Autotaxin–Lysophosphatidic Acid Pathway in Intraocular Pressure Regulation and Glaucoma Subtypes

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Purpose. To compare the levels of autotaxin (ATX), lysophosphatidic acid (LPA), and lysophosphatidylcholine (LPC) in the aqueous humor (AH) of healthy control subjects with those of patients with different subtypes of glaucoma, and also to investigate the relationship of the ATX–LPA pathway with IOP and subtype of glaucoma.

Methods. This study included 164 eyes of 164 consecutive cases of cataract and glaucoma surgery (37 healthy, 31 normal tension glaucoma, 49 primary open angle glaucoma, 28 secondary open angle glaucoma, and 19 exfoliation glaucoma). Aqueous levels of LPA, LPC, and ATX were quantified using liquid chromatography-tandem mass spectrometry and a two-site immunoenzymetric assay. The association between aqueous levels of ATX/LPA/LPC and IOP elevation in different glaucoma subtypes was investigated. The diagnostic values of indices of the ATX–LPA pathway were compared using receiver operating characteristic curve analysis.

Results. Notable increases in ATX/LPA/LPC levels in glaucoma patients were observed. The ATX–LPA pathway was significantly related to IOP elevation and the subtype of glaucoma, especially in SOAG and XFG patients, and the area under the curve was significant for discriminating glaucoma eyes from healthy eyes.

Conclusions. Bioactive ATX/LPA/LPC concentrations were present in aqueous humor, and higher ATX and LPA concentrations were significantly correlated with IOP in all study subjects. Furthermore, the ATX–LPA pathway was significantly related to glaucoma subtype. These results reveal the potentially important role of the ATX–LPA pathway for IOP regulation in healthy subjects and glaucoma patients.

Keywords: aqueous humor, autotaxin, lysophosphatidic acid, intraocular pressure, glaucoma subtypes

The glaucomas are a group of progressive optic neuropathies that are the second leading cause of blindness globally.1,2 Impaired aqueous humor (AH) drainage through the conventional outflow pathway is the primary cause of elevated IOP in glaucoma patients and also is an important risk factor in all glaucoma subtypes. In addition, lowering IOP delays loss of vision in glaucoma patients.3–5

Although we have learned much regarding the pathophysiology of open angle glaucoma (OAG), the biological basis for IOP elevation of individuals with open angles remains elusive. In particular, the cellular and molecular mechanisms that drive the increase in resistance to AH outflow are poorly understood. If a biological marker related to IOP elevation exists, it would provide a novel therapeutic target for the treatment of glaucoma.

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 (CTGF), myocilin, and several cytokines.4,6–14 These extracellular factors have been observed to influence trabecular meshwork (TM) tissue properties and cellular responses via different intracellular signaling pathways, including Rho/Rho-kinase (ROCK) signaling, Wnt, integrins, protein kinase c (PKC), bone morphogenetic proteins (BMPs)/SMADs, mitogen-activated protein (MAP) kinases, and others.15–17 It also has been reported that certain bioactive lipids, especially bioactive lysophospholipids, influence TM cell contractile and cell adhesive properties, and that they also have a role in the regulation of AH outflow dynamics and IOP elevation.18–21

Lysophosphatidic acid (LPA) is a simple phospholipid that induces many kinds of cellular responses including Rho GTPase-regulated cell adhesion, contraction, cellular proliferation, prevention of apoptosis, cell migration, cytokine and chemokine secretion, platelet aggregation, transformation of smooth muscle cells, and neurite retraction.22,23 Although LPA...
is known to be present in human AH, there is a paucity of information regarding the levels of LPA and its potential involvement in human IOP regulation. LPA is generated mainly from lysophosphatidylcholine (LPC) and predominantly through lysophospholipase D (lysoPLD) activity of the generating enzyme autotaxin (ATX). It has previously been reported that ATX levels in ocular hypertensive mice are increased, in contrast to the reduced levels of lysophospholipids. Further, primary open angle glaucoma (POAG) patients have higher lysoPLD activity of ATX, resulting in increased conversion of LPC into LPA, and that inhibition of ATX production decreases IOP in an animal model. These observations suggest the significance of ATX and possibly LPA in the regulation of AH outflow in glaucomatous eyes. However, previous studies have failed to determine the relationship between elevated levels of ATX and LPA in the AH of glaucoma patients, perhaps because quantitative measurement of ATX and LPA levels in the AH is difficult due to the small amounts that can be obtained from a sample of aqueous humor. To our knowledge, no study to date has directly addressed the role of the ATX/LPA pathway in regulating IOP and glaucoma subtypes in human subjects. The objective of the current study was to quantify aqueous levels of LPA, LPC, and ATX levels in healthy subjects and OAG patients. We further aimed to investigate the relationship between the ATX-LPA pathway with IOP and glaucoma subtypes.

**Methods**

**Patients and AH Samples**

AH samples were obtained from subjects who had undergone cataract or glaucoma surgery at the University of Tokyo Hospital. 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.

Glaucomatous patients who had undergone glaucoma surgery for OAG aged 20 years old or older were included. Patients were excluded who had other types of glaucoma including primary ACG and congenital/developmental glaucoma and a previous history of intraocular surgery other than uncomplicated small-incision cataract surgery. Each of the OAG patients was classified into one of four groups. POAG patients had a glaucomatous visual field or optic disc, as well as a normal angle with gonioscopy. If untreated IOP was below 21 mm Hg, the patients were defined as having normal tension glaucoma (NTG). Glaucoma patients with inflammatory diseases or chronic postoperative IOP elevation were diagnosed with secondary open angle glaucoma (SOAG), and those with exfoliative material were classified as having exfoliation glaucoma (XFG). Individuals with cardiovascular disease were excluded from this study. Cataract patients without systemic disease (other than hypertension and mild hyperlipidemia), ocular diseases other than cataracts, no history of ocular surgeries, or an IOP ≤21 mm Hg were included as controls. When both eyes of a patient met the inclusion criteria, only the eye treated first was included in the analysis.

IOP was measured using a Goldmann tonometer (Haag-Streit AG, Bern, Switzerland), and maximum preoperative IOP was evaluated within 3 months prior to surgery. For all participants, the anterior segment and optic disc were examined by glaucoma specialists using a slit-lamp biomicroscope and dilated fundoscopy to establish the diagnosis of glaucoma. Visual fields were performed using program 24-2.

**Swedish Interactive Threshold Algorithm (Humphrey Visual Field Analyzer; Carl Zeiss Meditec, Dublin, CA, USA), and they were considered to be glaucomatous if P < 0.05 and/or glaucoma hemifield test results were outside normal limits. The optic disc was considered glaucomatous if there was localized thinning of the neuroretinal rim or localized or diffuse retinal nerve fiber layer defects with ophthalmoscopy.**

**Preoperative AH samples were obtained at the initiation of the surgery before any incisional procedures through a limbal paracentesis using a syringe with a 30-gauge needle. Approximately 70 to 100 µL was collected in PROTEOSAVE CryoTubes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan), registered, and stored at −80°C until processing.**

**Measurement of Lysophospholipids Using LC-MS/MS**

Quantification of the lysophospholipids was performed as previously described in detail. Briefly, a total of 20 µL AH sample per patient was mixed with a 10-fold volume of methanol and an internal standard and then used for the LC-MS/MS analysis. Then, 20 µL methanol extract was separated using a Namspace LC (Shiseido, Tokyo, Japan) equipped with a C18 CAPCELL PAK ACR column (1.5 × 250 mm; Shiseido) using a gradient of solvent A (5 mM ammonium formate in water) and solvent B (5 mM ammonium formate in 95% [v/v] acetonitrile). Elution was sequentially ionized using an ESI probe, and the parent ion (m/z 380.2) and the fragment ion (m/z 264.2) were monitored in the positive mode using a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). 17:0-LPA and 17:0-LPC were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). LysoGP analyses were performed in the multiple reaction monitoring (MRM) mode: in positive ion mode for LPC and in negative ion mode for LPA. We recognized each LPA species, according to the previously described information about the MRM and collision energy of each species, considering the MRM together with the retention time. For each lysophospholipids class, 12 acyl chains (14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:3, 20:4, 20:5, 22:5, and 22:6) were monitored. We calculated the concentrations of lysophospholipids from the area ratio to the internal standard: 1 µM 17:0 LPA (for LPA) or 10 µM 17:0 LPC (for LPC species).

**Measurement of ATX**

ATX levels in the AH were determined using a two-site immunoenzymetric assay, as previously described in detail, in which the within-run and between-run coefficients of variation (CVs) were 3.1% to 4.6% and 2.8% to 4.6%, respectively. The assay reagents were compatible with a commercial automated immunoassay analyzer AXA system (Tosoh, Tokyo, Japan), which includes automated specimen dispensation, incubation of the reaction cup, a bound/free washing procedure, 4-methylumbelliferyl phosphate substrate dispensation, and fluorometric detection, and generates a result report.

**Statistical Analysis**

Statistical analyses were performed using JMP Pro 11 software (SAS Institute Inc., Cary, NC, USA). Clinical results were expressed as the mean ± SD. The Wilcoxon signed-rank test and χ² or Fisher's exact test were used for comparing two variables, and the Steel-Dwass test was used for multiple variables. Nonparametric tests were used as most clinical variables failed to satisfy normality or equality of variance assumptions, based on Kolmogorov–Smirnov and Levene tests. Correlations were computed using the Spearman correlation.
test. The independent effects of the LPC, ATX levels, and other background characteristics on IOP or glaucoma diagnosis were evaluated using simple and stepwise multiple linear regression analyses. Variance inflation factors (VIF) were calculated to check for multi-collinearity. Receiver operating characteristic (ROC) curves were used to describe the diagnostic values of indices of the ATX–LPA pathway to discriminate glaucomatous eyes from control eyes. Areas under the ROC curves (AUCs) with 95% binomial exact confidence intervals (CIs) were calculated. Actual values were used to create ROC curves, and covariates were not adjusted for. *P < 0.05 was regarded as statistically significant in all analyses.

**Results**

The study group consisted of 164 eyes: 37 healthy, 31 NTG, 49 POAG, 28 SOAG, and 19 XFG. Demographic characteristics of the study population are detailed in Table 1. All the subjects in the study were of Japanese ancestry. As expected, mean age was lower for SOAG than for control and XFG eyes (*P < 0.01) because of the late-onset nature of cataract and XFG. IOP was higher for SOAG and XFG (*P < 0.001), whereas there was no statistically significant difference in the number of glaucoma eye drops among POAG, SOAG, and XFG. SOAG and XFG demonstrated greater frequencies of previous phacoemulsification, with a higher incidence of steroid use for SOAG and older age of XFG.

**Comparison of IOP and ATX Levels in AH Between Groups**

Both mean IOP and ATX levels in the AH were significantly higher in the glaucoma group than in the control cataract group (Table 2). Comparison among glaucoma groups revealed that ATX levels were significantly higher in all groups than in controls, with SOAG and XFG patient groups showing significantly higher values compared with the NTG group. Pairwise differences in ATX levels between POAG and SOAG, and POAG and XFG failed to reach statistical significance (Fig. 1A).

Individual IOP and ATX levels in the AH were significantly correlated (Spearman’s correlation coefficient, *r* = 0.3559, *P < 0.0001; Fig. 2A). No significant correlation was found between IOP and ATX in blood plasma or between ATX levels in AH and plasma (data not shown).

**Comparison of IOP, LPA, and LPC Levels in AH Between Groups**

Total LPA and total LPC levels were significantly higher in the glaucoma group than in the control cataract group.

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**Table 1. Demographic Characteristics of the Study Population**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls</th>
<th>Glaucoma</th>
<th>P Value</th>
<th>NTG</th>
<th>POAG</th>
<th>SG</th>
<th>XFG</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>37</td>
<td>127</td>
<td>&lt;0.05†</td>
<td>31</td>
<td>49</td>
<td>28</td>
<td>19</td>
<td>&lt;0.05†</td>
</tr>
<tr>
<td>Sex (male: female)</td>
<td>12:25</td>
<td>67:60</td>
<td></td>
<td>10:21, 31:18</td>
<td>17:11</td>
<td>9:10</td>
<td>&lt;0.05†</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>73.6 ± 8.1§</td>
<td>68.7 ± 12.1</td>
<td>0.027‡</td>
<td>69.7 ± 9.2</td>
<td>69.8 ± 10.9</td>
<td>61.5 ± 15.8</td>
<td>75.1 ± 7.2§</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>Range</td>
<td>55 to 85</td>
<td>25 to 90</td>
<td></td>
<td>49 to 90</td>
<td>42 to 87</td>
<td>25 to 87</td>
<td>61 to 87</td>
<td></td>
</tr>
<tr>
<td>Preoperative IOP (mm Hg)</td>
<td>15.5 ± 2.9</td>
<td>21.9 ± 9.7</td>
<td>&lt;0.0001‡</td>
<td>15.6 ± 2.5</td>
<td>19.5 ± 6.8</td>
<td>31.0 ± 11.3</td>
<td>25.0 ± 10.3</td>
<td>&lt;0.0001‡</td>
</tr>
<tr>
<td>Range</td>
<td>10 to 24</td>
<td>10 to 43</td>
<td></td>
<td>12 to 21</td>
<td>10 to 40</td>
<td>14 to 50</td>
<td>12 to 45</td>
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<tr>
<td>MD (dB)</td>
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<td></td>
<td></td>
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<tr>
<td>Median</td>
<td>−15.2</td>
<td>−14.6</td>
<td>−16.5</td>
<td>−12.8</td>
<td>−15.9</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interquartile</td>
<td>−8.7 to −21.7</td>
<td>−6.7 to −20.4</td>
<td>−10.5 to −22.1</td>
<td>−6.4 to −20.0</td>
<td>−8.1 to −22.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glaucoma eye drops</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number, mean ± SD</td>
<td>0</td>
<td>3.2 ± 1.4</td>
<td>&lt;0.0001‡</td>
<td>2.4 ± 1.3</td>
<td>3.1 ± 1.5</td>
<td>3.9 ± 1.3</td>
<td>3.6 ± 0.8</td>
<td>&lt;0.0001‡</td>
</tr>
<tr>
<td>Range</td>
<td>0 to 0</td>
<td>0 to 6</td>
<td></td>
<td>0 to 5</td>
<td>0 to 5</td>
<td>0 to 6</td>
<td>2 to 5</td>
<td></td>
</tr>
<tr>
<td>Steroid eye drops</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betamethasone valerate, n (%)</td>
<td>0 (0)</td>
<td>6 (21)</td>
<td>NS</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (21)</td>
<td>0 (0)</td>
<td>&lt;0.0001‡</td>
</tr>
<tr>
<td>Fluorometholone, n (%)</td>
<td>0 (0)</td>
<td>3 (11)</td>
<td>&lt;0.0001‡</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (11)</td>
<td>0 (0)</td>
<td>&lt;0.0001‡</td>
</tr>
<tr>
<td>History of phacoemulsification, n (%)</td>
<td>0 (0)</td>
<td>29 (25)</td>
<td>&lt;0.0001‡</td>
<td>3 (10)</td>
<td>8 (16)</td>
<td>12 (43)</td>
<td>6 (33)</td>
<td>&lt;0.0001‡</td>
</tr>
</tbody>
</table>

* § Statistically significant difference from the SOAG group (Steel–Dwass test).
† Wilcoxon signed-rank test.
‡ Fisher’s exact test.

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**Table 2. ATX, Total LPA, and Total LPC Levels in Control and Glaucoma Subjects**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n = 37)</th>
<th>Glaucoma (n = 127)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATX (mean ± SD) (mg/L)</td>
<td>0.58 ± 0.13</td>
<td>0.94 ± 0.32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total LPA (mean ± SD) (nM)</td>
<td>37.6 ± 24.0</td>
<td>62.7 ± 40.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total LPC (mean ± SD) (nM)</td>
<td>41.1 ± 31.8</td>
<td>95.5 ± 123.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IOP (mean ± SD) (mm Hg)</td>
<td>15.5 ± 2.9</td>
<td>21.9 ± 9.7</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Total LPA was significantly higher in POAG, SOAG, and XFG groups than in the control group; however, there were no statistically significant differences between the control and NTG groups or between NTG and POAG groups. The SOAG and XFG groups showed significantly higher values of total LPA than the POAG group (Fig. 1B). The concentration of total LPC was significantly higher in POAG, SOAG, and XFG groups than in the controls (Fig. 1C). However, there were no significant differences between control and NTG, NTG, and POAG, or POAG and XFG groups. The frequency of outliers of LPC levels in the SOAG group was relatively high.

Individual IOP and total LPA in the AH were significantly correlated (Spearman’s correlation coefficient, $\rho = 0.3765$, $P < 0.001$; Fig. 2B) for all subjects. However, the correlation coefficient for the association between IOP and LPC was low ($\rho = 0.2990$, $P = 0.0039$; Fig. 2C). The correlations between total LPA and ATX, and total LPA and total LPC were statistically significant ($\rho = 0.5008$, $P < 0.0001$ and $\rho = 0.7462$, $P < 0.0001$, respectively; Figs. 2D, 2E).
Analysis of LPA and LPC Molecular Species in AH

There were significant differences in aqueous levels of LPA, LPC, and ATX in the four glaucoma groups evaluated. Because LPA is generated from LPC by ATX, we further focused on the LPA and LPC molecular species in the AH because the affinity of LPA/LPC for ATX is dependent on species.

For clarity, we divided the study cohort into three groups: control, a-POAG (NTG and POAG), and a-SOAG (SOAG and XFG). Both a-POAG and a-SOAG showed significantly higher levels of total LPA than the controls, with greater levels in the a-SOAG group than in the a-POAG for all species of LPA (Figs. 3A–3F). In the AH of a-SOAG subjects, LPA 16:0, LPA 18:1, and LPA 18:2 were the major LPA molecular species that showed differences in concentration compared with the controls (Fig. 3G). The absolute values of LPA species showed that 18:1 LPA was present at the highest concentration, followed by 18:2 LPA and 16:0 LPA in the AH of a-SOAG subjects.

To elucidate the reason for the increased LPA levels in AH, we first investigated the ATX-mediated pathway because LPA is thought to be mainly produced from LPC via ATX. The correlations between the ATX ATX level and each LPA species in the AH were found to be positively associated with ATX, whereas age, sex, and IOL status were not statistically significant (all $P > 0.05$). In multiple regression analysis, ATX was significantly associated with pre-IOP, diagnosis, and total LPA, and the level of total LPA was the most significant variable. In the second multivariate model with total LPA as the dependent variable, we found that ATX and total LPC were significant predictors, with sex, IOL status, pre-IOP, and diagnosis failing to reach statistical significance.

Glaucoma Diagnostic Ability of Indices in the ATX–LPA Pathway

The diagnostic values of indices of the ATX-LPA pathway were compared using ROC curve analysis (Table 5). Among the indices, ATX had the largest AUC for discriminating glaucomatous changes between all patients with glaucoma and healthy eyes (0.871; 95% CI, 0.812 to 0.9347), showing a significant difference compared with total LPA ($P = 0.0003$) and LPC ($P < 0.0001$). In subgroup analyses, the AUC of ATX in the a-SOAG (SOAG+XFG) group was larger than that in the a-POAG group (NTG+POAG). For discrimination between a-POAG and a-SOAG, total LPA had the largest AUC, showing a significant difference compared with total LPC ($P = 0.0328$); however, this was not significantly different from that of the ATX ($P = 0.3035$).
FIGURE 4. Correlation between LPA species, autotaxin, and LPC levels. (A–C) Correlations of aqueous concentrations of LPA species with aqueous autotaxin levels. (D–F) Correlations of aqueous concentrations of LPA species with aqueous LPC levels. (A, D) 16:0 LPA. (B, E) 18:1 LPA. (C, F) 18:2 LPA.

TABLE 3. Regression Analysis of Factors Associated With ATX and Total LPA in All Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Simple Analysis</th>
<th>Multiple Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B 95% CI β P Value</td>
<td>B 95% CI β P Value</td>
</tr>
<tr>
<td>ATX*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>−0.001 −0.006 0.004 −0.031 0.735</td>
<td>0.006 0.0011 0.0012 0.1735 0.018</td>
</tr>
<tr>
<td>Sex (male versus female; reference)</td>
<td>0.045 −0.059 0.120 −0.003 0.499</td>
<td>0.203 0.316 0.2500 0.001</td>
</tr>
<tr>
<td>IOL (phakia versus pseudophakia; reference)</td>
<td>0.066 −0.099 0.163 0.047 0.632</td>
<td>0.006 0.0011 0.0012 0.1735 0.018</td>
</tr>
<tr>
<td>Pre-IOP</td>
<td>0.016 0.010 0.022 0.430 &lt;0.001</td>
<td>0.006 0.0011 0.0012 0.1735 0.018</td>
</tr>
<tr>
<td>Diagnosis (control versus glaucoma; reference)</td>
<td>0.364 0.235 0.494 0.451 &lt;0.001</td>
<td>0.203 0.316 0.2500 0.001</td>
</tr>
<tr>
<td>Total LPA</td>
<td>5.909 4.582 7.237 0.625 &lt;0.001</td>
<td>6.103 3.7915 8.415 0.6486 &lt;0.001</td>
</tr>
<tr>
<td>Total LPC</td>
<td>1.457 0.903 1.971 0.436 &lt;0.001</td>
<td>−0.663981 −1.426982 0.099 −0.20313 0.087</td>
</tr>
<tr>
<td>Total LPA†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>0.00004 −5E−04 6E−04 0.011 0.890</td>
<td>0.0054 −0.0003 0.014 0.06 0.225</td>
</tr>
<tr>
<td>Sex (male versus female; reference)</td>
<td>−0.015 −0.027 −0.003 −0.19 0.015</td>
<td>0.0054 −0.0003 0.014 0.06 0.225</td>
</tr>
<tr>
<td>IOL (phakia versus pseudophakia; reference)</td>
<td>0.04 0.0259 0.0550 0.398 &lt;0.001</td>
<td>0.0054 −0.0003 0.014 0.06 0.225</td>
</tr>
<tr>
<td>Pre-IOP</td>
<td>0.002 0.0013 0.003 0.438 &lt;0.001</td>
<td>0.0054 −0.0003 0.014 0.06 0.225</td>
</tr>
<tr>
<td>Diagnosis (control versus glaucoma; reference)</td>
<td>0.025 0.0112 0.0391 0.271 &lt;0.001</td>
<td>0.0054 −0.0003 0.014 0.06 0.225</td>
</tr>
<tr>
<td>ATX</td>
<td>0.066 0.051 0.0810 0.625 &lt;0.001</td>
<td>0.033 0.023 0.043 0.313 &lt;0.001</td>
</tr>
<tr>
<td>Total LPC</td>
<td>0.292 0.2635 0.321 0.848 &lt;0.001</td>
<td>0.233 0.1938 0.2668 0.667 &lt;0.001</td>
</tr>
</tbody>
</table>

B, regression coefficient or partial regression coefficient; 95% CI, 95% confidence interval of partial regression coefficient; β, standardized partial regression coefficient; adjusted R², adjusted coefficient of multiple determination.

* Adjusted R² = 0.5001.
† Adjusted R² = 0.7698.
**TABLE 5.** Comparison of the Diagnostic Values of Indices of ATX–LPA Pathway Using ROC Curve Analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Versus a-POAG (95% CI)</th>
<th>Control Versus a-SG (95% CI)</th>
<th>a-POAG Versus a-SG (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATX</td>
<td>0.8527 (0.8120–0.9347)</td>
<td>0.8018 (0.7436–0.9083)</td>
<td>0.7459 (0.6349–0.8321)</td>
</tr>
<tr>
<td>Total LPA</td>
<td>0.7585 (0.6602–0.8534)</td>
<td>0.6909 (0.5733–0.7881)</td>
<td>0.8003 (0.6976–0.8744)</td>
</tr>
<tr>
<td>Total LPC</td>
<td>0.7220 (0.6197–0.8054)</td>
<td>0.6589 (0.5412–0.7598)</td>
<td>0.7322 (0.6262–0.8169)</td>
</tr>
</tbody>
</table>

Comparison of AUC

- ATX versus LPA: \( P = 0.0003 \)
- ATX versus LPC: \( P < 0.0001 \)
- LPA versus LPC: \( P = 0.2461 \)

**Correlation Between ATX–LPA Pathway and IOP Elevation and Glaucoma Subtypes**

In the present study, there were notable increases in aqueous humor levels of ATX, LPA, and LPC in glaucoma patients (Table 2). The higher ATX and LPA concentrations were significantly correlated with IOP elevation and therefore may be associated with the pathogenesis of IOP elevation. Although LPC concentrations in the AH of SOAG and XFG patients were significantly higher than in controls or NTG group, the difference in LPC concentration between glaucoma subtypes was not significant except for the SOAG subtype, in which the frequency of outliers was high. Disruption of the blood–aqueous barrier was suspected in SOAG/XFG because the SOAG group included various stages of primary disease such as uveitis, and postoperative conditions caused by diabetic retinopathy or ischemic retinal diseases (Fig. 1C). As shown in Table 3, IOP glaucoma diagnosis, and total LPA were statistically significant explanatory factors for ATX in the first multivariable model, and ATX and LPC were significant explanatory factors for LPA in the second model; this suggests an important role for the ATX/LPA pathway in the AH of glaucoma patients. Aqueous ATX and LPA levels were comparable to those in plasma; however, LPC levels were lower than those in plasma (Table 2). Thus, it can be that ATX is expressed and secreted by TM cells, we also observed that ATX expression is induced by dexamethasone challenge, which is known to be one of the major pathogenic factors of steroid glaucoma. Our study also revealed that the ATX/LPA pathway regulates the contractile and adhesive properties of TM cells and ECM production by TM cells. These results collectively suggest that the ATX/LPA has a role in the modulation of AH outflow and IOP in glaucomatous eyes.

**DISCUSSION**

Aqueous humor levels of ATX and LPA were significantly higher in OAG patients than in control patients. More importantly, these higher levels correlated with IOP elevation and were related to the subtype of OAG.

**ATX and LPA as Factors Modifying Outflow Pathway**

Several factors, such as TGF- \( \beta \), CTGF, endothelin-1, LPA, and S1P, have been suggested to have roles in the regulation of IOP because in vitro, ex vivo perfusion, and animal model studies have shown that they alter extracellular matrix (ECM) in the TM and decrease AH outflow facility. \(^8,11,18,24,34\) TGF- \( \beta \), CTGF, and endothelin-1 occur at higher concentrations in the AH of patients with POAG and XFG \(^12,35–37\) and influence the contractile properties of TM tissue, ECM synthesis, and actin cytoskeletal reorganization by activating Rho/ROCK signaling and other signaling pathways. \(^13,24,38\) However, there still is not a complete understanding of the mechanisms involved in IOP regulation or which factors in the AH have the principal role. In a previous study, we found that the TGF- \( \beta \) level in the AH of POAG patients was significantly higher than in normal controls undergoing cataract surgery but not significantly higher in the HA of SOAG or XFG patients, \(^59\) suggesting that the mechanisms that control IOP may differ among glaucoma subtypes.

LPA is a major lipid mediator and is associated with several cellular functions, including cell proliferation, differentiation, and ECM turnover. \(^40\) LPA receptors-1, -2, -3, and -4 are readily detectable in human TM cells, and LPA influenced the properties of the TM and SC cells in in vitro studies and decreased outflow facility in a perfusion study. \(^18,19,40,41\) Previously, Iyer et al. reported high lysoPLD activity, which was lower than those in plasma (Table 2). \(^42\) Thus, it can be inferred that LPA is detectable in human TM cells, and LPA influenced the contractile and adhesive properties of TM cells and ECM production by TM cells. These results collectively suggest that the ATX/LPA has a role in the modulation of AH outflow and IOP in glaucomatous eyes.

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surrised that the AH is more susceptible to changes in LPC levels than plasma, as observed in SOAG/XFG patients with higher LPC levels.

**Discrimination of Glaucoma Subtypes by ATX and LPA Species**

Among the observed molecular species of LPA, LPA16:0 and LPA18:1 were the dominant ones in a-SOAG (Figs. 3B, 3C, 3G), and their levels generally agreed with those of LPC species. ATX shows a preference for saturated LPC substrates with long acyl chains (18:0 <16:0 and 18:0 <18:1), indicating that these LPA species are probably produced within the AH and are not extravasated from plasma. This result is in good agreement with the results of AUC analysis and multiple linear regression analysis (Tables 3–5). Although ATX, total LPA, and total LPC all showed high diagnostic performance in their ability to discriminate between control and glaucoma patients, ATX had higher diagnostic performance than LPA or LPC in controls versus a-POAG (NTG and POAG) and controls versus a-SOAG (SOAG and XFG) (Table 5). However, total LPA showed a larger AUC in a-POAG than in a-SOAG, and we believe that this is in part explained by the dominance of ATX-preferred species of LPA in POAG. In addition, multiple linear regression analysis in glaucoma subjects revealed that ATX was a significant predictor for IOP (Table 4). Compared with previous studies, there was a relatively high value of lipids in the aqueous humor. We speculate that it is due, in part, to the difference of the methods of presentation. For example, Edwards et al. previously investigated phosphatidylcholine spectrum for aqueous humor samples from a control and from a POAG donor, and they reported the phosphatidylcholine value by picomolar (pM) for each species (i.e., 18:0/18:0 PC, 18:2/22:6 PC) with normalization by the total average protein in the aqueous humor (pmol per species/μg protein). In the present study, we have shown the LPC as a total LPC, which is the sum of each LPC species (i.e., 16:0/16:1/18:0/18:2 LPC); therefore, the total was summed into the nanomolar range. In the earlier study, it was reported that there were no significant differences between normal and POAG eyes, but this is in good accordance with our present result as there are no significant differences of LPC values between control and OAG eyes with lower IOP (data not shown). The present study is the first to investigate the quantification of lysosphospholipids in glaucoma subjects, especially for LPA, and including SOAG and XFG patients, the glaucoma subtypes with suspected disruption of the blood–aqueous barrier. These data collectively suggest that the high value of LPA is related to the upregulation of ATX and the LPA produced by ATX may have a prominent role in the pathogenesis of IOP elevation in POAG and SOAG. Moreover, we recently reported that there was significant ATX expression in conventional outflow pathway specimens from glaucoma patients, and ATX expression was upregulated by dexamethasone (Dex) treatment in human TM cells. In the study, we demonstrated that the fibrinotic response in TM cells was induced by Dex, possibly by the de novo production of LPA by ATX in the aqueous humor or outflow tissues.

Additionally, it is highly unlikely that LPC derived from plasma leakage into the anterior chamber is primarily responsible for IOP elevation, given the lack of statistical significance for this association in multivariable modeling. In the previous study, LPC2:2:6 in the blood was reported to be reduced in severe POAG. However, in the present study, LPC2:2:6 was not a significant species in the aqueous humor either in control or glaucoma eyes. In addition, Margolis et al. recently reported that the phospholipids within tissue of ciliary body (CB), AH, and blood and serum were consistent, and they have suggested CB could be the source for many but not all of the phospholipids in the AH. In that study, however, the composition of phospholipids in normal donor derived AH and CB organ culture from normal donor eyes was investigated. Further studies will be needed to elucidate the origin of LPA and LPC in glaucoma eyes. In future work, we plan to investigate the distribution of various molecular species of LPA and LPC in glaucoma subtypes.

There are some limitations in the current study. In the AH of glaucomatous patients, the administration of glaucoma medication was not discontinued or washed out preoperatively due to the advanced stage of glaucoma in the patients. The contribution of these medications to the results is not known. In addition, contamination from blood and contribution from different ocular region including lens, cornea, iris, or other anterior segment structures could be contributing, in part, to the higher value of phospholipids. However, we collected ocular fluid intraoperatively with careful procedure. After making a clear corneal incision, the blunt needle was inserted into the anterior chamber and aqueous fluid was aspirated. If the needle contacted ocular tissues such as iris, lens, or corneal endothelium, the sample was abandoned. Further, the significant correlation between ATX/LPA and IOP levels was observed only in a small number of patients; hence, caution should be exerted in interpreting the results. It would be of further interest to determine why and when ATX and LPA levels are increased during onset and progression of glaucoma.

In conclusion, ATX–LPA pathway activity appears to be associated with IOP elevation and the subtype of OAG. The current results should help facilitate a better understanding of the potential role of the ATX–LPA signaling pathway in glaucoma and the development of novel glaucoma therapeutics.

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**References**


