Comparative Phospholipid Profiles of Control and Glaucomatous Human Trabecular Meshwork

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Purpose. We compared phospholipid (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol) profiles of control and glaucomatous trabecular meshwork (TM) derived from human donors.

Methods. Control TM and most primary open angle glaucoma (POAG) TM were collected from cadaver donors. A select subset of POAG surgical TM samples also were collected for analyses. Lipid extraction was performed using a modification of the Bligh and Dyer method, protein concentrations were determined using the Bradford method, and for select samples confirmed with densitometry of PHAST gels. Lipids were identified and subjected to ratiometric quantification using a TSQ quantum Access Max triple quadrupole mass spectrometer with precursor ion scan (PIS) or neutral ion loss scan (NLS), using appropriate class specific lipid standards.

Results. The comparative profiles of phosphatidylcholine, phosphatidylserine, phosphoethanolamine, and phosphatidylinositol between control and glaucomatous TM showed several species common between them. A number of unique lipids in all four phospholipid classes also were identified in control TM that were absent in glaucoma TM and vice versa.

Conclusions. A number of phospholipids were found to be uniquely present in control but absent in glaucomatous TM and vice versa. Compared to a previous study of control and POAG blood, a number of these phospholipids are absent locally (TM), as well as systemically (in blood).

Keywords: lipidomics, mass spectrometry, trabecular meshwork, glaucoma, phospholipids

Elevated IOP and diurnal fluctuation in IOP are major risk factors for the development of glaucoma.[1] Lowering IOP is the only proven strategy for protecting the optic nerve from glaucomatous optic neuropathy. The IOP can be lowered by either decreasing aqueous production or increasing aqueous outflow. Aqueous outflow can be increased through two pathways: the trabecular meshwork (TM) pathway (also known as the conventional pathway) and/or the uveoscleral pathway. Many currently available medications, for example β-blockers and carbonic anhydrase inhibitors, decrease aqueous production, while others, such as prostaglandin analogues,[2] increase aqueous outflow via the uveoscleral pathway.[3] Analogs of prostaglandins, a class of lipids, were identified in the iris in 1955.[4] Synthetic prostaglandin analogs were developed and have been used as IOP lowering topical medications. This latter group of ocular drugs increase the aqueous outflow by targeting the uveoscleral pathway.[5] The development of additional drugs, which lower IOP, especially through increased aqueous drainage via the conventional outflow pathway, is needed.

A physiologic approach for lowering IOP is to increase outflow via the conventional pathway.[5,6] Apart from pilocarpine, a muscarinic agonist of inferior efficacy when compared to prostaglandins, no other drugs are available to enhance aqueous outflow via the TM pathway with less significant side effects. The critical barrier in developing new interventional strategies is the need for improved understanding of physiologic processes of the anterior segment and their aberration in the diseased state. Prostaglandin lipids are great regulators of outflow facility.[5,7] The receptors for prostaglandins have been found to be present in the uveoscleral pathway at a greater concentration than in the conventional pathway.[7] Other classes of endogenous lipids may exist that regulate outflow at coarse and fine regulatory levels. Furthermore, redundant endogenous regulatory lipids are expected to be present in the aqueous humor and TM, whose levels undergo an irreversible change in glaucoma in contrast with controls. We hypothesized that differences in age, race, individual health, and drug regimen will cause small variation in individual lipid species for the vast majority of lipids. We conjectured that there could be some differences in overall class of lipids, but only a small subset of lipids within any class is likely to show relatively large changes between control and glaucoma.

The lack of suitable methods to identify and quantify low amounts of lipids present in the TM is a critical barrier for high throughput lipid profiling in these samples. Most available methods require micromolar quantities for lipid analyses, whereas TM presents possibly only nanomolar to picomolar, and perhaps even lower concentrations. The advent of mass spectrometry, and the recent developments of bioinformatic approaches and databases have eliminated these critical barriers.[8–11] We present the results of profiling for four phospholipids classes, namely phosphatidylcholines (PCs), phosphatidylserines (PSs), phosphatidylethanolamines (PEs), and phosphatidylinositols (PIs), in the TM, and their comparative analyses between glaucomatous and control donors.

Methods

Tissue Procurement

Donor tissues were used following the institutional review board approved protocols and adhering to the tenets of the Declaration of Helsinki. Control TM and a subset of primary open-angle glaucoma (POAG) TM were collected from cadaver donors, a limited subset of POAG surgical specimen (acquired under institutional review board approved/exempted protocols) also were used for these studies. Cadaver donor tissues were sourced from Midwest Eye-Banks, Lions Eye Bank (Miami, FL), National Disease Research Interchange (NDRI; Philadelphia, PA), and Mundorf Eye Institute (Charlotte, NC). Use of tissues for analyses and donors are detailed in Supplementary Table S1. The postmortem time ranged from 3 to 16 hours. Control TM was stored briefly in Optisol at 4° and then used for lipid extraction. POAG TM tissues were stored at −80°C until time of use. Totals of 16 control and 16 glaucomatous TM were used for these studies. The mean age of control donors was 57.8 ± 11.7 years and the mean age of glaucomatous TM donors was 64.3 ± 9.8 years (Supplementary Table S1), and both sexes were included for these studies. Trabecular meshwork tissue was isolated using established procedures, as performed in our previous reports.[12] Cadaver TM tissue was received before arrival of information about the donor medical history. Although lipid extraction and mass spectrometry were performed on all tissues received, approximately 40% donor TM tissues were not used after mass spectrometry for further bioinformatic analyses due to lack of sufficient donor information.

Lipid Extraction

Isolated trabecular meshwork tissue was subjected to an alternating cycle of immersion in liquid nitrogen and 37°C, followed by extraction of lipids using a modified Bligh and Dryer method.[13] The organic phase with extracted lipids was dried in a Speed-Vac (Model 7810014; Labconco, Kansas City, MO). Samples were flushed with argon gas to prevent oxidation. Corresponding aqueous phase extracted proteins were subjected to determination of concentration using Bradford's method.[14] A subset of protein samples also were subjected to densitometric quantification using bovine serum albumin as a standard (amino acid quantified) after electrophoretic separation on a PHAST (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gel system[15] All extractions and subsequent handling were made using glass vials, and polyvinyl plastic was avoided completely. We also added a PC control standard during tissue homogenization, determined its recovery in an aliquot, and used for the calculation of total recovery of this standard for each extraction to ensure >99% recovery of added standard during extraction. The procedures to determine extraction efficiency are similar to that which were performed for our published corneal lipid extraction and analyses.[16]

Mass Spectrometric Analysis

A triple quadrupole electrospray mass spectrometer (TSQ Quantum Access Max; Thermo Fisher Scientific, Pittsburgh, PA) was used for analysis of lipids in infusion mode using TSQ Tune software that is part of the Xcalibur 2.3 software package. Extracted lipids were dried and resuspended in LC-MS grade acetonitrile: isopropanol (1:1). Samples were infused with a flow rate of 10 μL/min and analyzed for 1.00 minute with a 0.500-second scan. Scans typically ranged from 200 to 1000 m/z unless specified otherwise. A peak width was set at 0.7 and collision gas pressure was set at 1 mTorr. Sheath gas (nitrogen) was set to 20 arbitrary units. Auxillary gas (Argon) was set to 5 arbitrary units. For analyses of different phospholipid classes, collision energy, spray voltage, and ion mode were set (Table 1) based on previous studies.[8,17,18] Precursor ion scans (PIS) were performed where the daughter ion of choice is selected in the third gate (Q3) and the correlating parent ion in the first gate (Q1, from which the daughter ion originated) is scanned. This enables all lipid parent masses to be determined. Neutral loss scans (NLS) were performed, which correlated precursor and daughter ions in first (Q1) and third (Q3) gates for a definite mass (e.g., 87.1 for PSs). For TM tissue, at least $n = 16$ donors each for control and glaucomatous case were used for each of the four phospholipid classes analyzed. Class-specific lipids were quantified using class-specific quantitative lipid standards (Table 1). Approximately 10 scans each with and without internal standard (usually in the range of 0.1–5 pmol) were performed for each sample. Ratiometric quantification was achieved using the MZmine 2.9 program. Lipid concentration was normalized to protein amount determined from the corresponding aqueous phase as described above.

Data Analysis

A representative spectra for each sample was inspected carefully and manually by two independent observers from 10 spectra collected for each sample with and without the internal standard (a total of 20 spectra), and then was used for further analyses. Spectra were converted to netCDF files from Thermo RAW files using Xcalibur 2.3 software suite, subsequently imported into MZmine 2.9,[19] and subjected to noise removal and analyses. A selected subset of data also was subjected to analysis in SimLipid version 3.1. Briefly, the following steps were used for quantification using MZmine 2.9. Thermo RAW files were converted into netCDF files using the Xcalibur software, and then imported into the MZmine program interface. Original RAW files always were retained. Imported spectra were filtered by the Scan-by-Scan methods from 200 to 1000 m/z for PE, PI, and PS, and from 400 to 900 m/z for PC. Masses were detected in centroid mode, and noise levels were kept at 1.00 E0 for all phospholipid classes. Noise was removed after the chromatogram was constructed. The minimum span time was set at 0:01 minutes with a mass tolerance of 1.00 m/z. Noise was removed manually, up to and including all detected masses E3 for positive ion mode and E2 for negative ion mode. Isotopic peak correction was done with a mass tolerance of 2.00 m/z with a retention time tolerance of 1.00 minute with a monotonic shape, a maximum charge of 1, and the representative isotope as the lowest m/z. Identification was done against a custom database created from the LipidMaps Database (LMSD) with parameters of 1.00 m/z tolerance and a retention time of 1.00 minute. The identification obtained indicated positions of double bonds and other features of lipids as present in the database, and has been retained in our Tables. However, our triple quadrupole data cannot verify all the detail features. Thus, what we indicated in the Tables are the lipids as in the database that has been matched by MZmine 2.9. We defined unique when a given lipid species was found in only one group (control or POAG) and with a frequency ≥2 donors. For common lipid species the presence was recorded in both groups with at least a frequency of ≥2 in one group.

All unique lipid experimental readings (the amount of lipid species) were found to be significantly different from 0.0 by one-sample *t*-test ($P \leq 0.05$). The common lipid species (Supplementary Tables S2–S6) had statistically significant differences between the control and POAG by ANOVA. Scheffe's post hoc test showed that controls were statistically different from POAG ($P \leq 0.05$).

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Table 1. Analytical Parameters and Internal Standards for Class-Specific Lipids Quantification

Lipid Class	Name of the Lipid Standard	Ion Mode	Scan Type	Daughter Ion Mass, m/z	CE, V	Parent Mass of the Standard	Catalog Number*
Phosphocholine (PC)	1,2-ditridecanoyl-sn-glycero-3-phosphocholine	+	PIS	184	35	649.89	850340
Phosphoserine (PS)	1,2-dioleoyl-sn-glycero-3-phospho-L-serine	–	NLS	87.1	24	810.03	840035
Phosphoethanolamine (PE)	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	–	PIS	196	50	744.04	850725
Phosphoinositol (PI)	1,2-dioleoyl-sn-glycero-3-phospho-(1′-myo-inositol)	–	PIS	241	45	880.15	850149

* All standards were procured from Avanti Polar Lipids (Albaster, AL) unless stated otherwise.

Results

We obtained lipid profiles for all four classes of phospholipids using established parameters (Table 1) and infusion mode. A representative PC profile for TM without (Fig. 1A) and with (Fig. 1B) ratiometric standard from a control donor has been shown. The cumulative data for each of the four phospholipid classes were analyzed further using Excel macros that were written in-house[20] to determine the presence of common and unique lipid species in control and glaucomatous samples. Phospholipids species were considered unique and retained only when they occurred in any one donor group with a frequency of $\geq$2. For common species, their presence in at least one group was $\geq$2, even if their donor frequency in the other group was only 1. The total amount of phospholipids in all four classes normalized to total amount of proteins in the corresponding aqueous phase extractions have been presented in Table 2. All phospholipid classes showed lower total amounts in glaucomatous TM compared to controls except PEs and PIs (Table 2).

PCs of TM

Six unique PCs were found most frequently in control TM: PC(13:0/20:4[5Z,8Z,11Z,14Z]) in 3 donors of both sexes with ages ranging from 35 to 72 years, PC(15:0/18:2[9Z,12Z]) in 3 donors of both sexes with ages ranging from 51 to 72 years, and PC(16:0/20:5[5Z,8Z,11Z,14Z,17Z]) in 2 male donors 54 to 64 years old. All of these donors were not taking statins. PC(22:6[4Z,7Z,10Z,13Z,16Z,19Z]/22:6[4Z,7Z,10Z,13Z,16Z,19Z]) and PC(18:1[11Z)]/22:6[4Z,7Z,10Z,13Z,16Z,19Z]) were found in 5 and 6 donors of both sexes with ages ranging from 35 to 67 years; PC(20:3[8Z,11Z,14Z]/0:0) was found in two male donors ages ranging from 54 to 55 years, which included donors on or without statins. There were no uniquely found PC species in glaucomatous tissues (species that occurred with a donor frequency of greater than 1). Compared to a small number of unique PC species, several PC lipids were common to normal and glaucomatous TM with a high frequency of occurrence (Table 4). A total of 165 PCs was common between control and glaucomatous TM (Supplementary Table S3). TM donors that showed unique species did not suffer from hyperlipidemia, cholesterolemia, or diabetes unless stated otherwise. All donor ages specified here refer to age at the time of TM collection.

PSs of TM

We found no unique PS species in the control TM tissues (donor frequency of higher than 1, Table 3). A single unique PS species, PS(O-16:0/15:0), was found in glaucomatous TM in 2 donors encompassing both sexes, age range 55 to 59 years without hyperlipidemia and diabetes (Table 3). A number of PS species were common with high frequency (Table 4) between control and POAG TM, totaling 140 PS lipid species (Supplementary Table S4).

PEs of TM

No unique PE species were found either in control or in the POAG TM (Table 3). The number of common PE species in TM was 143 (Table 4, Supplementary Table S5).

PIs of TM

Two unique PI species were identified in the control TM: PI(17:1[9Z]/22:2[13Z,16Z]), found in 2 donors of both sexes, ages ranging 48 to 65 years, and PI(20:3[8Z,11Z,14Z]/0:0) in 3 donors of both sexes. All these control donors were nonstatin users. Six PI species were found in glaucomatous TM: PI(14:1[9Z]/20:5[5Z,8Z,11Z,14Z,17Z]), found in two donors of both sexes, ages range 51 to 57 years, and without hyperlipidemia or diabetes; PI(15:0/20:5[5Z,8Z,11Z,14Z,17Z]) in 2 male donors, ages range 50 to 67 years; PI(16:0/22:2[13Z,16Z]) in 3 donors of both sexes, ages range 50 to 61 years; PI(16:1[9Z]/22:2[13Z,16Z]) in 3 donors of both sexes ages range 57 to 67 years, and without hyperlipidemia or diabetes; PI(20:1[11Z]/22:2[13Z,16Z]) in 4 donors of both sexes, ages range 55 to 67 years; and PI(22:2[13Z,16Z]/22:4[7Z,10Z,13Z,16Z]) was found in 3 male donors, ages range 50 to 67 years. All these POAG donors were without hyperlipidemia or diabetes (Table 3). The number of common PI species in TM with a high frequency of occurrence were far greater than the unique PI species in control or POAG group (Table 4), totaling 124 (Supplementary Table S6).

Discussion

In POAG, aqueous outflow is impeded secondary to increased resistance at the level of the TM. This results in elevated IOP, which in turn causes optic nerve damage. Prostaglandins (PGs) were identified in the iris and named irin in 1955, and were characterized initially as a smooth muscle contracting substrate.[4] A long period of continued research in PG analogs resulted in the development of topical glaucoma medications (such as latanoprost and travoprost) that are among the most effective IOP reducing medications used in the treatment of glaucoma.[21]

The effect of other classes of lipids on IOP or outflow facility has not been well studied. Using controlled rodent experiments, it has been shown that increasing dietary omega-3 can reduce IOP due to increased outflow facility.[22] Interactions of a few non-PG lipids have been known to be involved in the regulation of aqueous humor flow and integrity of TM tissue. For example, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) decrease aqueous humor outflow.[23]

Phospholipids are the main components of the membrane lipid bilayer that assist in creating cell boundaries as well as integrity of the cells for life processes. Membrane phospholipids create a hydrophobic environment for transmembrane protein function and communication. Some membrane lipids

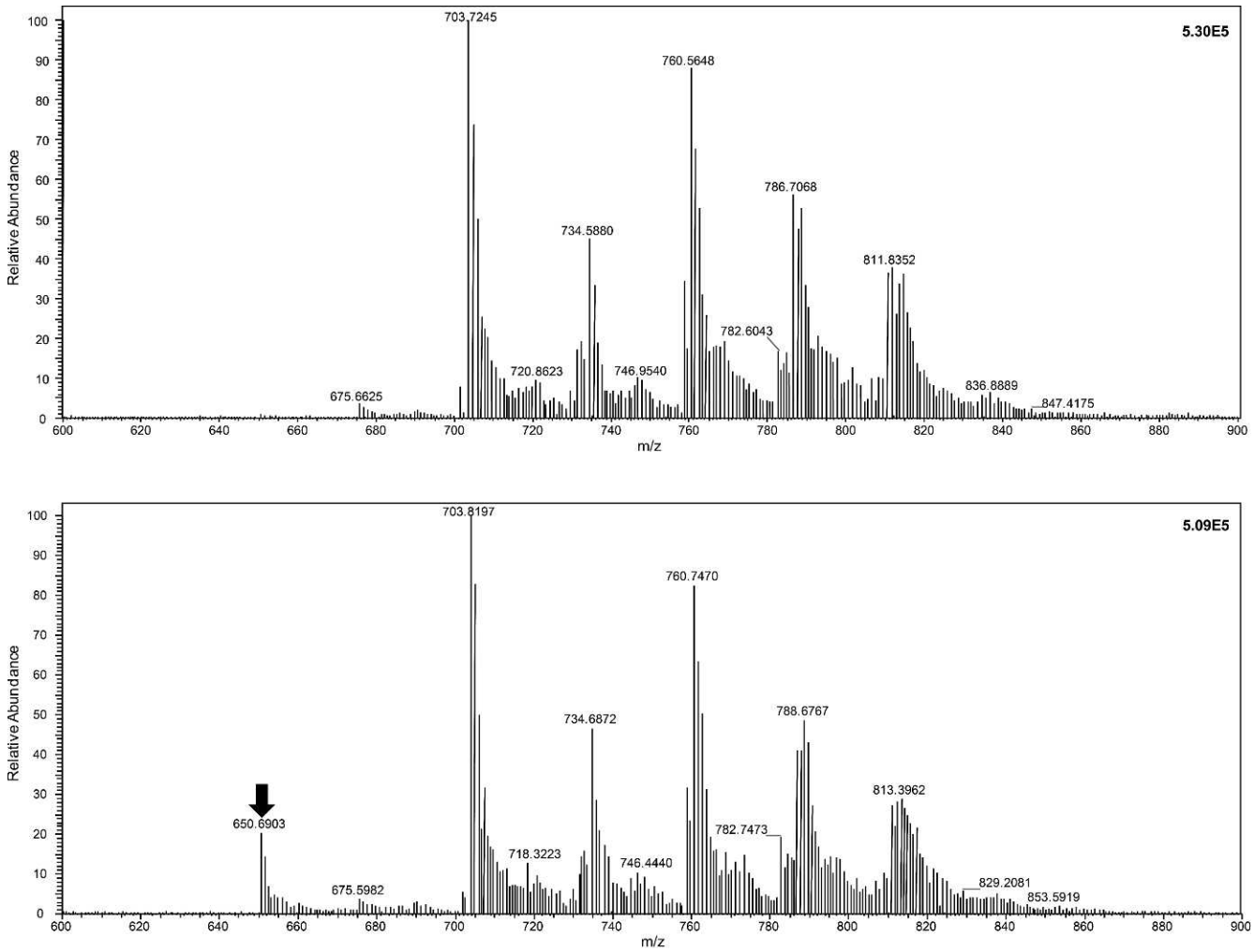

Figure 1. Representative electrospray ionization tandem mass spectrometric analysis of PC class of lipids extracted from human TM in positive-ion mode. (**A**) Representative PIS scanning of m/z 184.0 corresponding to PC class. (**B**) Representative PIS as above with internal standard addition (*arrowhead*; m/z ratio of 650.6) enabling ratiometric quantification of all identified lipids in PC class. The experimental conditions for PIS are listed in Table 1. Parent ion scan for m/z 600 to 900 has been shown.

are part of lipid second messengers, which are metabolized by enzymatic activity from phospholipid precursors.[24] PIs is one of the known classes of phospholipids that undergoes significant changes during the administration of PG analogs for IOP lowering.[25,26]

Endogenous lipids in the anterior chamber may be involved in regulation of aqueous outflow, outflow facility, and IOP. The previous techniques, such as various forms of chromatography and nuclear magnetic resonance, necessitated knowledge of several different chemistries due to different chemical behavior of different lipid species even within a class, and the requirement of large amounts (microgram quantities) of lipids for identification and characterization. Recent advances in mass spectrometry enable overcoming these two hurdles toward high throughput determination of identities of phospholipids of all four classes (PC, PS, PE, and PI) in the TM.

In our investigation, the TM tissue used consisted of surgical and cadaver eye derived samples received from local as well as secondary sources. Sourcing of TM samples from distant locations contributed to significant unavoidable transit times and storage in PBS or Optisol GS. Our control analyses with cornea[16] and other anterior chamber tissue (data not shown) from mammalian model systems (porcine, bovine, and a select subset of humans) showed across the board decrease of phospholipid species, but no selective absence or appearance of a phospholipid species as a function of storage up to a week at 4°C in PBS or Optisol GS (Bhattacharya SK, Aribindi K, Crane AC, unpublished observations, 2012). Three other factors are intrinsically likely to affect the lipid profiles broadly apart from biochemical individuality of the donors: (1) confounding factors of diseases or disorders that are well known to affect lipid profiles, such as hyperlipidemia; (2) use of drugs by the donors, such as statins, and (3) confounding factor of diseases that are not lipid-related per se, such as diabetes. Previously, use of statins has been found not to affect the phospholipid

Table 2. Total Average Protein Normalized Phospholipids in the Trabecular Meshwork

	Phospholipids, pmol/μg Protein	
	Control	**Glaucomatous**
Phosphatidylcholines	6351.5	4184.3
Phosphatidylserines	3602.1	563.9
Phosphatidylethanolamines	1073.8	2126.4
Phosphatidylinositols	179.0	567.4
All Phospholipids (total)	11,206.4	7441.9

Table 3. Unique Phosphoslipid Species* Identified in Control and Glaucomatous Trabecular Meshwork

	m/z*	Average Lipid Amount, pmol per Species/ µg Protein	Donor Frequency	LIPIDMAPS ID†	PUBCHEM ID	HMDB Accession Number‡
Phosphatidylcholines						
Control trabecular meshwork						
PC(13:0/20:4[5Z,8Z,11Z,14Z])	739.20	3.53	3	LMGP01011356	123060644	
PC(15:0/18:2[9Z,12Z])	742.15	4.37	3	LMGP01010543	7983030	HMDB07940
PC(16:0/20:5[5Z,8Z,11Z,14Z,17Z])	779.89	0.31	2	LMGP01010633	7983102	HMDB07984
PC(18:1[11Z]/22:6[4Z,7Z,10Z,13Z,16Z,19Z])	830.62	5.90	6	LMGP01010847	7983334	HMDB08090
PC(22:6[4Z,7Z,10Z,13Z,16Z,19Z]/ 22:6[4Z,7Z,10Z,13Z,16Z,19Z])	878.51	0.01	5	LMPG01011119	7983606	HMDB08748
PC(20:3[8Z,11Z,14Z]/0:0)	545.48	0.05	2	LMGP01050133	123061568	
Phosphatidylserines						
Glaucomatous trabecular meshwork						
PS(O-16:0/15:0)	708.49	4.87	2	LMGP03020005	123063653	
Phosphatidylinositols						
Control trabecular meshwork						
PI(17:1[9Z]/22:2[13Z,16Z])	901.97	0.02	2	LMGP06010243	123065262	
PI(20:3[8Z,11Z,14Z]/0:0)	622.82	0.23	3	LMGP06050021	123066183	
Glaucomatous trabecular meshwork						
PI(14:1[9Z]/20:5[5Z,8Z,11Z,14Z,17Z])	826.38	0.57	2	LMGP06010100	123065119	
PI(15:0/20:5[5Z,8Z,11Z,14Z,17Z])	842.07	0.01	2	LMGP06010123	123065142	
PI(16:0/22:2[13Z,16Z])	890.54	2.18	3	LMGP06010168	123065287	
PI(16:1[9Z]/22:2[13Z,16Z])	888.33	3.30	3	LMGP06010192	123065211	
PI(20:1[11Z]/22:2[13Z,16Z])	944.83	0.28	4	LMGP06010523	123065542	
PI(22:2[13Z,16Z]/22:4[7Z,10Z,13Z,16Z])	966.67	0.33	3	LMGP06010754	123065773	

* A representative mass/charge ratio is presented (variations in m/z were reconciled by MZmine 2.9).

† The lipid species identification is based on Lipidmaps database, used as a *.csv file for bioinformatic analyses with MZmine 2.9 program.

‡ The HMDB identifiers report following details for identified lipids. Localization, extracellular membrane and membrane component; functions, membrane integrity, cell signaling, energy source, and energy storage.

profiles in the lens and conjectured not to affect that in the ocular tissues.[27] Consistent with our hypothesis, careful analyses of statin users reflect very little changes in phospholipids systemically,[28] supporting that phospholipids probably are not affected significantly in statin users. We also found only small variations in individual lipids due to differences in age, race, individual health, and drug regimen of the donors for the vast majority of lipids (Tables 3, 4; Supplementary Tables S3–S6). Compared to common lipids (Supplementary Tables S3–S6) we found only a small subset of lipids to be unique in the control or POAG group (Table S3). On the other hand we found decreased total PS and increased total PE lipids in glaucoma compared to controls (Table 2). All phospholipid classes showed lower total amounts in POAG compared to control TM with two exceptions; that is, PEs and PIs, which were higher in glaucoma compared to controls, supported by the 2-way ANOVA analysis (Scheffe's post hoc analysis, Table 2).

We have determined the endogenous phospholipids present in TM using triple quadrupole mass spectrometry in parent-ion and neutral loss scan modes with parameters that have been well established in the lipid field.[8] Such approaches recently have been made to characterize red blood cells from POAG patients,[29] and also for determining the composition of phospholipids of the optic nerve.[30,31] PC (22:6[4Z,7Z,10Z,13Z,16Z,19Z]/22:6[4Z,7Z,10Z,13Z,16Z,19Z]) was found uniquely in control TM tissues (Table 3). The result is consistent with a previous study in which the level of PC (22:6[4Z,7Z,10Z,13Z,16Z,19Z]/22:6[4Z,7Z,10Z,13Z,16Z,19Z]) systemically decreases in the blood as the severity of glaucoma increases in POAG patients.[29,30] Phospholipid species PC (18:1[11Z]/22:6[4Z,7Z,10Z,13Z,16Z,19Z]) also have been identified uniquely (Table 3) in control TM as well as red blood cells of control donors.[29,30] Taken together with recorded stiffness or rigidity of glaucomatous TM cells compared to controls,[32] a reduction of observed PCs in the glaucomatous TM cells is indicative of altered cell membrane fluidity in glaucoma.

Our results presented here provide insight that tissue metabolism and, consequently, the cell biology in glaucoma is altered as well. Negatively charged PS lipid vesicles[33] could be expected to maintain the TM beams. The observation of lowering of several PS species in the glaucomatous TM would be consistent with the hypothesis that the PS lipid vesicle formation that helps maintain TM beams is destabilized in glaucoma (Table 3, Supplementary Table S4). In contrast to PS, several of the PE species in the glaucomatous TM have elevated levels compared to controls, for example, NAPE(18:1[9Z]/16:1[9Z]/18:0), PE(10:0/10:0), and PE(12:0/12:0) (Table 3, Supplementary Table S5). The glaucomatous or control TM tissues do not show any appreciable differences in levels of free choline or free ethanolamine levels (Bhattacharya SK, unpublished observations, 2012). Thus, the altered levels of phospholipid conversion or biosynthetic enzymes are other possibilities.[34,35] PS to PE conversion could be contributed by PS synthases (PSS1, PSS2), phosphatidylethanolamine N-methyltransferase (PEMT) or phosphoserine decarboxylase (PSD).[35] The activity of PEMT necessitates S-Adenosylhomocysteine (AdoHcy). Elevated AdoHcy homolog/product homocysteine (Hcy) in POAG TM has been reported.[36] We hypothesize that decreased PS and increased PE levels in glaucomatous TM are due to impaired expression or activity of any or all of these enzymes (PSS1, PSS2, PEMT, and PSD; Fig. 2) that must be investigated in future. Our data demonstrated the utility of our analyses. Our data clearly now allude toward these testable hypotheses. The design of these studies is the comparison of control with that of glaucoma. Thus, most changes as a result of glaucoma have been captured in our results. These results

Table 4. Common Lipid Species Between Control and Glaucomatous Trabecular Meshwork

		Normal			Glaucoma		
Lipid Species*	m/z†	Av. Normalized Lipid Amount, pmol per Species/μg Protein	SD	Donor Frequency	Av. Normalized Lipid Amount, pmol per Species/μg Protein	SD	Donor Frequency
PC(O-16:0/2:0)	523.11	57.30	11.09	14	15.27	27.43	9
PC(12:0/14:1[9Z])	646.72	24.48	3.90	13	7.72	19.03	7
PC(10:0/10:0)	565.77	34.54	4.06	12	5.78	8.89	10
PC(O-14:0/2:0)	495.27	55.21	7.84	12	3.65	5.85	9
PC(6:0/6:0)	453.30	26.48	4.90	11	6.71	11.54	12
PC(16:0/3:0)	551.46	32.71	4.02	11	21.53	42.62	8
PC(16:0/5:0)	579.40	37.29	4.65	11	21.11	36.30	8
PC(12:0/18:2[9Z,12Z])	701.63	161.66	52.16	11	13.30	28.78	7
PC(10:0/25:0)	775.81	61.05	10.65	11	71.28	124.62	6
PC(12:0/13:0)	635.53	59.28	11.36	11	9.25	7.63	5
PC(22:0/22:4[7Z,10Z,13Z,16Z])	893.37	12.58	1.87	11	18.61	28.96	4
PC(16:0/18:1[9Z])	760.21	261.60	42.31	10	470.48	1345.17	13
PC(10:0/4:0)	481.45	26.53	4.12	10	11.74	31.02	10
PC(11:0/11:0)	592.73	36.20	5.46	10	12.51	25.32	10
PC(22:4[7Z,10Z,13Z,16Z]/0:0)	572.27	32.74	4.00	10	17.25	34.51	9
PC(6:2[2E,4E]/6:2[2E,4E])	445.10	28.71	4.83	10	3.10	3.91	9
PC(10:0/22:0)	733.21	83.18	14.40	10	147.33	182.79	8
PC(14:0/20:4[5Z,8Z,11Z,14Z])	753.57	15.02	2.73	10	43.68	57.28	8
PC(18:0/18:0)	789.01	135.43	31.15	10	374.06	571.39	8
PC(18:4[9E,11E,13E,15E]/0:0)	514.77	12.44	1.98	10	7.68	8.05	5
PC(12:0/17:2[9Z,12Z])	687.28	7.05	1.51	10	23.67	47.34	4
PC(16:0/20:3[5Z,8Z,11Z])	783.96	14.93	3.60	10	115.29	190.66	4
PC(12:0/18:3[6Z,9Z,12Z])	698.34	30.90	3.71	10	0.00	0.00	3
PC(17:1[10Z]/0:0)	506.77	12.34	1.46	10	5.16	0.00	1
PS(19:0/22:1[11Z])	858.71	44.90	95.98	15	0.75	1.10	13
PS(17:2[9Z,12Z]/0:0)	506.79	10.88	18.60	14	1.96	3.95	12
PS(20:0/22:1[11Z])	873.62	58.93	115.30	14	6.67	11.58	8
PS(12:0/0:0)	441.14	47.08	61.43	13	3.48	3.86	16
PS(13:0/22:1[11Z])	775.16	63.12	114.59	12	6.92	14.38	9
PS(20:4[5Z,8Z,11Z,14Z]/0:0)	545.32	46.82	61.20	11	5.98	17.10	16
PS(13:0/22:2[13Z,16Z])	773.17	26.08	54.30	11	2.03	3.56	14
PS(13:0/22:0)	777.05	111.34	190.87	11	10.23	25.56	13
PS(17:0/22:1[11Z])	831.28	66.64	170.16	11	3.57	6.26	13
PS(22:0/0:0)	581.82	24.78	66.99	11	3.43	4.64	10
PS(17:0/14:1[9Z])	719.84	22.79	37.26	11	4.39	6.58	10
PS(22:4[7Z,10Z,13Z,16Z]/ 22:6[4Z,7Z,10Z,13Z,16Z,19Z])	884.25	127.06	178.09	11	5.97	8.52	8
PS(14:1[9Z]/0:0)	467.20	3.28	7.10	11	3.33	4.49	7
PS(12:0/17:0)	693.42	14.01	29.09	11	2.62	5.00	6
PS(14:0/0:0)	469.73	59.21	76.98	10	1.81	2.32	13
PS(16:0/16:0)	734.95	13.44	25.13	10	2.86	4.53	12
PS(10:0/10:0)	567.19	75.66	111.68	10	1.80	2.01	11
PS(20:0/0:0)	553.22	13.00	27.03	10	2.07	2.67	10
PS(18:0/22:1[11Z])	845.72	49.66	86.76	10	3.15	5.73	10
PS(13:0/22:6[4Z,7Z,10Z,13Z,16Z,19Z])	764.93	9.02	18.92	10	2.47	5.88	9
PS(22:6[4Z,7Z,10Z,13Z,16Z,19Z]/ 22:6[4Z,7Z,10Z,13Z,16Z,19Z])	879.00	124.45	188.34	10	1.56	2.83	9
PS(12:0/17:1[9Z])	691.34	2.09	4.40	10	1.57	3.64	8
PS(20:0/22:4[7Z,10Z,13Z,16Z])	867.63	27.46	47.64	10	2.14	2.00	8
PE(20:0/20:2[11Z,14Z])	799.47	29.37	47.08	16	75.60	95.11	8
PE(21:0/22:0)	845.80	7.83	15.55	14	36.60	67.64	14
PE(13:0/22:4[7Z,10Z,13Z,16Z])	725.04	13.86	21.74	14	38.84	58.76	11
PE(17:0/14:1[9Z])	675.64	9.30	17.57	13	13.84	25.24	11
PE(16:0/20:4[5Z,8Z,11Z,14Z])	739.14	10.50	19.55	13	25.81	17.28	7
PE(26:2[5Z,9Z]/26:2[5Z,9Z])	963.79	20.03	24.09	12	19.47	15.36	7
PE(13:0/18:3[6Z,9Z,12Z])	671.07	0.36	0.95	12	2.44	1.83	4
PE-NMe(16:0/16:0)	704.97	12.18	17.98	11	6.72	10.32	13
PE(13:0/20:2[11Z,14Z])	701.02	1.29	2.11	11	7.51	5.88	11

Investigative Ophthalmology & Visual Science

Table 4. Continued

Lipid Species*	m/z†	Normal: Av. Normalized Lipid Amount, pmol per Species/µg Protein	Normal: SD	Normal: Donor Frequency	Glaucoma: Av. Normalized Lipid Amount, pmol per Species/µg Protein	Glaucoma: SD	Glaucoma: Donor Frequency
PE(16:0/18:1[9Z])	716.89	6.92	8.13	11	10.95	8.59	10
PE(13:0/22:2[13Z,16Z])	728.50	14.95	26.93	11	16.13	21.43	9
PE(12:0/14:0)	607.48	3.89	5.61	11	3.89	4.20	8
PE(17:0/22:2[13Z,16Z])	785.19	7.19	13.06	11	18.27	17.40	8
NAPE(18:1[9Z]/16:1[9Z]/18:0)	981.97	10.96	20.68	10	20.02	21.28	10
PE(24:0/20:0)	859.55	22.67	35.70	10	27.86	25.59	10
PE(17:0/20:0)	761.87	20.52	40.11	10	35.13	35.20	9
PE(18:0/20:0)	775.65	39.40	52.63	10	104.58	138.29	9
PE(18:0/22:6[4Z,7Z,10Z,13Z,16Z,19Z])	791.94	16.48	21.83	10	41.83	47.27	9
PE(22:1[11Z]/22:2[13Z,16Z])	853.87	0.81	2.51	10	2.74	3.45	8
PE(17:0/22:1[11Z])	787.07	5.40	11.57	10	25.64	26.54	7
PE(20:5[5Z,8Z,11Z,14Z,17Z]/ 22:5[7Z,10Z,13Z,16Z,19Z])	811.42	22.16	20.45	10	53.99	61.12	7
PE(20:2[5Z,8Z]/18:0)	771.63	14.75	17.79	10	4.02	3.66	4
PI(12:0/18:2[9Z,12Z])	778.50	1.16	1.67	10	5.30	4.75	9

* The lipid species identification is based on Lipidmaps database, used as a *.csv file for bioinformatic analyses with MZmine 2.9 program.

† A representative mass/charge ratio from normal samples is presented (variations in m/z were reconciled by MZmine 2.9). Average normalized values for only those species with a frequency of ≥10 in control group have been presented (other identified species are listed in Supplementary Tables), SDs are absent for some lipids due to lack of presence in all samples.

possibly may be embedded with data that may predict a predisposition to glaucoma, which we expect to be highly convoluted. Control experiments with animal models (for example, DBA/2J mice) are under progress to capture the later changes.

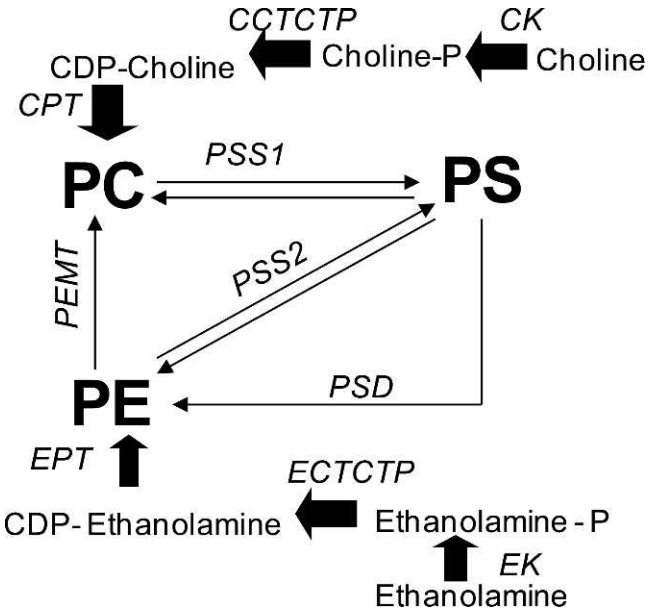

Figure 2. A schematic diagram of potential conversion cycle of phospholipids in the TM. The conversion enzymes have been *italicized*. CK, choline kinase; CCTCTP, phosphocholine cytidylyltransferase; CPT, cholinephosphottransferase; PSS1&2, PS synthase 1 and 2; PEMT, phosphatidylethanolamine N-methyltransferase; EPT, ethanolamine-phosphotransferase; PSD, phosphoserine decarboxylase; ECTCTP, phosphoethanolamine cytidylyltransferase; EK, ethanolamine kinase.

The triple quadrupole instrument used here has a resolution of 1 atomic mass unit (at 0.7 FWHM), which is a first pass attempt to profile all phospholipid species. Further high-resolution mass spectrometry will enable these identifications with greater conformity. In the future, the unique species identified here will need to be characterized carefully using different collision energies and high-resolution mass spectrometry. A comprehensive comparison with blood and other tissues, including TM and aqueous humor, will provide insight about systematic and local changes that may occur in glaucoma as well as complications from confounding factors. Blood plasmalogen changes, specially PC and PS, have been shown to be predictors of occurrence, progression, and severity of glaucoma. Future work will reveal biologic consequences of local alteration of phospholipids in the TM region and insight into their contribution in pathology. Profiling of lipids also will be expected to help expanding databases (perhaps infinitesimally incremental), and will enable the synthesis of interesting lipid species and their screening to determine the biologic roles that they might have in the anterior segment of the eye.

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