficient to show the effects of statins on TGF-β (Fig. 6), we hypothesized that the regulation of Smad phosphorylation, which occurs within a short period of time, is more likely to be the mechanism rather than the regulation of gene expression. What made Rho signaling more interesting in the context of our present investigation was that the Rho signaling pathway is involved in glaucoma development. The Rho protein plays an important role in regulating aqueous outflow in the trabecular meshwork and therefore in maintaining adequate intraocular pressure, which is compromised in glaucoma (Rao et al., 2001). To test our hypothesis in the future, we will first determine Rhoa activation in cells exposed to TGF-β alone and to TGF-β with statins. We will use a ROCK (a downstream effector of Rhoa) inhibitor to test whether Rhoa signaling is required for the regulation of TGF-β2 by statins.

The two main glial cells of the human ONH LC are astrocytes and LC cells. Similar to astrocytes, LC cells can respond to glaucomatous stimuli, resulting in ECM remodeling (Kirwan et al., 2005; Quill et al., 2011). In vitro, recombinant TGF-β2 also induces fibronectin and PAI-1 expression and Smad2/3 phosphorylation in LC cells (Zode et al., 2011). Considering this functional similarity between astrocytes and LC cells, it would be interesting also to address the effects of statins on LC cells in a future study.

Our study has limitation to note. Since we used 5 μg/mL concentration of statins which affected cell viability by 15–17%, our result should be interpreted with caution.

In conclusion, we have shown the antifibrotic effects of statins in human astrocytes of the ONH and identified TGF-β2 as a mediator of statin action. This evidence is in line with the reported beneficial effects of statins on glaucoma. Given that TGF-β2 expression is increased in glaucomatous astrocytes and that TGF-β2-mediated ECM remodeling is a pathological feature of glaucomatous ONH, our current study sheds light on the mechanism underlying the beneficial effects of statins on glaucoma. Future analysis of the in vivo effects of statins on ECM remodeling should be performed to translate these findings into clinical practice.

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**Conflict of interest**

The authors report no conflicts of interest.

**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2017.08.004.

**References**


