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Aqueous Humor Phospholipids of DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ mice

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Abstract

Purpose—To compare phospholipid profiles [phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI)] of normotensive and hypertensive aqueous humor (AH) from DBA/2J and compare them with phospholipid profiles of DBA/2J-*Gpnmb*⁺/SjJ mice.

Methods—AH was obtained from young-normotensive DBA/2J, old -hypertensive DBA/2J mice, young and old DBA/2J-*Gpnmb*⁺/SjJ mice (aging control). Lipids were extracted using modified Bligh and Dyer method and subjected to mass spectrometric identification using appropriate class-specific lipid standards and ratiometric quantification. Corresponding aqueous phase (of extraction) protein concentrations were measured using Bradford method.

Results—The total amount of phospholipids showed a decrease in the hypertensive state compared to normotensive state. The total PE and total PS contributed over 50% of the total amount. Total PS showed a remarkable decrease in hypertensive compared to normotensive state. In contrast, total PE in the hypertensive stage presented an increase in amount. Unique lipid species were found encompassing all four phospholipid classes in normotensive as well as in the hypertensive state. Several phospholipid species were found common to both states but with remarkable differences in amount in individual states. The ratio of lysophospholipids to total phospholipids is significantly reduced in the hypertensive state. Commensurate with reduced level of lysophospholipids, we found an increased level of lysophospholipase D (Autotaxin) in the hypertensive state. The difference of total phospholipids between young and old was 35.4% in DBA/2J group but 10% in DBA/2J-*Gpnmb*⁺/SjJ mice.

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Conclusion—The significant change of phospholipids including lysophospholipids was found commensurate with the elevated intraocular pressure (IOP).

Keywords

lipidomics; phospholipid; mass spectrometry; aqueous humor; glaucoma

1. Introduction

Glaucoma, a group of progressive and irreversible blinding diseases affect over 65 million individuals worldwide[1]. Elevation of IOP is the major risk factor. The aberrant increased resistance to aqueous humor (AH) outflow at the level of trabecular meshwork (TM) is attributed to elevated IOP in glaucoma[2]. The AH dynamics is altered in most glaucoma if not all glaucoma prior to further pathologic changes. The diurnal fluctuations in IOP have been reported to be increased in primary open angle glaucoma (POAG) as well as in other types of glaucoma[3]. The obstruction to AH outflow at the TM has been found to be segmental in glaucoma[4]. Deposits of proteins and mucopolysaccharide found in the pathologic state is consistent with segmental blockage of AH outflow at the TM[5]. The segmental blockage of AH outflow is not consistent with (or correlates with) acellularity of the TM regions. Careful studies of cadaver glaucomatous donor eyes without prolonged glaucoma shows lack of correlation with loss of cellularity with TM regions with blocked flow. The location of the resistance to TM outflow is not entirely clear as yet but some studies now attribute the most resistance to a layer of Schlemm's canal (SC)[6,7]. The pressure fluctuations or altered fluid dynamics is expected to affect the cell membranes and are likely to be associated with changes in membrane phospholipids. The cells in the irido-corneal angle, ciliary body, TM or that of SC layer have not been investigated in sufficient detail yet either in model systems or in humans to provide insight into the changes in their membranes or their phospholipid composition. The AH would be in a dynamic state for carrying biomolecules secreted by the ciliary body as well as all other living and metabolically active constituents of anterior chamber. Whereas the protein composition of AH in normal control and glaucoma donors has been subject to multiple studies, the lipid composition has been subjected to scant studies[8,9].

Could the composition of AH and, in particular, phospholipids undergo changes during the transition from normotensive to hypertensive state? This could be best addressed by model systems due to ethical considerations precluding AH accumulation from ocular hypertensive individuals without giving them any prior treatment.

In DBA/2J, a mouse of glaucoma, the transition from a normotensive to a hypertensive state is spontaneous[10,11]. A great degree of variability with respect to onset as well as magnitude of elevation in IOP is found in DBA/2J mice. A substantial number of DBA/2J mice develop IOP elevation with open irido-corneal angle without discernable pigmentary dispersion or anterior chamber morphological changes, despite that this strain is attributed to anterior segment dysgenesis and pigmentary dispersion[11]. Owing to these features (spontaneous IOP elevation, with intact anterior chamber and open angle) as well as the availability of a genetically matched mouse strain (DBA/2J-*Gpnmb*⁺-Sj/J)[12], AH of the DBA/2J mice offers a suitable system to study its phospholipid composition. We present

here a comparison of phospholipids of AH among young-normotensive DBA/2J, old - hypertensive DBA/2J mice, young and old DBA/2J-*Gpnmb*⁺/SjJ mice.

2. Method

2.1 Animals

All animal protocols were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) and in accordance with the ARVO statement for use of animals in ophthalmic and vision research. Breeding pairs of DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ mice originally from The Jackson Laboratory (Bar Harbor, ME) were housed in the McKnight vivarium at the University of Miami Miller School of Medicine. Mice ages and gender used are as indicated in individual experiments and n=40 for each time point unless stated otherwise.

DBA/2J mice were distributed into the normotensive or hypertensive group based on IOP. IOP 15mm of Hg was considered as being normotensive and IOP 18mm of Hg was deemed as hypertensive. According to what we found before, the elevation of IOP in DBA/2J mice is asynchronous, so only those with a daily measurement of IOP consistently higher than 17mm of Hg were enrolled. In our previous study we also found that most of the lower IOP (15mm of Hg) was from younger DBA/2J mice (6 months), and the higher IOP (18mm of Hg) was from older DBA/2J mice (8 months). Thus normotensive and hypertensive DBA/2J mice often have an age gap of two months or more. To rule out the effect of aging superimposition on effect of elevated IOP, we used DBA/2J-*Gpnmb*⁺/SjJ mice as an aging control. All the IOP measurements were performed using a TonoLab instrument (Colonial Medical Supplies, NH) two times a day (in the morning and in the afternoon).

2.2 Aqueous Humor Procurement and Lipid Extraction

DBA/2J mice were anesthetized by an intraperitoneal injection of a cocktail of Ketamine and Xylazine. A drop of tetracaine hydrochloride ophthalmic solution (0.5%, Bausch & Lomb, USA) was used to anesthetize the ocular surface prior to AH procurement. Normotensive and hypertensive AH was obtained from the anterior chamber of DBA/2J mice via paracentesis using a syringe (catalog no. 7635-01 Hamilton, Reno, Nevada) with a small 33 gauge removable needle (701RN, catalog no. 7803-05 Hamilton). A total of ~3 µl of AH was collected per mice and used for phospholipid extraction using modified Bligh and Dyer extraction method[13,14]. Phospholipids extracted in the lower organic phase were dried using a Speed-Vac (Model 7810014; Labconco, Kansas City, MO), and then flushed with argon gas for oxidation prevention. Proteins procured from the upper aqueous phase were quantified using a suitable modification of Bradford's method.

2.3 Mass Spectrometric Analysis

Identification and quantification of phospholipids: phosphatidylcholines (PCs), phosphatidylserines (PSs), phosphatidylethanolamines (PEs), and phosphatidylinositols (PIs) were performed. Dried lipid samples were re-suspended in LC-MS grade Acetonitrile: Isopropanol (1:1). Samples were infused with a flow rate of 0.3µl/min, using Triversa

Nanomate (Advion Inc., Ithaca, NY), a chip-based electrospray ionization machine controlled with Chipsoft 8.3.3 version software. PC sprays were performed in positive ion mode with voltage of 1.6 kV and gas pressure of 0.2 psi; while PE, PS, and PI sprays were performed in negative ion mode with voltage of 1.3 kV and gas pressure of 0.6 psi. TSQ Quantum Access Max Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Pittsburgh, PA) controlled by the vendor supplied XCalibur 2.3 software suite was used for the following analysis. Samples were analyzed for 1.00 minute with 0.5 second scans. A full width at half maximum (FWHM) was set at 0.4. The collision gas pressure was set at 1.2 mTorr and argon (auxiliary gas) was set to 5 arbitrary units. The scans ranged from 200 to 1000 m/z. The parameters and quantitative phospholipid standards for quantifying each specific phospholipid were used based on previous studies[14,15]. Briefly, PCs were analyzed in positive ion mode with a parent ion scan (PIS) of 184 m/z at 35 V, PE was analyzed in negative ion mode with a PIS of 196 m/z at 50 V, PI was analyzed in negative ion mode with a PIS of 241 m/z at 45 V, and PS was analyzed in negative ion mode with a neutral loss scan (NLS) of 87.1 m/z at 24 V. The 0.1% formic acid (FA) was used as an additive method for analyses of lipids in the positive ion mode only (that is, for analyses of PC but not for PS, PE or PI). We used a PC standard concentration as 2pmol/μl, and 5pmol/μl, for PE, PS, and PI standard ensuring that the standard peak would be a significant peak (>30% relative abundance) in comparison to the most intense sample peak. A blank [Acetonitrile/Isopropanol (50:50)] was run before and after each sample ensuring absence of sample peaks in the blank. The blanks showed a maximum intensity of E3 in positive and E2 in the negative ion mode respectively. Thus peaks with intensity below or at E3 for PC and at or below E2 for PE, PS, and PI were treated as noise and removed as noise correction. E refers to the relative abundance of ion current or intensity. E is the base (=10) of the exponent of the peaks within the spectra.

2.4 PHAST Gel and Western Blot

AH from DBA/2J mice (normotensive and hypertensive) and DBA/2J-*Gpnmb*⁺/SjJ (younger and older) mice were collected. In addition to a Bradford assay[16], protein concentration of AH was quantified using the PHAST gel fractionation (GE Healthcare Bio-Sciences, Pittsburgh, PA) and densitometry analysis with amino acid quantified bovine serum albumin (BSA) run on separate lanes as a standard[17]. For the Western blot of lysophospholipase D (also known as Autotaxin as well as lysoPLD), about 15 μg of protein from AH was loaded onto a 4–20% gradient gel along with a Sea Blue Plus 2 (Invitrogen Corporation, Carlsbad, CA) protein marker, immunoblotted to a PVDF membrane and subsequently probed with Autotaxin antibody (Cayman Chemical, Ann Arbor, MI). Since presence of Autotaxin has been previously shown in the human AH[18] therefore we used human normal and glaucomatous AH as controls (2.5μg per lane). Coomassie Blue staining of the gel has been used for demonstration of the equal protein amount loading.

2.5 Enzyme-linked immunosorbent assay analysis (ELISA)

ELISA analysis was performed using AH from DBA/2J mice (normo- and hyper-tensive; 1 μg proteins) and from human subjects (control and glaucoma; 2.5 μg proteins) for the quantification of Autotaxin following established protocols[19]. Briefly, AH were added to wells of a 96 well plate (Corning Costar 9018 plate; catalog number 44-2504; eBioscience

Inc., San Diego, CA), and then incubated for 20 min at room temperature, after washing with phosphate buffered saline (PBS), 1% BSA incubation for 1h was used for blocking. The wells were incubated for 1 h with the Autotaxin antibody (Cayman Chemical, Ann Arbor, MI) and subsequently with a secondary antibody coupled with alkaline phosphatase for 1h. The wells were then washed with PBS and incubated with a phosphatase substrate (100 μ l /well) in a diethanolamine buffer pH 7.5. The absorbance was measured at 405 nm on microplate reader (BioTek Synergy HT, BioTek Instruments, Inc., Winooski, VT). Results were presented as mean $\pm$ standard deviation and analyzed using the two tailed one-sample t-test: * $p < 0.05$.

2.6 Data Analysis

Representative spectra from 10 technical repeats were reviewed and selected carefully by two independent observers. Mass spectrometric data was imported into the MZmine 2.9 program, analyzed using a database downloaded from LipidMaps (www.lipidmaps.org) and then subjected to ratiometric quantification in two steps according to established procedures in previous studies[14,20]. 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 ± 1.00 m/z, and a maximum charge of 1. All unique lipid amounts (amount of lipid species pmol/ μ g protein) were subjected to one sample t-test compared to 0.0 and were considered significantly different if the results so indicated ($P < 0.05$). ANOVA tests were performed to determine statistical differences between the lipids species (for common lipids) in the normotensive and hypertensive states. The Scheffe's post hoc test was performed to confirm that they were statistically different ($P < 0.05$).

3. Results

We used DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ mice of 3 and 6 months (referred to as young) and 8–10 months (referred to as old) animals. All older DBA/2J had elevated IOP (> 18 mm of Hg) and all younger DBA/2J had normal IOP (< 17 mm of Hg).

3.1 IOP elevation in DBA/2J mice

The DBA/2J mouse develops an elevated IOP somewhat asynchronously. The cohort of mice used for our measurement showed elevated IOP at a minimum age of 6.5 months or higher. Our current study was not designed to isolate mice > 6.5 months where the IOP remains > 17 mm of Hg. DBA/2J mice usually developed an elevated IOP at 8 or past 8 months (a limited subset may not develop high IOP) of age, while IOP in DBA/2J-*Gpnmb*⁺/SjJ mice remained < 15 mm of Hg from 3–12 months of age (Fig. 1A, B). We measured IOP elevation separately in both genders, consistent with past studies. Slight and statistically insignificant variation was found in the IOP level between male and female mice or in the right or left eye (Fig. 1 C, D).

3.2 Lipid changes between normotensive and hypertensive states

The current analysis is invasive and endpoint was extractive mass spectrometry of normotensive and hypertensive states of DBA/2J mice. The normotensive state was

comprised of mice at 3 and 6 months of age (young DBA/2J) and hypertensive state was represented by 8 and 10 month old mice (old DBA/2J mice). We found a decrease in total AH phospholipids in the hypertensive state (Fig. 2A). A decreased total PS and increased PE were found in the hypertensive state compared to that in the normotensive state (Fig. 2A). The number of total, common and unique lipids in each state that contributed to the total amount has been presented (Fig. 2B). As noted above, the PS and PE underwent a significant decrease and increase in hypertensive state respectively, there were 12 unique PS and PE species in the normotensive state that were below the sensitivity limit of detection in the hypertensive state (Fig. 2B; Supplementary Tables S1, S2). About 25 PS and PE unique species were found in the hypertensive state (Fig. 2B; Supplementary Tables S1, S2). Several phospholipids of four classes were found to be common between normotensive and hypertensive states (Figures 3, 4). Most common lipids showed level differences between normotensive and hypertensive states (Figs. 3, 4).

3.3 Lysophospholipids and lysophospholipase D enzymes between normotensive and hypertensive states

The lysophospholipids are formed from phospholipids when tissues are subjected to a variety of stress or are under elevated hydrolytic conditions. We determined the level of lysophospholipids to total phospholipids in normotensive and hypertensive states respectively (Fig. 5A, B). Ratio of lysophospholipids to total phospholipids is significantly reduced in the hypertensive compared to the normotensive state (~20%) even though the total lipid amount is also reduced in the former. Interestingly the lysophosphatidylserines (LysoPS) showed a significant decrease (~10%), the largest decrease in lysolipid species among all four classes (Fig. 5A, B). The lysoethanolamine (lysoPE) species on the other hand showed a modest increase (>2%) in the hypertensive state. Commensurate with reduced level of lysophospholipids we found an increased level of Autotaxin in the AH of hypertensive animals (Fig. 5C). In parallel, the glaucomatous human AH was also found to have an elevated level of Autotaxin compared to controls (Fig. 5D), consistent with a previous report[18]. The hypertensive animals showed greater amount of proteins in the AH compared to normotensive mouse (Fig. 5E) and is consistent with previous reports of increased AH protein content in human glaucoma. The Western blot analyses of Autotaxin is consistent with the ELISA (Fig. 5F)

3.4 Lipid changes between DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ

Modern day DBA/2J mice are progenies of original D2 mice[12]. DBA/2J-*Gpnmb*⁺/SjJ mice are descendants of a cross between DBA/2J mice and an estranged line of original D/2 (or DBA/2J) which was separated in 1983. Thus they are genetically matched but not identical. We attempted to determine the ratio of total unique to total phospholipids between young and old DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ (Fig. 6A). The DBA/2J-*Gpnmb*⁺/SjJ (Fig. 6A) where the comparison is between the old and young AH alone showed far less variability (10%) compared to DBA/2J mice with far greater variability (35.4%) where the comparison is between old, hypertensive versus young, normotensive (Fig. 6A). These results suggests that aging alone demonstrate less variability in lipids (~10%) than in the DBA/2J (35.4%), where aging and ocular hypertension occurs simultaneously (Fig. 6A). The young versus old ratio of PCs was 38.5 and 31.5% respectively for DBA/2J and

DBA/2J-*Gpnmb*⁺/SjJ (Fig. 6A). For rest of the three classes of phospholipids (PS, PE and PI), the ratio was smaller than 10% in DBA/2J-*Gpnmb*⁺/SjJ, but much higher in DBA/2J group (Fig. 6 A). Since PC lipids showed the largest aggregate difference in unique lipids for young versus old in both DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ species, we looked into individual species (carbon chain length 20–31) in all four groups (Fig. 6B), which did not show any discernable difference in the amount in the hypertensive state for PC species compared to all other three groups.

4. Discussion

Many early clinical observers have documented remission or slowing of glaucoma progression due to lowering of IOP[21]. Pharmacological strategies for lowering IOP involves two approaches, reducing AH generation or increasing AH outflow. The lowering of IOP remains thus far the only proven intervention strategy that confers neuroprotection from glaucomatous optic neuropathy[22,23]. “Irin”, a substance discovered in iris[24] was later identified as a member of eicosanoid subclass of lipids, a prostaglandin, and has been proven to be one of the most pronounced drugs to increase AH outflow and lower IOP[25,26]. Early clinical observers noted a greatly increased quantity of proteinaceous materials, which they called albuminoids or proteids or blood proteids at that time and inorganic salts in glaucoma[27]. They also noted that acute glaucoma AH becomes turbid and coagulates more readily. In acute glaucoma they noted that the increase of albuminoids is greater than in chronic glaucoma[27].

The generation and nature of AH had gone through controversies during early years of investigation about AH. The AH was initially believed to be the dialysate of blood from the ciliary body processes until the experimental evidence proved otherwise[28]. That AH is a dynamic fluid with constant generation and exit rather than a once filled liquid in the eye during birth came to realization around 1560 [29]. AH exit routes were established based on transport of small molecules[30] and slit lamp examination[31]. Generation of AH in the ciliary body was established based on a series of experiments[32–35]. The aqueous phase and most inorganic salts, some peptides and lipids (such as arachidonic acid and prostaglandins) are actively secreted by the ciliary body[36,37]. However, the composition of metabolites including lipids in the AH, their variations in individuals or with physiological states and their origin still remains to be investigated.

Phospholipids of the AH have been previously investigated in a number of organisms[38–40]. However, the major analytical methods posed limitations in unequivocal identification and quantification of lipids in the AH. The chromatographic methods necessitated large amounts of materials and nuclear magnetic resonance (NMR) methods required purified compounds to identify the lipids and quantify them in the AH. The previous analyses[38–40] also was limited and failed to identify a large repertoire of lipids due to their sensitivity limits of detection beyond the threshold necessary for AH lipid detection. Advances in mass spectrometry have eliminated these barriers[20,41]. As noted above, the altered AH dynamics or pressure fluctuations should be experienced by the cell membranes within the anterior segment tissue and likely to cause interchange of phospholipids in the AH. The identification of phospholipids changes in the AH will provide clues to possible molecular

events occurring during the transition from a normotensive to a hypertensive state. Such investigations will also provide tools for future non-invasive assessments of molecular events during normotensive to hypertensive states, for example fluorescent analogs of lipids can show their location and interaction changes.

Advancement in mass spectrometric techniques now allows identification and quantification of lipids even from tiny volumes (1–6µl) of AH rendering studies in small animal models feasible. We have used DBA/2J for AH phospholipid studies. The transition from normotensive to hypertensive states in DBA/2J mice is spontaneous. A conisogenic strain DBA/2J-*Gpnmb*⁺/SjJ which is readily available from Jackson Laboratory, harbor a functional allele of *Gpnmb* and do not develop elevated IOP and glaucoma (although they exhibit a mild iris stromal atrophy) is thought to be a genetically matched control for DBA/2J mice. John and colleagues have described their studies that DBA/2J mice homozygous for wild-type versions of *Gpnmb* and *Tyrp1* gene has been found to lack glaucoma[12]. Thus, the spontaneous transition to hypertensive states and a potential aging control in DBA/2J-*Gpnmb*⁺/SjJ mice, render DBA/2J as an attractive model system to study AH changes in the hypertensive state and potentially deduct age-related metabolic changes. The DBA/2J mice has been attributed to pigmentary dispersion, anterior segment dysgenesis and frequently angle closure with advanced age[42]. In previous studies, slight variation in IOP has also been detected between male and female DBA/2J mice[10,11]. DBA/2J mice demonstrate tremendous variability with respect to onset and severity of IOP elevation as well as pigment dispersion and anterior segment dysgenesis. In our colony, almost all DBA/2J mice that develop elevated IOP between 6.5–9.5 months of age were found to retain open angle, which was assessed using non-invasive optical coherence tomography (Supplementary Figure 1). We also assessed the pigmentary dispersion using a double gonio lens anterior segment microscope and found a subset of DBA/2J mice where IOP is elevated with little or no pigmentary dispersion. We also found mice 8 months old where IOP remains 17 mm of Hg but their numbers were very few. We found many mice in our cohort where IOP is considerably elevated (>18 mm of Hg) at age 6.5 months and a substantial number of these mice had minimal or no pigmentary dispersion.

A decreased total PS and increased PE was found in the hypertensive state compared to that in the normotensive state (Fig. 2A), which is consistent with similar observations in human AH between control and POAG donors[15]. The number of common, total and unique lipids in each state that contributed to the total amount has been presented in Fig. 2B. Our goal is to understand the biological changes associated with the transition from normotensive to hypertensive states that herald in the phospholipid amount changes. A change in interconversions or alteration in the balance between production and consumption may manifest in observed changes. It has been shown that PS and PE can be produced individually through different routes from ethanolamine or choline [43,44]. There is also another predominant mechanism involved in the biosynthesis, which is the conversion between PS and PE [45,46]. PE can be produced by the decarboxylation of PS [47] (Fig. 7). We found a remarkable decrease in PS in hypertensive compared with the normotensive state, and, conversely, an elevated level of PE was found in the hypertensive state, which can be attributed to an altered PS to PE interconversion in the hypertensive state. Our data

presented here showed that PE (21:00/22:00) increased but PS (21:00/22:00) decreased in DBA/2J mice from normotensive to hypertensive state (Fig. 3, 4). PS (18:0/18:1(9Z)) and PS (20:1(11Z)/0:0) were present in normotensive AH but absent in hypertensive AH. While the opposite was found true for PE (18:0/18:1(9Z)) and PE (20:1(11Z)/0:0) (Supplementary Tables S1, S2). The enzymes phosphatidylserine synthase 2 (PSS2) and phosphatidylserine decarboxylase 1 (PSD1) or phosphatidylserine decarboxylase 2 (PSD2) are considered critical enzymes for the conversion of PS to PE in human and mice [45,48,49]. We conjectured that decreased PS and increased PE levels in hypertensive AH could be due to over-activation of one or all of these enzymes (Fig. 7).

Overall the total phospholipid amount was reduced in hypertensive AH (Fig. 2A), similar to that in human POAG AH compared to controls[15]. However, the level of lysophospholipids was also reduced in hypertensive state. The ratio of lysophospholipids to total phospholipids showed a significant 20% reduction in the hypertensive compared to the normotensive state (Fig. 5A, B). The LysoPSs contributed as the most significant fraction to undergo a ~10% decrease, while lysoPEs showed a 2% increase (Fig. 5 A, B) in the hypertensive state. Extraction processes may contribute to differences in total lipids as well as lysophospholipids, however, all mouse samples (normotensive and hypertensive states DBA/2J as well as DBA/2J-*Gpnmb*⁺/SjJ) were extracted using the same procedure and the addition of an internal standard suggested >99% recovery in all extractions. Thus the observed differences are consistent with real differences in samples rather than extraction artifacts. Lysophospholipids are often formed due to stress or elevated hydrolytic conditions. The amount of lysophospholipids decreased in the hypertensive state compared to normotensive state. A decreased generation or increased consumption of lysophospholipids will be explicable for this occurrence. Autotaxin, an extracellular lysophospholipase D in plasma, has been demonstrated can convert lysophospholipids into bioactive lysophosphatidic acid (LPA). Our experimental findings are consistent with an increased level of Autotaxin in the AH of hypertensive DBA/2J mice (Fig. 5C), compared to normotensive DBA/2J. This is commensurate with reduced levels of lysophospholipids and we conjecture this being the reason of decreased amount of lysophospholipids in the hypertensive state (Fig. 5 A, B). This is consistent with elevated Autotaxin level observed in human POAG AH[18].

We have utilized DBA/2J-*Gpnmb*⁺/SjJ mice that does not develop an elevated IOP or glaucomatous neurodegeneration for determination of an approximate effect due to aging. As noted above, DBA/2J-*Gpnmb*⁺/SjJ mice is genetically matched but not genetically identical to DBA/2J mice. The Jackson Laboratory, describes that the DBA/2J-*Gpnmb*⁺/SjJ mice was generated by a wild type allele of *Gpnmb* (which is present in the mutant strain DBA/2J-Dtnbp1sdy/J), into DBA/2J for a minimum of six generations. The sandy mutation (DBA/2J-Dtnbp1sdy/J) occurred in 1983 prior to the appearance of the *Gpnmb*R150X mutation in the Jackson Laboratory DBA/2J production colony. To generate DBA/2J-*Gpnmb*⁺/SjJ strain, mice from DBA/2J-Dtnbp1sdy/J were crossed to “modern” DBA/2J mice, and progeny were selected for the *Gpnmb* wild type allele. The ratio of total unique to total phospholipids between young and old mice showed less variability in lipids due to aging in DBA/2J-*Gpnmb*⁺/SjJ (~10%) than in DBA/2J mice (35.4%) (Fig. 6A). The ratio of

PC was 38.5% and 31.5% (comparison of young and old) for DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ respectively (Fig. 6A). For PS, PE and PI, the ratio for DBA/2J-*Gpnmb*⁺/SjJ mice was less than 10% (Fig. 6A). The PC lipids thus showed the largest difference for young versus old in both DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ species. However, individual PC species with carbon chain length of 20–31 in all four groups, that is young and old DBA/2J as well as that for DBA/2J-*Gpnmb*⁺/SjJ (Fig. 6B), did not show any discernable difference in the hypertensive state in DBA/2J mice, suggesting greater complexity for lipids as any potential contributor towards pathologic state. It is likely that *Gpnmb* is not the only gene or region of DNA that is altered in the DBA/2J-*Gpnmb*⁺/SjJ mice. A near identical control would be a DBA/2J mouse where a wild type *Gpnmb* copy has been knocked in. In addition the function of *Gpnmb* gene product remains unknown and thus an understanding towards its contribution to lipid metabolism. Our investigation has suggested an early and significant difference between AH metabolites including lipid differences between DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ mice. Thus while utilization of DBA/2J-*Gpnmb*⁺/SjJ mice in study design offers additional insight, the best aging control for hypertensive DBA/2J mice is age-matched DBA/2J mice without ocular hypertension. The tremendous heterogeneity in DBA/2J mice allows isolation of very minimal or no pigmentary dispersion (using non-invasive anterior segment microscopic assessment) with open angle (non-invasive optical coherence tomographic assessment) hypertensive mice and age-matched similar non-hypertensive mouse for proper comparison.

Our study presented here provides some important insight, in that, AH phospholipids levels are indeed reduced in the hypertensive state. If they continue to remain reduced in mice with drug treated post-IOP elevation state then that would be similar to what has been observed between human control and POAG AH[15]. We also found an overall decreased PS and increased PE species in hypertensive compared to normotensive state, this is also similar to human control and POAG AH[15] as well as TM[14]. We found decreased lysophospholipids in the hypertensive AH that logically support at least one possibility to have increased level of Autotaxin in the hypertensive AH, which was experimentally found to be true. A proteomic investigation of AH protein levels between control and glaucomatous AH arrived at the same conclusion[18]. The analyses of lysophospholipids provided additional insight into PS being the most significantly decreased class where as a modest increase in lysoPEs. Our comparison of DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ demonstrates larger phospholipid changes in the older DBA/2J mice that also suffer from ocular hypertension compared to young than that in DBA/2J-*Gpnmb*⁺/SjJ suggesting ocular hypertension brings greater changes in addition to aging. However, these studies also suggest need for a better aging model that may serve as an appropriate aging control and comparative studies in model systems for drug induced post-IOP elevation state in addition to normotensive and hypertensive states. Our designs for such studies are under progress.

In summary, our studies present here shows a decreased total phospholipids, decreased and increased phosphatidylserines and phosphatidylethanolamines, reduced lysophospholipids and increased levels of Autotaxin in the AH of hypertensive state. In human POAG AH independent detection of elevated Autotaxin suggests that even in drug induced post IOP elevation state in vivo some of the underlying aberrant biochemical processes may continue.

In the future, localization of lipids identified from profiling studies and the determination of their role in those locations will provide insight into early changes that may be concomitant with onset of pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The total amount of all four phospholipid classes decreased in the hypertensive state.
- Unique lipid species were found in normo- and hypertensive state.
- Lysophospholipids ratio significantly decreased in hypertensive state.
- Significant change of phospho- and lyso-lipids was commensurate with elevated IOP.
- Phospholipidome analyses expand understanding about their role in IOP homeostasis.

Figure 1. Intraocular pressure (IOP) of young and old DBA/2J and DBA/2J-*Gpnmb*⁺/SjJ mice
Average IOP and standard deviation from a week of daily measurements for the indicated month (at the beginning week of the month) has been presented (n = 40). IOP measurements were carried out twice daily. We considered 3 to 6 month younger and 8 to 12 month older group of mice, respectively. Only 8 to 10 month old hypertensive mice were used for lipid analyses. (A) IOP of right eye (OD), and, (B) IOP of the left eye (OS) as indicated. In DBA/2J mice, IOP was 15 mm of Hg before 6 months and increased to >17mm of Hg from 8 months onwards, regardless of gender. In DBA/2J-*Gpnmb*⁺/SjJ mice IOP was always 15 mm of Hg irrespective of age or gender. (C) IOP of right (OD) or (D) left eye indicated as OS of DBA/2J separated gender wise as indicated. Symbols are as indicated.

Figure 2. Protein normalized total phospholipid amount and total species counts (unique, common and total)
(A) Total phospholipids of all four classes: phosphatidylcholines (PC), phosphatidylserines (PS), phosphatidylethanolamines (PE), and phosphatidylinositols (PI), normalized to corresponding aqueous extraction phase total protein between DBA/2J AH normotensive (3–6 months) and hypertensive state (8–10 months) (hollow and filled bars as indicated) respectively (*p < 0.05). (B) The number of total, common, and unique phospholipid species in each class as indicated in normotensive and hypertensive states.

Figure 3. Heat map of common PC and PS species between AH of DBA/2J normotensive and hypertensive states

(A) Fold change in average normalized amount (pmol/ μ g) of common PC species (ratio of hypertensive to normotensive state). (B) Fold change in average amount of common PS species between normotensive and hypertensive phases. Scale bar depicts the range. The actual values are as indicated. The fold change is the ratio relative to normotensive state drawn on a $\log_2$ scale. A negative sign precedes before modulus where the denominator is higher indicating higher amounts are present in normotensive than hypertensive state. Scale bar of fold change used for given lipid species are as indicated. Asterisk indicates a fold change ≥ 5.0 .

Figure 4. Heat map of common PE and PI between AH of DBA/2 mice normotensive and hypertensive states

(A) Fold change in average normalized amount (pmol/μg) of common PE species (ratio of hypertensive to normotensive state). (B) Fold change in average amount of common PI species between normotensive and hypertensive states. The fold change is the ratio relative to normotensive state drawn on a log₂ scale. A negative sign precedes before modulus where the denominator is higher indicating higher amounts are present in normotensive than hypertensive state. Scale bar of fold change used for given lipid species are as indicated. Asterisk indicates a fold change > 5.0.

Figure 5. The protein normalized amounts of lysophospholipids and enzyme lysophospholipase D (Autotaxin) between normotensive and hypertensive state DBA/2J AH
 The protein normalized lysophospholipids in pmol per μg of protein (lysoPC, lysoPS, lysoPE, and lysoPI) and phospholipids (PC, PS, PE and PI) have been represented by grey and black bars respectively as indicated. (A) The total lyso- and phospholipids in the normotensive state. The percentage of lysolipid to the total phospholipids is as indicated above each bar for that lipid class. (B) The total lyso- and phospholipids in the hypertensive state. The percentage of lysolipid to the total phospholipids is as indicated above each bar for that lipid class. (C) The Western blot of DBA/2J AH ($\sim 15\mu\text{g}$ per lane) probed with anti-Autotaxin antibody. A panel with partially transferred gel subjected to Coomassie blue stain to demonstrate equal protein loading has been presented. Normotensive and hypertensive states are indicated. (D) The Western blot probed with Autotaxin antibody using human control and glaucomatous AH. Coomassie blue stained gel after partial transfer has been presented as a second panel. (E) A representative Coomassie blue stained gel of equal volume of DBA/2J AH ($5\mu\text{l}$) from normotensive and hypertensive state as indicated. (F) The enzyme-linked immunosorbent assay (ELISA) using anti-Autotaxin antibody using DBA/2J (two states are as indicated) and human AH (normal and glaucoma) as indicated. The results are

mean $\pm$ standard deviation from three independent experiments. Statistically significant difference (*P 0.05) was derived from a two tailed paired Student's t-test.

Figure 6. The percentage of total unique to total lipids between young and old animals in DBA/2J and DBA/2J-Gpnmb⁺/SjJ mice AH

The percentage of unique lipid species to that of the total species of a class or for all classes has been presented here. The number of unique species between young and old animals for a given class or for all phospholipids was determined. The total unique species (number of unique species in young+ that in the old animals) expressed as a percentage of total species (unique in young+ unique in old+ common species) for each class or that encompassing all classes were determined. (A) The ratio of total unique (see Venn diagram on the side) to total phospholipids between young and old DBA/2J (diamonds) and DBA/2J-Gpnmb⁺/SjJ (squares) has been presented. The total, PC, PS, PE and PI are represented by black, red, blue, aqua and green respectively. (B) Protein normalized amounts (pmol/μg) for unique PC species in DBA/2J and DBA/2J-Gpnmb⁺/SjJ young and old AH indicated. Scale bar indicates the range of amounts.

Figure 7. A schematic diagram of conversion cycle of phospholipids

The enzymes and their localization have been depicted. PS: phosphatidylserine; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PSD1: phosphatidylserine decarboxylase 1; PSD2: phosphatidylserine decarboxylase 2; PEMT: phosphatidylethanolamine N-methyltransferase; PSS1&2: phosphatidylserine synthase 1 and 2; CEPT1: choline/ethanolamine phosphotransferase 1; CDP-Choline: cytidine 5diphosphocholine; CDP-Ethanolamine: cytidine diphosphate ethanolamine.