**Role of the Autotaxin-LPA Pathway in Dexamethasone-Induced Fibrotic Responses and Extracellular Matrix Production in Human Trabecular Meshwork Cells**

Megumi Honjo,1 Nozomi Igarashi,1 Junko Nishida,1 Makoto Kurano,2,5 Yutaka Yatomi,2–4 Koji Igarashi,5 Kuniyuki Kano,5,6 Junken Aoki,3,6 and Makoto Aihara1

1Department of Ophthalmology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
2Department of Clinical Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
3CREST, Japan Science and Technology Corporation (JST), Saitama, Japan
4Department of Clinical Laboratory, The University of Tokyo Hospital, Tokyo, Japan
5Bioscience Division, Reagent Development Department, AIA Research Group, TOSOH Corporation, Kanagawa, Japan
6Laboratory of Molecular and Cellular Biochemistry, Graduate School of Pharmaceutical Sciences, Tohoku University, Miyagi, Japan

Correspondence: Makoto Aihara, Department of Ophthalmology, University of Tokyo School of Medicine, 7-3-1 Hongo Bunkyo-ku, Tokyo 113-8655, Japan; aihara-rky@umin.net.

Submitted: August 14, 2017
Accepted: November 22, 2017

**PURPOSE.** Dexamethasone (Dex) regulates aqueous humor outflow by inducing reorganization of the cytoskeleton and extracellular matrix (ECM) production. Rho kinase (ROCK) has an important role in this process, but the upstream pathway leading to its activation remains elusive. The purpose of the study was to determine the role of autotaxin (ATX), an enzyme involved in the generation of lysophosphatidic acid (LPA), in the Dex-induced fibrotic response and ECM production in human trabecular meshwork (HTM) cells.

**METHODS.** The expression of ATX in specimens from glaucoma patients was investigated by immunohistochrometry. Regulation of ATX expression and the changes in actin cytoskeleton, ECM production, myosin light chain (MLC) and coflin phosphorylation, ATX secretion, and extracellular matrix production in human trabecular meshwork cells were determined by immunofluorescence, real-time quantitative PCR, immunoblot, and the two-site immunoenzymometric and lysoPLD assays.

**RESULTS.** Significant ATX expression was found in conventional outflow pathway specimens from glaucoma patients. Dex treatment induced increases in ATX mRNA levels, protein expression, and secretion in HTM cells in association with reorganization of cytoskeleton and ECM accumulation. Significant suppression of these aforementioned changes was observed after ATX/LPA-receptor/ROCK inhibition as well as suppression of fibrotic changes and MLC and coflin phosphorylation in HTM cells.

**CONCLUSIONS.** The results of this study, including the robust induction of ATX by Dex treatment, in association with fibrotic changes and ECM production in HTM cells, collectively suggest a potential role for ATX-LPA pathway in the regulation of aqueous humor outflow and IOP in glaucomatous eyes.

Keywords: trabecular meshwork cells, autotaxin, dexamethasone, lysophosphatidic acid, extracellular matrix

Elevated intraocular pressure (IOP) is the foremost risk factor for all glaucoma subtypes and lowering IOP delays loss of vision in glaucoma patients.1–5 Physiologically, IOP is derived from the outflow resistance of aqueous humor (AH) at the iridocorneal angle, which constitutes the conventional outflow pathway consisting of the trabecular meshwork (TM), the juxtacanalicular connective tissue (JCT), Schlemm’s canal (SC), the distal collector channels, aqueous veins, and intrasceral/episcleral veins. The increased resistance in this pathway is known to be the primary cause of elevated IOP in glaucoma patients.2–4 While many therapeutic agents are effective in reducing IOP, curiously, none have been developed on the basis of the biological mechanisms that have been reported to lead to IOP elevation. Consequently, an agent may reduce IOP but have no effect on the cause of its initial increase.

Previous studies have shown that AH derived from glaucoma patients contains elevated levels of various bioactive factors including transforming growth factor-β (TGF-β), endothelin-1, connective tissue growth factor, and several other cytokines.1–13 These extracellular factors have been observed to influence TM tissue properties and cellular responses in the context of AH outflow resistance and elevation of IOP via different intracellular signaling pathways, including Rho/Rho-kinase (ROCK) signaling, Wnt, integrins, PKC, BMPs/SMADs, MAP kinases, and others. The mechanisms involve the regulation of the contractile properties of TM cells, extracellular matrix (ECM) turnover, adhesive interactions, permeability, and survival of outflow pathway tissues and cells.1,3,12,14,15 Changes in stiffness and metabolic activity of TM tissue reportedly caused by alterations in cellular contraction and oxidative damage are associated with increased resistance to
AH outflow and elevated IOP. These observations indicate that altered levels of one or more secreted factors by autocrine and paracrine actions can modulate IOP by altering the cellular characteristics of the AH outflow pathway, including the TM, SC, and JCT, with subsequent effects on AH outflow resistance. We hypothesized that biological markers exist in the pathways leading to raised IOP and that these could be actioned to reduce IOP elevation and its disease-causing sequelae.

Lysophosphatidic acid (LPA) is a simple phospholipid but yet induces many types of cellular responses including Rho GTPase-regulated cell adhesion, contraction, cellular proliferation, cell migration, cytokine and chemokine secretion, and neurite retraction. LPA also influences the contractile platelet aggregation, transformation of smooth muscle cells, and neurite retraction. LPA is derived, under various conditions both in cells and biological fluids, mainly from lysophosphatidylcholine (LPC) and predominantly through the lysophospholipase D (lysoPLD) activity of ATX. ATX is a secreted glycoprotein, widely present in biological fluids, and originally isolated from the supernatants of melanoma cells as an autocrine motility stimulation factor. It has previously been reported that ATX levels in AH of hypertensive mice and in primary open angle glaucoma (POAG) patients are increased, and inhibition of ATX production decreases IOP in an animal model.

Changes in the cytoskeletal organization and fibrotic response of human TM cells (HTM cells) are thought to be responsible for open angle glaucoma pathologies. Dexamethasone (Dex) has a wide variety of effects on the HTM, of which changes in cytoskeletal reorganization, fibrotic response, and cross-linked actin network (CLAN) formation are of particular interest. The Rho GTPase–ROCK signaling pathway is an important modulatory agent of Dex-induced cytoskeletal changes in HTM cells. We, therefore, hypothesized that the ATX-LPA pathway is one potential pathway mediating Dex-induced cytoskeletal and fibrogenic changes in HTM cells. Here, using immunohistochemistry, we report for the first time that ATX is upregulated in glaucomatous human specimens of the outflow pathways. We also revealed that Dex treatment induced significant ATX expression as well as fibrotic and cytoskeletal changes in HTM cells, and significant suppression of these changes was observed after ATX/LPA-receptor/ROCK inhibition.

**METHODS**

**Reagents and Antibodies**

1-Myristoyl (14:0)-LPC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Rat monoclonal antibody (clone 4F1) against ATX were purchased from MBL (Nagoya, Japan). Human recombinant transforming growth factor-β2 (TGF-β2), dexamethasone, K116425, rhodamine-phalloidin, anti-β-smooth muscle actin (anti-β-SMA), anti-β-actin, anti-collagen type 1, and anti-fibronectin antibodies were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). S28286 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Y-27632 (Calbiochem, Gibbstown, NJ, USA), rabbit anti-phospho-MLC, anti-phospho-collin, peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA), Halt Protease and the Phosphatase Inhibitor Single-use Cocktail (Pierce Biotechnology, Rockford, IL, USA), ECL Advance Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, USA), WB Stripping solution (Nacalai Tesque, Kyoto, Japan), RIPA buffer, Halt Protease and Phosphatase Inhibitor and bicinechonic acid protein assay kit, Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific K.K., Kanagawa, Japan), choline oxidase (Asahi Chemical, Tokyo, Japan), horseradish peroxidase (Toyobo, Osaka, Japan), TOOS reagent (Dojindo Laboratories, Kumamoto, Japan), PrimeScript RT reagent kit, and SYBR Premix Ex Taq II (TaKaRa BIO, Inc., Shiga, Japan) were obtained from the indicated commercial sources. Other chemicals were purchased from Wako Chemicals (Wako Pure Chemical Industries, Ltd., Osaka, Japan), unless otherwise stated.

**Patients and Tissue Samples**

Glaucomatosus patients who had undergone glaucoma surgery for open angle glaucoma (OAG), aged 20 years or older, were recruited. All OAG patients were classified into four groups. Patients with statistically higher IOP and normal IOP without any cause were diagnosed with POAG and normal tension glaucoma (NTG), respectively. Patients with inflammatory diseases were diagnosed with secondary open angle glaucoma (SOAG) and those with pseudoxfiliation materials were classified as exfoliation glaucoma. In patients who underwent trabeculectomy, TM and sclerocorneal specimens were obtained from the 10- to 11-o’clock upper corneoscleral limbus at the time of surgery. The specimens were immediately fixed in 10% buffered neutral formalin and embedded in OCT compound (Tissue-Tek OCT Compound, Sakura Finetek Japan Co., Ltd., Tokyo, Japan). Control specimens were collected from anterior segment of age-matched normal cornea donor eyes, obtained from the Rocky Mountain Lions Eye Bank (Aurora, CO, USA) (Amercia). The eyes were obtained and managed in compliance with the Declaration of Helsinki. Next, 10-μm-thick cryosections were cut at −20°C and stored until processing.

**Measurement of ATX**

The ATX levels in the conditioned medium of cultured TM cells were measured by using a slightly modified reagent consisting of a sensitive anti-ATX monoclonal antibody (R10.1–R10.23 monoclonal antibody combination), using a two-site immunoenzymetric assay, as previously described in detail, in which the within-run and between-run coefficients of variation were 3.1% to 4.6% and 2.8% to 4.6%, respectively. The assay reagents were compatible with a commercial automated immunoassay analyzer ALA-system (Tosoh, Tokyo, Japan), which includes automated specimen dispensation, incubation of the reaction cup, a bound/free washing procedure, 4-methylumbelliferyl phosphate substrate dispersion, and fluorometric detection, and generates a result report.

**Measurement of lysoPLD Activity**

ATX activity was measured as lysoPLD activity, as previously described. Briefly, lysoPLD activity was assessed by measuring choline liberation from the substrate LPC. The reactions were performed in 100 μL aliquots; the samples (20 μL) were incubated with 2 mM 1-myristoyl (14:0)-LPC in the presence of 100 mM Tris-HCl (pH 9.0) for 3 hours at 37°C. Liberated choline was detected by using an enzymatic photometric method with choline oxidase, horseradish peroxidase, and TOOS reagent as a hydrogen donor. The choline concentration was estimated with an absorption spectrometer.

**Preparation of Human TM Cells**

Primary HTM cells were isolated from human donor eyes and characterized as described previously. Only well-character-
ized normal HTM cells from passages three through eight were used for subsequent studies. All in vitro experiments were performed after overnight serum starvation and cells were treated with the indicated concentrations of ATX inhibitor, ROCK inhibitor, and LPA receptor antagonist simultaneously with Dex stimulation, with the addition of the indicated concentration of fetal bovine serum (FBS).

**Immunofluorescence**

To determine the distribution profile of ATX in the conventional AH outflow pathway, corneoscleral specimens of patients who underwent trabeculectomy (from three NTG, three POAG, and three SOAG patients) were immunostained with ATX antibody as previously described. Tissue sections were incubated with rat monoclonal anti-ATX antibody and then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody. TM cells were plated on glass coverslips and stained for ATX and F-actin with phalloidin-then secondary antibody.

**RNA Extraction and Real-Time Quantitative PCR (Real-Time qPCR)**

To measure ATX and other ECM gene expression profiles in HTM cells, total RNA was extracted from cultured cells with TRizol Reagent (Life Technologies, City, State, USA), and cDNA was synthesized from the isolated messenger RNA (mRNA) with a PrimeScript RT reagent kit. Serum-starved (for 24 hours) confluent cultures of HTM cells were treated with Dex (100 nM) for 24 hours and subjected to real-time qPCR under serum-free conditions. mRNA levels were quantified by qRT-PCR of cDNA with SYBR Premix Ex Taq II and the Thermal Cycler Dice Real-Time System II (TaKaRa Bio, Inc.) with the ΔΔCt method, as previously described. The sequences of PCR primers used were as follows: GAPDH: forward 5′-GAGTCAAGGGATTTGTCGT-3′ and reverse 5′-TGATTTGGAGGGGTATCCTG-3′; ATX: forward 5′-ACACGGAGGATGGCTGAT-3′ and reverse 3′-AGAGGTCCAGGCTGTTTGA-3′; fibronectin: forward 5′-AACCAATTCTTGGAGCAGG-3′ and reverse 5′-CCATAAAGGGCAACCAAGG-3′; COL1A1: forward 5′-CACCTTACGCTTGTGAG-3′ and reverse 5′-CTTTGATTTTGGAGGGATCTCG-3′; and COL4A1: forward 5′-GTGACATTAGCTGAGTCAGG-3′ and reverse 5′-CCATTGTGCTT-3′.

**Western Blot Analysis**

The expression of phosphorylated myosin light chain (MLC) and coflin was determined by using Western blot analysis, as previously described. Briefly, after serum starvation, cells were stimulated with Dex and/or inhibitors, and cell lysates were collected. Protein concentrations of the cell lysates were measured by using a bicinchoninic acid protein assay kit, and equal amounts of protein samples were separated on 4% to equal amounts of protein samples were separated on 4% to equal amounts of protein samples were separated on 4% to 12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked, incubated with rabbit polyclonal antibody phospho-MLC or phospho-cofilin antibody, and then with a peroxidase-conjugated secondary antibody, visualized by using ECL Advance Western Blotting Detection Reagent, and imaged with a luminescent image analyzer. All membranes were stripped of antibodies by using WB Stripping solution and incubated with mouse monoclonal antibody β-actin (1:1000), and subsequently with H goat anti-mouse IgG antibody (1:2000) as a loading control. Densitometry of scanned films was performed by using ImageJ 1.49 (http://imagej.nih.gov/ij/) provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), and results are expressed relative to the loading control (β-actin).

**Study Approval**

Ethical approval was obtained from the institutional review boards of The University of Tokyo for human aqueous humor samples. All of the procedures conformed to the Declaration of Helsinki. Written informed consent was obtained from each participating patient.

**Statistical Analysis**

Statistical analyses were performed by using JMP Pro 11 software (SAS Institute, Inc., Cary, NC, USA). All experimental data in vitro are shown as the mean ± SE unless otherwise noted. Differences in the data among the groups were analyzed by 1-way ANOVA and Tukey’s test as a post hoc test. P values less than 0.05 were regarded as statistically significant in all analyses.

**RESULTS**

**Increased ATX Expression in the Trabecular Meshwork Specimens of Glaucoma Patients**

Based on the increase in ATX levels in the AH in POAG and SOAG patients in our ongoing study (Honjo M, et al., unpublished data, 2017), we investigated for ATX in the outflow pathway by immunohistochemistry. We found increased ATX expression in the TM of trabeculectomy specimens from glaucoma patients (Fig. 1). In SOAG patients, specimens showed intense immunostaining of ATX in both the corneoscleral trabecular meshwork (CS-TM) and JCT, which was distributed at more moderate levels in POAG patients than in SOAG patients. Staining was weaker in the SC region than in the TM and JCT regions. Specimens from control and NTG eyes did not show significant ATX expression.

**Increased ATX Expression in Cultured Human Trabecular Meshwork Cells**

Our clinical study indicated a strong association between ATX, LPA, and LPC levels and IOP or glaucoma subtypes (Honjo M, et al., unpublished data, 2017). Since LPA is produced from LPC by ATX, we investigated which factor acts as the primary signal to activate the ATX-LPA signal cascade and increase outflow resistance and IOP. Accordingly, we investigated whether ATX, LPA, and LPC levels are increased in TM cells by glaucoma-like stress. First, we evaluated the changes in ATX mRNA expression after a challenge with Dex, which has been implicated in the pathobiology of elevated IOP in steroid glaucoma. Dex treatment significantly increased mRNA expression of ATX compared with FBS (-) (Fig. 2A). Further increases in ATX expression were observed when serum starvation was prolonged. Therefore, in the present study, further experiments with TM cells were performed under the 2% FBS condition, unless otherwise stated.

Immunostaining of cultured HTM cells further confirmed the increase in ATX expression at 24 hours after Dex (100 nM) treatment (Fig. 2B). Before Dex treatment, ATX was weakly localized to the cytoplasm, but after Dex treatment, ATX localized to the perinuclear region with a vesicular/punctate distribution, in good agreement with previous studies.
As the mean total LPC level in glaucoma patients, especially those with SOAG, was significantly higher than that of controls (Honjo M, et al., unpublished data, 2017), we investigated the effect of Dex on ATX expression in the presence of 0, 0.1 μM, and 1 μM LPC in TM cells. RT-qPCR confirmed that although ATX mRNA expression increased with increasing Dex concentration, LPC addition had no significant effect on ATX mRNA expression at any of the Dex concentrations tested (Fig. 3A).

We also measured ATX secretion and lysoPLD activity in the conditioned media of cultured cells. Dex treatment (500 nM) significantly increased lysoPLD activity and ATX secretion in the conditioned media (*P < 0.05) (Figs. 3B, 3C). LPC (0.1 μM and 1 μM) had no effect on lysoPLD activity or ATX secretion in the conditioned media. Unless otherwise stated, all other experiments were performed with 100 nM or 500 nM Dex and 0.1 μM LPC.

Dex Treatment Leads to Increased ATX Levels and Actin Cytoskeletal Reorganization in HTM Cells

To obtain further insight into potential functional interactions between the ATX and LPA pathways as well as their downstream signaling, we quantified the effects of the ATX inhibitor S32826 (1 μM) or HA130 (1 μM), the LPA receptor antagonist Ki16425 (10 μM), and Y27632 (10 μM), a Rho-associated protein kinase (ROCK) signaling pathway inhibitor, on Dex-induced ATX upregulation. S32826 and HA130, but not Ki or Y27632, inhibited Dex-induced increases in ATX mRNA expression in HTM cells and lysoPLD activity in conditioned media (Figs. 4A, 4B). In these experiments, we also recorded changes in actin cytoskeletal organization following upregulation of ATX in HTM cells, using immunohistochemistry (Fig. 5). Dex treatment induced a robust...
FIGURE 3. Increased ATX expression and activity with or without LPC in Dex-treated human trabecular meshwork cells. (A) ATX expression in HTM cells treated with Dex 100 nM or Dex 500 nM (D100, D500) and LPC (0, 0.1, 1 μM) for 24 hours. ATX mRNA levels in cells were quantified by RT-qPCR. (B) ATX protein in the conditioned media was measured by a two-site immunoenzymetric assay. (C) LysoPLD activity in the conditioned media. *P < 0.05, **P < 0.01, ***P < 0.001. Data are representative of three independent experiments.

FIGURE 4. The effects of inhibition of ATX on cellular responses in Dex-treated human trabecular meshwork cells. (A) The effects of S32826, Ki16425, or Y-27632 on ATX, COL1A1, COL4A1, and fibronectin (FN) mRNA expression induced by Dex100 (D100) in HTM cells were evaluated by RT-qPCR. (B) LysoPLD activity in the conditioned media was also evaluated after treatment of HTM cells with Dex 100 nM, Dex 500 nM or Dex 100 nM and S32826, Ki16425, or Y-27632. *P < 0.05, **P < 0.01. Data are representative of three independent experiments.
increase in actin stress fiber formation, in association with ATX upregulation around the nucleus. Actin stress fiber formation was particularly increased in cells in which ATX expression was especially high. In HTM cells treated simultaneously with Dex and S32826, neither ATX expression nor actin stress fiber formation was increased. Conversely, after simultaneous treatment with Dex and Y27632, Dex-induced upregulation of ATX expression was still observed, but cytoskeletal reorganization was decreased.

To determine whether the ATX-LPA pathway is involved in Dex-induced changes in the actin cytoskeleton, we evaluated the status of phosphorylation of cofilin and MLC by immunoblot analysis. As shown in Figure 6, Dex (100 nM) increased the levels of phosphorylated MLC (p-MLC) and phosphorylated cofilin (p-cofilin); and S32826, Ki16425, and Y27632 inhibited the increase.

**Dex-Induced Fibrogenic Activity in HTM Cells**

Since fibrogenic activity of TM cells is putatively involved in the pathogenesis of glaucomatous change in TM, we further investigated the role of Dex-induced ATX in modulating fibrogenic activity in HTM. Real-Time qPCR revealed that Dex treatment significantly upregulated the mRNA expression of COL1A1, COL4A1, and fibronectin in HTM cells (Fig. 4A). S32826 significantly inhibited Dex-induced mRNA upregulation of COL1A1 and COL4A1 in the HTM cells. Y27632 significantly inhibited Dex-induced mRNA upregulation of COL1A1, COL4A1, and fibronectin in the HTM cells.

We also evaluated protein expression of α-SMA, collagen-1, and fibronectin by immunohistochemistry in HTM cells (Fig. 7). Since ROCK is also upregulated by TGF-β2, we used its level as a positive control. Both Dex and TGF-β2 treatment induced significant increases in α-SMA, fibronectin, and collagen expression in HTM cells. S32826 abolished Dex-mediated increases in α-SMA expression and inhibited the expression of fibronectin and collagen-1; however, it had little effect on the
changes induced by TGF-β2. The ROCK inhibitor Y-27632 significantly inhibited Dex-induced and TGF-β2-induced increases in α-SMA, fibronectin, and collagen expression, although the inhibition of TGF-β2-induced HTM responses was incomplete (see Discussion).

**DISCUSSION**

Previous work has shown that cytoskeletal changes correlate with increased ECM production and fibrogenic activity of HTM cells upon impairment of AH outflow following TM obstruction and IOP elevation. Notably, these changes appear to involve the Rho/ROCK pathway, which has been implicated in the control of the contractile and biomechanical properties of cells and tissues. In the present study, immunostaining revealed significant ATX expression in conventional outflow pathway specimens of glaucoma patients. ATX was upregulated and secreted from HTM cells following Dex treatment as demonstrated by immunofluorometry, qRT-PCR, immunoblot analysis, and two-site immunoenzymetric and lysoPLD assays.

Dex treatment induced α-SMA upregulation, fibrotic changes, and cofilin and MLC phosphorylation in TM cells, while these cytoskeletal and fibrogenic changes and production and overexpression of ECM were inhibited by an ATX inhibitor, a ROCK inhibitor, and an LPA-receptor inhibitor. These observations suggest that activation of the ATX-LPA pathway induces cytoskeletal changes and increases fibrogenic activity in the conventional outflow pathway, leading to IOP elevation in OAG patients. Thus, the ATX-LPA pathway has potential as a therapeutic target for the regulation of IOP and treatment of glaucoma.

**Presence and Induction of ATX in TM**

We found increased protein expression of ATX in the TM of surgical trabeculectomy specimens from POAG and SOAG patients (Fig. 1). This suggests the potential involvement of the ATX-LPA pathway in the pathogenesis of glaucoma, especially in SOAG patients. Therefore, we further investigated the possible role of the ATX/LPA pathway in HTM cells. Recently, it has been reported that several factors secreted by TM and SC
cells via the action of autocrine and paracrine factors influence their characteristics and modulate AH outflow and IOP.51 By in vitro studies, we showed that HTM cells express and secrete ATX upon Dex treatment (Figs. 2A, 2B, 3C), while the addition of LPC in the medium did not influence the expression or secretion of ATX or lysoPLD activity (Figs. 3A, 3B).

Cytoskeletal and ECM Changes Associated With ATX in TM Cells

Treatment with Dex caused cytoskeletal actin changes including polymerization of F-actin microfilaments and increased expression of α-SMA, as shown in Figures 5 and 7. After Dex treatment, most F-actin fibers appeared to be reorganized into bundles in close proximity to other fibers. After Dex exposure, we observed increased ECM mRNA expression of COL1A1 and COL4A1, in addition to increased expression of ECM proteins collagen I and fibronectin, and elevated expression of α-SMA (Figs. 4A, 7). ROCK inhibitor Y27632 significantly inhibited Dex-induced ECM mRNA upregulation in the HTM cells, in agreement with the results of a previous report.7 The higher expression of these factors together could substantially increase the stiffness of the cell substrate, promoting colocalization of α-SMA to stress actin fibers, as observed and previously discussed.48,52 The increased expression of α-SMA and ECM proteins caused by Dex suggests transdifferentiation of HTM cells into myofibroblast-like cells expressing fibrogenic and fibroblast-like markers, as recently reported by others.48,53 Furthermore, fibronectin is reported to induce α-SMA expression via positive feedback,24 thus, the increased fibronectin expression might facilitate fibrogenic changes of HTM cells. Inhibition of ATX was associated with a significant decrease in Dex-induced lysoPLD activity in conditioned media, while neither an LPA receptor antagonist nor a ROCK inhibitor showed any effect (Fig. 4B). Both ATX inhibition and an LPA receptor antagonist (LPAIR) significantly blocked Dex-induced MLC phosphorylation and cofilin phosphorylation, as did the ROCK inhibitor (Fig. 6), which is known to affect the regulation of AH outflow.55 These observations are supported by earlier reports that the ATX/LPA pathway modulates signal transduction through LPAR1 and LPAR3, and subsequently affects several downstream kinases including the Rho/ROCK pathway, p38 MAPK, and PKC.56,57 LPAR1, the LPA receptor responsible for LPA-driven cell motility, is reported to be predominantly expressed in HTM cells.26 Signaling by the LPA through LPAR1 and the downstream effector ROCK are presumed to modulate various cellular functions.58 In a previous study, we reported that ROCK inhibition releases cytoskeletal tension and increases AH outflow.14 Indeed, one ROCK inhibitor is now clinically available as an IOP-lowering drug in Japan.59 Thus, it seems obvious that the ATX-LPA pathway plays a crucial role in TM outflow regulation as a part of upstream signaling for the Rho/ROCK pathway. However, further study is needed to investigate and elucidate the interplay between ATX, LPA, LPAR, and Rho/Rho-kinase (ROCK) pathway in the TM.

Most of the responses of Dex-induced HTM cells were similar to those invoked by TGF-β2, including the effects of the latter on TM cell cytoskeletal activity, ECM synthesis, MLC phosphorylation, and α-SMA expression. Both Dex-induced and TGF-β2-induced α-SMA and fibronectin/collagen expression were significantly inhibited by the ROCK inhibitor, but not by ATX inhibition, as expected (Fig. 7). It is reasonable that the ROCK inhibitor failed to attenuate fully TGF-β2-induced HTM responses because TGF-β2 is known to evoke several signaling pathways other than the Rho/ROCK pathway.83 Intriguingly, the ROCK inhibitor was also less effective in blocking Dex-induced increases in α-SMA, collagen, and fibronectin expression in the HTM than the ATX inhibitor (Figs. 4A, 7). This might be because ATX can regulate several cytokines and evoke signals other than those of the Rho/ROCK pathway.60-62

In addition, increased levels of LPA have been shown to induce profibrotic TGF-β signaling via G protein-coupled receptor (GPCR) signaling.63,64 Therefore, in future studies investigating the role of the ATX-LPA pathway in the regulation of AH outflow and IOP, we plan to undertake a detailed assessment of the balance and interactions between the TGF-β2 and ATX-LPA pathways as well as their involvement in ocular hypertension and etiology of glaucoma.

One limitation of the present study was its nearly exclusive focus on the responses of HTM cells. Thus, it will be important to investigate the regulation of Schlemm’s canal permeability in future studies.55 Another limitation was that it was impossible for us to investigate ATX expression in specimens from healthy controls under the same condition with glaucoma specimens. Instead, we collected control specimens from donor eyes from a different ethnic group; therefore, we have to be careful in interpreting the results from the present study, which should be clarified in future studies. Additionally, further studies should be performed to determine more precisely the role of ATX-LPA receptor interactions in the outflow pathway and obtain direct evidence for increased AH outflow resistance or IOP in experimental animal models or perfusion studies.

In summary, to our knowledge, this is the first report to demonstrate expression of ATX proteins in the human outflow pathway and HTM cells, and their inhibitory effects on Dex-induced fibrotic and cytokinetic changes in HTM cells. Our study showed upregulated expression of ATX in human outflow specimens, especially in hypertensive glaucoma subtypes, suggesting that the upregulated ATX-LPA pathway plays a role in IOP elevation. HTM cells treated with Dex exhibited ATX mRNA and protein expression and secreted ATX into the medium, and showed fibrotic and cytoskeletal changes. Inhibition of ATX and the LPA receptor suppressed Dex-induced effects, such as fibronectin and COL upregulation and cytokinetic changes, but not TGF-β2-induced effects, in TM cells. Therefore, this study suggests that Dex-induced fibrotic and cytokinetic changes are mediated, at least in part, by the ATX-LPA pathway. Finally, the study suggests a potential role of the ATX-LPA signaling pathway in the regulation of the conventional outflow pathway in glaucoma, suggesting that its manipulation could be used to prevent disease progression in the glaucomatous TM.

Acknowledgments

The authors thank Yasuko Kuwabara and Fujie Takeda for their help with cell culture and immunohistochemistry.

Supported by Japan Society for the Promotion of Science (JSPS) Grant No. 15K10854 (MH), a Grant-in-Aid for Scientific Research on Innovative Areas 15H05906 (YY), and CREST from JST.

Disclosure: M. Honjo, None; N. Igarashi, None; J. Nishida, None; M. Kurano, None; Y. Yatomi, None; K. Igarashi, None; K. Kano, None; J. Aoki, None; M. Aihara, None

References


45. Honjo M, Kameda T, Kawai T, Yoshimura N, Arai M. Potential role of Rho-associated protein kinase inhibitor Y-


