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Research paper

Phospholipid profiles of control and glaucomatous human aqueous humor

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ABSTRACT

To compare phospholipid (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol) profiles of human control and glaucomatous aqueous humor (AQH).

AQH samples were procured during surgery from human POAG and control subjects ($n = 15$ each). Samples were used following institutional review board approved protocols and adhering to the tenets of the Declaration of Helsinki. Lipid extraction was performed using a modification of the Bligh and Dyer method, protein concentrations were determined using the Bradford's method, and select samples were confirmed with Densitometry of PHAST gels. Lipids were identified and subjected to ratiometric quantification using a TSQ Quantum Access Max triple quadrupole mass spectrometer utilizing precursor ion scan (PIS) or neutral ion loss scan (NLS) using appropriate class specific lipid standards in a two step quantification process.

The comparative profiles of phosphatidylcholines, phosphatidylserines, phosphatidylethanolamines and phosphatidylinositols between control and glaucomatous AQH showed several species common between them. A number of unique lipids in all four phospholipid classes were also identified in control eyes that were absent in glaucomatous eyes and vice versa.

A number of phospholipids were found to be uniquely present in control, but absent in glaucomatous AQH and vice versa. Compared with a previous study of control and POAG red blood cells, a number of these phospholipids are absent locally (AQH).

1. Introduction

The glaucomas are a group of diseases that cause irreversible blindness frequently associated with an elevated intraocular pressure (IOP). Primary open angle glaucoma (POAG) is one of the most common forms. An estimated 60.5 million people worldwide suffer from glaucoma [1]. Elevation of IOP occurs due to pathologically increased resistance to aqueous humor drainage [2]. Lowering IOP is the only proven strategy for protecting the optic nerve from glaucomatous optic neuropathy. In addition to elevated IOP, diurnal fluctuation in IOP has also been found to be a risk factor for glaucoma development [3]. POAG is most frequently associated with increased elevated IOP [4] and more frequent fluctuation in IOP [3]. The aqueous humor is actively produced by ciliary epithelium [5,6] and exits through the structures in the anterior chamber [4]. The elevated IOP is thought to be due to impeded outflow. The outflow is reduced in glaucoma due to increased resistance to outflow at the trabecular meshwork (TM), a filter like structure responsible for fluid flow regulation. The exact factor responsible for this increased resistance in the TM in glaucoma is poorly understood. Changes in the TM extracellular matrix (ECM) [7,8] and the intrinsic elastic modulus of TM both at tissue and cell levels [9–11] have been demonstrated in glaucoma compared to physiologic conditions.

The main drainage pathway of aqueous humor (AQH) lies in the anterior chamber, which consists of the trabecular meshwork and the Schlemm's canal [12]. Two mechanisms of lowering IOP are decreasing the production of aqueous humor or increasing the outflow of aqueous humor. Aqueous outflow can be increased through two known pathways – the trabecular meshwork or the conventional, and the uveosceral pathway. A number of pharmacological factors

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modulate aqueous outflow, for example, β-blockers and carbonic anhydrase inhibitors are known to decrease aqueous humor production. Another group of pharmacological factors, single classes of lipids known as prostaglandins were originally found to exist endogenously. These lipids were discovered in the iris and as a result were named irin [13,14]. Although they were found to have IOP lowering ability by increasing aqueous humor outflow via the uveoscleral pathway, they also have significant side effects [13–18]. Further research into their mechanism of action provided insight into the existence of prostaglandin receptors in the uveoscleral pathway with greater concentration compared to that in the conventional pathway [19].

As noted above, the reason behind increased TM resistance in POAG remains poorly understood. The endogenous factors that regulate TM cell behavior and homeostasis also remain poorly understood. Importantly the presence of endogenous lipids in the AQH other than prostaglandins remains to be investigated. TM is constantly bathed in AQH and therefore it is plausible that factors in the AQH may play a modulatory role for TM cell behavior. Existence and the identification of different classes of endogenous lipids in the AQH largely remain to be investigated. A significant compositional change in the AQH may change the state of TM cell behavior and health of TM tissue. Until recently, suitable methods applicable to mixed lipids in very low amounts present in the AQH were critical barriers for their identification and simultaneous quantification. Recent developments in tandem mass spectrometry, bioinformatics and lipid databases have largely eliminated these barriers [20–24]. We present the results of profiling for four phospholipids classes namely: phosphatidylcholines (PCs), phosphatidylserines (PSs), phosphatidylethanolamines (PEs) and phosphatidylinositols (PIs) in the aqueous humor and their comparative analyses between glaucomatous and control donors.

2. Materials and methods

2.1. Aqueous humor procurement

Control and POAG AQH were procured during glaucoma and cataract surgeries following institutional review board approved protocols and principles outlined in the Declaration of Helsinki were adhered to. A total of 15 control and 15 glaucomatous AQH samples (Supplemental Tables S1 and S2) were included for these studies. All AQH samples were immediately stored at −80 °C until time of use. The mean age of donors was 69.8 ± 9.5 years and both genders were included for these studies.

2.2. Lipid extraction

Aqueous humor samples were subjected to extraction of lipids using suitable and minimal modification of Bligh and Dyer method [25,26]. The lower organic phase containing the extracted lipids was isolated and solvent dried with a Speed-Vac (Model 7810014; Labconco, Kansas City, MO). Samples were subsequently flushed with argon gas to prevent oxidation. Proteins recovered from the corresponding upper aqueous phase were quantified using Bradford's method [27]. A subset of protein samples were also subjected to densitometric quantification using bovine serum albumin (BSA) as a standard (amino acid quantified) after electrophoretic separation on a PHAST (GE Healthcare Bio-Sciences AB, Sweden) gel system [28]. We also repeated protein estimations using an amino acid analyzer after overnight digestion in hydrochloric acid following previously published protocols [29]. The protein amounts determined using amino acid analyzer was utilized in normalization of lipids per amount of proteins. In order to determine and ensure extraction efficiency, ovine wool cholesterol (molecular mass 386.7; catalog no. 700000; Avanti Polar Lipids, Albaster, AL) [30] was premixed with AQH prior to extraction. All extractions and subsequent handling was made using glass vials to avoid contaminating impurities.

2.3. 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 Xcaliber 2.3 software package. Extracted lipids were dried and re-suspended in LC–MS grade acetonitrile: isopropanol (1:1). Samples were infused with a flow rate of 10 μl/min and analyzed for 1.00 min with a 0.500 s scan. Scans typically ranged from 200 m/z to 1000 m/z unless specified otherwise. A peak width was set at 0.7 and collision gas pressure was set at 1mTorr. Sheath gas (nitrogen) was set to 20 arbitrary units. Auxiliary gas (Argon) was set to 5 arbitrary units. For analyses of different phospholipid classes collision energy, spray voltage, and ion mode were set based on previous studies [20,24,26,31]. Control and POAG AQH, $n = 15$ each, were utilized for each of the four phospholipid classes analyzed. Class specific lipids were quantified using class specific quantitative lipid standards in two steps [20]. In the first step the most abundant lipids of the class were quantified using a class specific lipid standard and in the second step, the quantification values determined using the first step were used for quantification

Table 1
Unique phospholipid species in control and glaucomatous aqueous humor.

	m/z[a]	Average lipid amount (pmol per species/μg protein)	Donor frequency	LipidMaps ID[b]	Pubchem ID
Phosphatidylethanolamines					
Control aqueous humor					
PE-NMe2(O-16:0/O-16:0)	692.55	0.002	2	LMGP02040007	14714332
PE(18:1(9E)/18:1(9E))	743.46	25.085	7	LMGP02010039	14714000
Glaucomatous aqueous humor					
PE(15:0/20:3(8Z,11Z,14Z))	727.53	26.092	4	LMGP02010466	123061712
Phosphatidylinositols					
Control aqueous humor					
PI(20:2(11Z,14Z)/22:4(7Z,10Z,13Z,16Z))	938.93	0.119	6	LMGP06010555	123065574
Glaucomatous aqueous humor					
PI(16:1(9Z)/22:4(7Z,10Z,13Z,16Z))	884.56	0.008	6	LMGP06010193	123065212
PI(O-18:0/0:0)	587.67	0.003	3	LMGP06060002	123066196

[a] A representative mass/charge ratio is presented (variations in m/z was reconciled by MZmine 2.9).
[b] The lipid species identification is based on LipidMaps database, used as a*.csv file for bioinformatic analyses with MZmine 2.9 program. Average standard non-normalized dataset is presented here.