Investigative Ophthalmology & Visual Science, Vol. 32, No. 3, March 1991

Membrane Phospholipids of the Ocular Tunica Fibrosa

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The authors compared porcine corneal and scleral membrane phospholipids determined with use of ^{31}P nuclear magnetic resonance (NMR). These tissues make up the tunica fibrosa (outer coat) of the eye. Since the sclera, unlike the cornea, does not possess an epithelium or an endothelium, comparative analysis of these tissues included examination of the cornea with and without its epithelium and endothelium. The phospholipids quantified include: phosphatidylcholine, lysophosphatidylcholine, phosphatidylcholine plasmalogen, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylethanolamine plasmalogen, phosphatidylserine, sphinogmyelin, phosphatidylinositol, phosphatidylglycerol, cardiolipin, and an uncharacterized phosphatide that accounts for 1.5%–3.5% of the detected phospholipids. Metabolic indices, comprised of individual or grouped metabolites, were calculated to further compare and contrast metabolites and to provide pathway-specific metabolic interrelations for each set of phospholipids from cornea and sclera. Significant differences exist between the corneal stroma and the sclera in 9 of the 12 phospholipids, whereas differences exist between the whole cornea and the sclera in 7 of the 12 phopholipids. Invest Ophthalmol Vis Sci 32:625–632, 1991

The ocular tunica fibrosa is the outermost coat of the eye and consists principally of collagen. The anterior one-sixth of the tunica fibrosa is composed of the transparent avascular cornea and the posterior five-sixths is composed of the white, minimally vascularized sclera. In addition to the cornea refracting light that enters the eye and the sclera that limits the entrance of light, the cornea and sclera provide a tough ocular coat and maintain the shape of the eye.

There is an exigency for further development of biochemical methods used to distinguish between benign and malignant processes to reduce the urgency for clinical intervention. There is also a mandate to develop methods used to determine disease progression and pathogenesis–especially in those cases where methods of pathologic and clinical evaluation are inconclusive. The advent of in vivo magnetic resonance imaging (MRI) and the ultimate potential for in vivo magnetic resonance spectroscopy (MRS) addresses these demands. Thus, there is a need to document the resonance signals that contribute to the magnetic resonance spectrum and to identify those significant spectral differences seen between benign and malignant ocular tissues.

This study was done to determine the similarities and differences in the phospholipid metabolite profiles of the tissues that make up the tunica fibrosa (cornea and sclera) of the eye with the use of ^{31}P NMR and to develop and use metabolic indices to compare and contrast pathway-specific metabolic interrelationships for phospholipids.

Materials and Methods

Corneal and Scleral Samples

Freshly enucleated porcine eyes (n = 40) were obtained from a local abattoir and maintained in physiologic saline solution. Analysis of cornea and sclera was conducted on 10 samples that consisted of four tissue specimens each. After the surface of the globe is irrigated with fresh physiologic saline, extraocular tissues, eg, conjunctiva, Tenon's capsule, and extraocular muscles, were excised from the globe surface with a #15 Bard Parker scalpel (Becton, Dickinson and Co., Rutherford, NJ). Corneas were excised with a #15 Bard Parker scalpel, the anterior chamber was entered, and the cornea was cut circumferentially with fine corneal—scleral scissors. The excised corneas were then rinsed in fresh physiologic saline and frozen in liquid nitrogen. The remainder of the globes were quartered and the optic nerve and nerve head excised. The iris and ciliary body were removed, and the chorioretinal tissue was scraped and removed with a #15 Bard Parker scalpel. The specimens were then immersed in liquid nitrogen. No more than 3.5

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Supported by grants from the Max Goldenberg Foundation and Pfizer, Inc.

Submitted for publication: September 13, 1989; accepted October 9, 1990.

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min elapsed from the beginning to the end of the surgical procedure(s) and subsequent immersion of specimens into liquid nitrogen.

Since the cornea is composed of two surface layers (anterior epithelium and posterior endothelium), that are not seen on the sclera, a comparative study of these tissues that make up the tunica fibrosa must include analyses with and without these layers. Thus, an additional group of porcine corneas were designated for dissection, which included removal of the epithelial and endothelial layers. Since removal of these layers permits the potential for metabolic damage to the underlying stromal tissue, it is prudent to use an additional control in studies with such dissection. A randomized sampling technique was used: the corneal epithelium and endothelium tissues were measured and, along with corneal stroma, compared with the whole cornea. Metabolic damage to the stromal tissue could be estimated with a standard summation technique,[1] and fidelity of the dissection technique could be verified. Quantitatively, the addition of the membrane phospholipids of these three tissues, with the use of the techniques previously described[1], showed a close correlation between their totals and those analyses of the whole cornea (Table 1). The dissection method did not significantly alter stromal phospholipid concentrations.

On corneas designated for stromal samples, the epithelium was scraped and denuded with a #15 Bard Parker scalpel, and immediately after removal, the epithelial cell mass was frozen in liquid nitrogen. The underlying stroma was wiped with a cotton-tipped applicator to completely remove the rest of the epithelial cells. Corneas were then excised from the globe with a scalpel as described in the above procedure for corneal excision. The rest of the cornea, composed of stroma and endothelium, was placed endothelial side-up on a Petri dish of fresh physiologic saline. With the scalpel, the endothelium was denuded from the surface of Descemet's membrane and immediately immersed in liquid nitrogen. To ensure complete removal of the endothelium, a cotton-tipped applicator was used to wipe the surface of Descemet's membrane. Histologic sections of randomly selected corneas, prepared as described[1], showed rare epithelial and endothelial cells. Immediately after removal of epithelial and endothelial cells, the porcine stroma was immersed in liquid nitrogen. No more than 2 min elapsed from the beginning to the end of the above surgical procedures and subsequent immersion of the epithelial, endothelial, and stromal samples in liquid nitrogen. Analysis of epithelium and endothelium was conducted on three samples of 30 tissue specimens each; analysis of corneal stroma was conducted on 10 samples of four tissue specimens each.

Phospholipid Extraction

A modified Folch extraction was performed on 10 samples of each tissue.[2] Liquid-nitrogen-frozen tissues were pulverized to a fine powder with a liquid-nitrogen-chilled stainless steel mortar and pestle maintained in a liquid nitrogen bath. Tissues were weighed and extracted by 20 weight-volumes (g-ml) of chloroform/methanol, 2/1 v/v. Each sample, with one liquid phase, was filtered. The liquid extract was washed with 0.2 volume 0.1 M KCl and allowed to separate thoroughly (about 24 hr). The chloroform phase was recovered and evaporated with a rotary evaporator at 37°C. Lipid extract quantities of 0.01–10 mg were used per analysis. No further refinement was required for tissue extract phospholipid profiles.

Phospholipid analytical reagent preparations of high generic purity or known composition were obtained from Sigma Chemical Co. (St. Louis, MO), P-L Biochemicals, Inc. (Milwaukee, WI) and Life-Science Resources (Milwaukee, WI).

The analytical medium for the ^{31}P magnetic resonance spectroscopic phospholipid profile analysis was generated from two reagents: reagent-grade chloroform and reagent-grade methanol that contained dissolved cesium EDTA salts at pH 6.0. The cesium salts of EDTA were generated by the titration of a 0.2 M suspension of the free acid with the hydroxide to a pH of 6.0, at which point the EDTA was in solution. (The EDTA was titrated from the free acid with care so that, at the end point, only the required number of cation equivalents were seen for each equivalent of EDTA, and the solution contained no excess extraneous salts, such as chlorides. It is important not to compromise the cation scrubbing action of the EDTA with excess cations.) One milliliter of the EDTA solution was dissolved in 4 ml methanol to prepare the final methanol reagent. In sealed bottles, the preparation is stable indefinitely at 24°C.[3]

For ^{31}P magnetic resonance analysis, 0.01–10 mg of prepared sample lipid is dissolved in 2.0 ml reagent chloroform that contains 5% benzene-d_6. (The prepared sample lipids must be free of excess solvents and must not be contaminated with excessive amounts of paramagnetic cations or free radicals. The benzene-d_6 provides the reference signal for magnetic resonance field-frequency stabilization.) To this solution, 1 ml of the methanol reagent is added, and the mixture is gently stirred. Two liquid phases will be obtained–a major chloroform phase and a small water phase. The sample is placed in a 10-mm NMR sample tube, where it separates within 1 min. The sample tube turbine is adjusted so that only the chloroform phase is sensed by the receiver coil of the

NMR spectrometer. Precipitated solids should not be evident.

^{31}P NMR Spectroscopy

The NMR spectrometer used in this investigation was a multinuclear GE 500 NB (GE Medical Systems, Fremont, CA) system that operates at 202.4 MHz for ^{31}P. This system is interfaced to an Oxford Instruments 500/52 (Oxford Instruments Ltd., Oxford, England) magnet and cryostat that has an operating magnetic field of 11.75 Tesla. The spectrometer is equipped with deuterium field-frequency stabilization and an automatic field-homogeneity adjustment capability that continually adjusts the room-temperature shim coils of the spectrometer to improve field homogeneity during data acquisition. Because the resonance signals obtained with the described phospholipid assay procedure are narrow, these last two features and an ambient temperature stable to within 1° C are essential for the maintenance of signal resolution during long-term signal averaging of dilute samples.

For phospholipid analysis, analytical samples in standard 10-mm (spinning) NMR sample tubes were spun at 16 Hz during the analytical period. PCA-extract samples were filtered through glass wool into a microcell assembly that was not spun during the analytical period. Samples were analyzed with proton broad-band decoupling to eliminate ^{1}H-^{31}P NMR multiplets. Under these conditions, each spectral resonance corresponds to a single phosphorus functional group that represents a single phosphatic metabolite or generic phospholipid species.

Chemical shift data are reported relative to the usual standard of 85% inorganic orthophosphoric acid;[4,5] however, the primary internal standard was a naturally occurring phospholipid derivative, glycerol 3-phosphorylcholine glycerol 3-phosphorylcholine [GPC]; chemical shift, −0.13 δ). GPC is soluble in chloroform solutions, although it can be extracted with water, and it exhibits a chemical shift that is solvent-independent under the usual analytical circumstances used in NMR.[6] Chemical shifts adhere to the convention of the International Union for Pure and Applied Chemistry and are reported in the field-independent units of δ.

Spectrometer conditions used for detection of phospholipids were: pulse sequence, one pulse; pulse width, 18 μ sec, which corresponds to a 45° spin-flip angle· acquisition delay, 500 μ sec; cycling delay, 500 m sec; number of acquisitions, 512 for whole cornea, stroma, and sclera, and 20,000 for epithelium and endothelium; number of data points per free-induction decay, 4096; acquisition time, 1.02 sec; sweep width, ±1000 Hz. The total time per analysis was 13 min. In addition, a computer-generated filter time constant that introduces 0.6 Hz line broadening was applied. Data reductions that included peak area and chemical shift measurements and spectral curve analysis were calculated with the computer of the spectrometer.

With the NMR scan conditions, relative saturation effects among the phospholipids is not detectable, although this may change under different conditions. To compensate for relative saturation effects among the various phosphorus signals detected in a single ^{31}P NMR spectroscopic profile, the NMR spectrum must be standardized against measured amounts of tissue-profile metabolites wherever these are known. The procedures needed to carry out this calibration, so that an accurate quantitative measurement is obtained from the ^{31}P NMR spectral profile, have been described.[3,7,8]

Data Analyses

Phospholipid concentrations in mean relative mole percentages were computed[9] for all detected resonances in the corneal and scleral tissues. Initially, a one-way analysis of variance was performed. For those resonances where statistically significant differences were seen ($F < 0.05$), a Scheffé range test was carried out with a probability < 0.05 accepted as significant. In those instances that involved tissue groups with missing values, where the Scheffé range test could not be applied, the student t-test was used, with the probability of the t value being based on a two-tailed test of significance.

In addition to the measured differences in tissue biochemical levels, the analyses included metabolic indices to which the one-way analysis of variance and the Scheffé range test was applied. These indices compare and contrast metabolites or groups of metabolites and provide more pathway-specific metabolic interrelations for discussion.[10] For purposes of statistical analysis, missing values represent resonance signals that lie below the limits of detection.

Metabolic indices for the phospholipids are defined as follows: Plas A, PE plas + PC plas; Plas B, PE + PC; Unsat, (Plas A)/(Plas B); Unsat C, (PC plas)/(PC); Unsat E, (PE plas)/(PE); Lecithin, PC + PC plas; Chol A (all choline-containing P-lipids), PC + PC plas + SPH; Chol B (all other P-lipids detected exclusive of lyso derivatives), PI + PS + PE + PE plas + CL + PG; Choline, (Chol A)/(Chol B); Chol/Eth, Lecithin/(PE + PE plas); Outside, PC + SPH; Inside, PS + PE; Leaflet, (Outside)/(Inside); (PC)/(PE); (SPH)/(PS); An/Neut (ratio of anionic P-lipids to neutral-ionic P-lipids), (PI + PS + CL + PG)/(PC + PC plas + SPH + PE + PE plas); Lyso A, LPC + LPE; Lyso B, PC + PE; Lysprop, (Lyso A)/(Lyso B); (LPC)/(PC); (LPE)/(PE).

Results

The ^{31}P NMR phospholipid analyses from whole cornea, corneal stroma (cornea without epithelium or endothelium), and sclera yield well-resolved signals (Fig. 1) and quantitative differences among the tissues (Table 2). Curve resolution was used to analyze the region of signal congestion in the chemical shift range between 0.20 δ and −0.20 δ (Fig. 2). Twelve phospholipid signals were detected and quantitated for porcine whole cornea, corneal stroma, and sclera (Table 2).

An unidentified phospholipid was detected (Fig. 1 and Tables 1 and 2). This phospholipid is spectroscopically identical to the major phospholipid detected in the human lens, which had been identified by thin-layer chromatographic techniques as sphingomyelin.[11] By ^{31}P spectroscopy, this phospholipid is not identified as sphingomyelin. Further, it cannot be saponified to yield phosphodiester polar head-group fragments, which suggests that it is either a phosphorylsphingosine derivative or a plasmalogen. It is not, however, the ethanolamine plamalogen, since the unidentified phospholipid is ninhydrin negative[11] and exhibits a ^{31}P chemical shift that is distinct from that of the ethanolamine plamalogen (Tables 1 and 2). Full characterization awaits the unidentified phospholipid isolation and functional group analysis.

Relative to the whole cornea, the phospholipid profile of the stroma differs, with an elevation in PC, PE plas, and SPH and a diminution in PE, PS, U, PG, and CL, and differs from the sclera with an elevation in PC plas, PE plas, PS, and U and a diminution in PC, PE, PI, and PG. Relative to the stroma, the profile of the sclera differs, with an elevation in PC plas, LPE, PE plas, PS, and U, and a diminution in PC, LPC, PE, and PI.

Relative to whole cornea, the phospholipid indices of the stroma differ with respect to Lecithin, Chol A, Chol B, Choline, Chol/Eth, Outside, Inside, Leaflet, PC/PE, SPH/PS, and An/Neut, and differ from the sclera with respect to all the indices, except Leaflet, SPH/PS, An/Neut, Lyso A, Lysprop, and LPC/PC

Table 1. Phospholipid mole percentages of porcine corneal tissues determined by ^{31}P NMR

	Mole-percent			
Phospholipid	*Epithelium*	*Stroma*	*Endothelium*	*Whole cornea*
PC	38.2	52.5	55.5	48.1
LPC	0.0	1.4	0.0	1.1
PC plas	0.6	1.6	2.9	1.6
PE	17.7	8.4	9.6	10.5
LPE	2.1	0.8	0.0	1.4
PE plas	7.1	8.4	11.6	7.6
PS	10.0	7.2	4.5	9.4
SPH	15.1	13.7	4.3	11.1
PI	5.6	4.7	7.2	4.9
U	0.0	0.0	0.0	1.5
PG	0.4	0.0	0.0	0.6
CL	3.2	1.2	3.8	2.1

Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PC plas, phosphatidylcholine plasmalogen; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PE plas, phosphatidylethanolamine plasmalogen; PS, phosphatidylserine; SPH, sphingomyelin; PI, phosphatidylinositol; U, uncharacterized phospholipid; PG, phosphatidylglycerol; CL, cardiolipin.

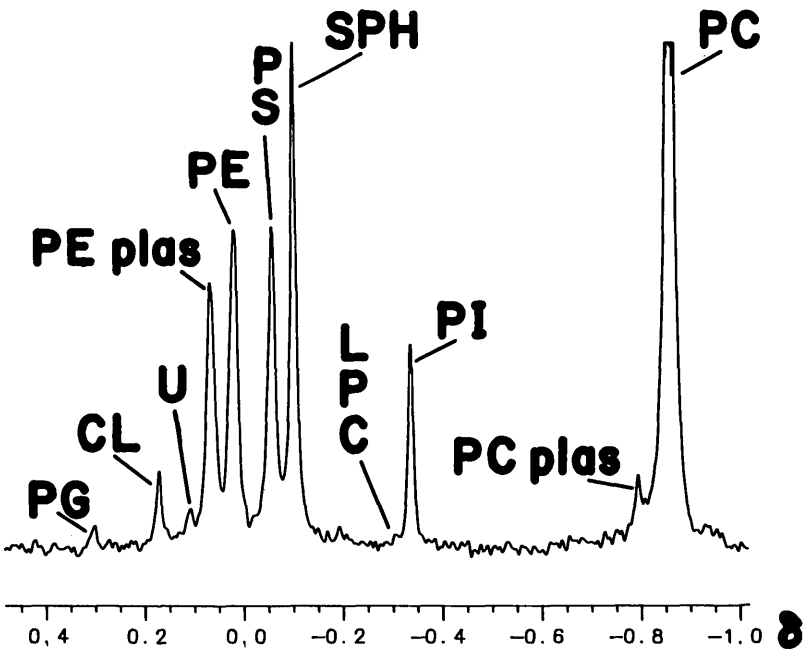

Fig. 1. ^{31}P NMR membrane phospholipid profiles of the porcine whole cornea: phosphatidylcholine (PC), phosphatidylcholine plasmalogen (PC plas), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), phosphatidylethanolamine plasmalogen (PE plas), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), sphingomyelin (SPH), phosphatidylinositol (PI), an uncharacterized phospholipid (U), cardiolipin (CL). In this illustration, the PC signal is off scale so that the minor phospholipid signals can be observed.

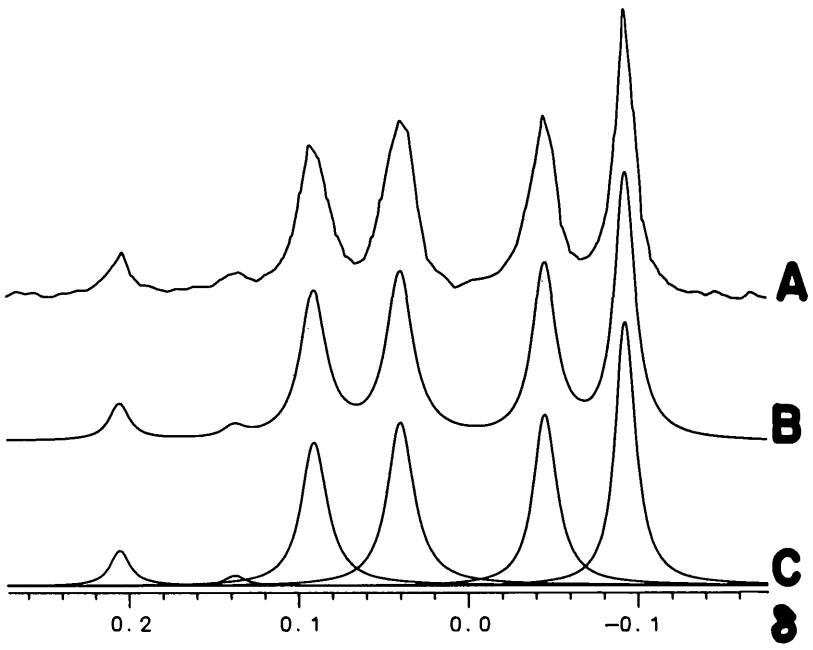

Fig. 2. Curve resolution of the ^{31}P NMR phospholipid spectral region between 0.20 δ and −0.20 δ. The curve resolution analysis is a component subroutine of the NMR spectrometer's operating software. Its use improves the precision of data obtained in crowded regions of the spectrum. Trace A is the plot of the experimental spectrum, B, the trace of the calculated spectrum, and C, the plots of the individual component lines.

(Table 3). Relative to the stroma, the sclera differs, with respect to all indices except Inside, PC/PE, Lyso A, and LPC/PC.

Discussion

Interest in phospholipid ^{31}P NMR analysis arises from its potential as a profiling tool for the determination and characterization of tissues, particularly for the characterization of diseased relative to healthy tissues.[7,10,12-14] Phospholipid ^{31}P NMR spectroscopy offers the potential for the examination of changes in tissue function that are associated with altered membrane phospholipid profiles and for evaluation of the consequences of tissue dysfunction on membrane biochemistry. The phospholipids may be key to the disorders of the cornea[16] and sclera. Although there are studies that report corneal[17-22] and scleral[17,20,21,23] phospholipid values, analyses were limited to detection of a few metabolites when compared with this analytical technique. In this study, phospholipids have been quantitated in the whole cornea, the stroma, and the sclera to provide baseline data for eventual work that involves pathologic tissue.

Analyses of corneal and scleral tissue phospholipids were performed by thin-layer chromatography.

Table 2. ^{31}P chemical shifts and mole-percentages of porcine corneal and scleral tissue membrane phospholipids

Phospholipid	*Chemical shift (δ)*	*Mole-percent (Means ± SE)*		
		Whole cornea	*Stroma*	*Sclera*
PC	−0.84	48.1 ± 0.81	52.5 ± 0.34*	37.6 ± 0.31†‡
LPC	−0.28	1.1 ± 0.22	1.4 ± 0.08	0.7 ± 0.16‡
PC plas	−0.78	1.6 ± 0.10	1.6 ± 0.14	3.1 ± 0.20†‡
PE	−0.01	10.5 ± 0.41	8.4 ± 0.20*	6.3 ± 0.31†‡
LPE	0.43	1.4 ± 0.37	0.8 ± 0.12	2.7 ± 0.51‡
PE plas	0.07	7.6 ± 0.28	8.4 ± 0.15*	16.5 ± 0.76†‡
PS	−0.05	9.4 ± 0.44	7.2 ± 0.19*	11.3 ± 0.43†‡
SPH	−0.09	11.1 ± 0.24	13.7 ± 0.40*	12.6 ± 0.27
PI	−0.37	4.9 ± 0.25	4.7 ± 0.13	3.7 ± 0.19†‡
U	0.10	1.5 ± 0.16	—*	3.7 ± 0.35†‡
PG	0.52	0.6 ± 0.10	—*	—†
CL	0.18	2.1 ± 0.06	1.2 ± 0.05*	1.9 ± 0.24

Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PC plas, phosphatidylcholine plasmalogen; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PE plas, phosphatidylethanolamine plasmalogen; PS, phosphatidylserine; SPH, sphingomyelin; PI, phosphatidylinositol; U, uncharacterized phospholipid; PG, phosphatidylglycerol; CL, cardiolipin.

* Corneal stroma compared to whole cornea ($P < 0.05$).
† Sclera compared to whole cornea ($P < 0.05$).
‡ Sclera compared to corneal stroma ($P < 0.05$).

Table 3. Porcine cornea and sclera tissue membrane phospholipid metabolic indices

Phospholipid metabolic index	*Index value (Means ± SE)*		
	Whole cornea	*Stroma*	*Sclera*
Plas A	9.2 ±0.27	10.0 ±0.15	19.6 ±0.59†‡
Plas B	58.6 ±1.1	60.9 ±0.4	43.8 ±0.4†‡
Unsat	0.16 ± 0.004	0.16 ± 0.003	0.45 ± 0.01†‡
Unsat C	0.03 ± 0.002	0.03 ± 0.003	0.08 ± 0.005†‡
Unsat E	0.72 ± 0.03	1.00 ± 0.02	2.65 ± 0.18†‡
Lecithin	49.7 ±0.87	54.1 ±0.41*	40.7 ±0.37†‡
Chol A§	60.8 ±0.71	67.9 ±0.52*	53.3 ±0.22†‡
Chol B§	35.1 ±0.70	29.9 ±0.44*	39.6 ±0.94†‡
Choline§	1.74 ± 0.05	2.27 ± 0.05*	1.35 ± 0.04†‡
Chol/Eth§	2.76 ± 0.08	3.24 ± 0.08*	1.80 ± 0.09†‡
Outside§	59.2 ±0.64	66.2 ±0.48*	50.2 ±0.17†‡
Inside§	19.9 ±0.74	15.7 ±0.31*	17.5 ±0.52†
Leaflet§	2.99 ± 0.12	4.24 ± 0.11*	2.87 ± 0.08‡
PC/PE§	4.59 ± 0.16	6.27 ± 0.16*	6.03 ± 0.29†
SPH/PS§	1.19 ± 0.06	1.90 ± 0.09*	1.12 ± 0.03‡
An/Neut	0.22 ± 0.009	0.16 ± 0.003*	0.22 ± 0.004‡
Lyso A	2.6 ±0.57	2.3 ±0.14	3.4 ±0.06
Lyso B	58.6 ±1.1	60.9 ±0.4	43.9 ±0.5†‡
Lysprop	0.04 ± 0.010	0.04 ± 0.002	0.08 ± 0.015‡
LPC/PC	0.02 ± 0.005	0.03 ± 0.002	0.02 ± 0.004
LPE/PE	0.15 ± 0.043	0.10 ± 0.013	0.43 ± 0.09†‡

Index definitions: Plas A (PE plas + PC plas); Plas B (PE + PC); Unsat [(Plas A)/(Plas B)]; Unsat C [(PC plas)/(PC)]; Unsat E [(PE plas)/(PE)]; Lecithin (PC + PC plas); Chol A [(all choline-containing P-lipids), PC + PC plas + SPH]; Chol B [(all other P-lipids detected exclusive of lyso derivatives), PI + PS + PE + PE plas + CL + PG]; Choline [(Chol A)/(Chol B)]; Chol/Eth [Lecithin/(PE + PE plas)]; Outside (PC + SPH); Leaflet [(Outside)/(Inside)]; An/Neut [ratio of anionic P-lipids to neutral-ionic P-lipids, (PI + PS + CL + PG)/(PC + PC plas + SPH + PE + PE plas)]; Lyso A (LPC + LPE); Lyso B (PC + PE); Lysoprop [(Lyso A)/(Lyso B)].

* Corneal stroma compared to whole cornea ($P < 0.05$).

† Sclera compared to whole cornea ($P < 0.05$).

‡ Sclera compared to corneal stroma ($P < 0.05$).

§ Indices of the phosphatide metabolite set.

In general, these data have similarities to the data of this study, but certain assumptions must be made that preclude reasonable comparisons, eg, that the phophatidylethanolamine designated in an earlier study[21] includes the contribution of PE plas, and the unidentified phospholipid F of this earlier work is PC plas. Moreover, the ^{31}P NMR methods of this study have shown the presence of previously uncharacterized phospholipids in significant quantities (Tables 1 and 2). All these factors render problematic, rigorous quantitative comparisons to earlier work.

The metabolic indices (Table 3) facilitate the interpretation of the metabolites detected by ^{31}P NMR (Table 2). The indices are useful to analyze aspects of phosphatide metabolism that vary with tissue metabolism and to identify those specific enzymatic systems responsible for the observed variation in metabolite levels. For example, with the exclusion of the corresponding lyso derivatives, the metabolic indices Plas A, Plas B, Unsat, Unsat C, and Unsat E are a measure of the relationship of the more-reduced enol-ether-containing plasmalogens to their more oxidized ester-containing analogs. Plas A represents the sum of the detected plasmalogen, PE plas + PC plas in the current case. Plas B represents the sum of their ester-containing analogs, PE + PC, and Unsat represents the ratio of these, with Unsat C and E representing the more restrictive cases that compute the separate ratios that correspond with the choline and ethanolamine derivatives, respectively. Collectively, these indices reflect the contribution of the metabolic pathways responsible for the metabolism of the ester-containing phosphatides and their corresponding enol ethers.

In all instances, the sclera shows a greater plasmalogen content relative to the corneal tissues examined, with the ethanolamine derivative as the primary phospholipid responsible for the tabulated differences (Table 2). From these points of view, the stroma is similar to the whole cornea, and the epithelium and endothelium contribute little variation to the overall corneal phospholipid profile.

The ratio of Plas A to Plas B (Unsat) is a measure of the balance resident within the total ester/enol-ether metabolic system. The magnitudes of the Unsat indices indicate the relative position of the metabolic equilibrium points. When considering this, the metabolic balance of the sclera is in the direction of the enol-ether relative to those of the corneal tissues. The scleral system is relatively reduced with respect to corneal tissues because it contains more of the enol-ether-containing plasmalogens and less of the ester-containing analogs.

The restricted Unsat indices, Unsat C, the choline index, and Unsat E, the ethanolamine index, identify the contribution each generic phospholipid system

makes to the overall metabolic balance point. A priori, it might be anticipated that the ethanolamine phosphatide system is primarily responsible for the position of the balance point, particularly because the relative concentrations of the ethanolamine components are high. Moreover, this system varies significantly between the sclera and corneal tissues. The choline index, however, also varies significantly between sclera and the corneal tissues. Both indices nearly triple in magnitude, which indicates parallel degrees of plasmalogen production for both choline and ethanolamine phosphatides in sclera relative to the corneal tissues.

Lecithin represents the sum of detected phosphatidylcholines exclusive of the lyso derivatives (PC + PC plas). This index is lower in the sclera relative to the corneal tissues, and reflects, primarily, the relatively lower PC content of the stroma and not its elevated PC plas content. Among the corneal tissues, the index is highest in the stroma and is attributable to an elevated PC content; the PC plas content is the same among the corneal tissues.

The indices Chol A, Chol B, Choline, Chol/Eth, Outside, Inside, Leaflet, PC/PE, and SPH/PS primarily address the issue of membrane leaflet asymmetry.[24] Chol A is the sum of all choline-containing phospholipids, exclusive of the lyso derivatives; Chol B is the sum of all other phospholipids detected, exclusive of the lyso compounds. Choline is the ratio of Chol A to Chol B. The sclera is unique among the tissues examined and is relatively poorer in the choline phospholipids and richer in other phospholipids than the other fine tissues examined. Choline, the ratio index, is particularly sensitive to this variation. Among the corneal tissues, the stroma is unique. The stroma shows a relatively high Choline index, which is a reflection of an elevated Chol A index and a diminished Chol B index. The index Chol/Eth is similar to Choline, but it restricts the comparison to the phosphatidylcholines and the phosphatidylethanolamines, again, with the exception of the lyso derivatives. From this more restricted point of view, the sclera is unique among the tissues. The elevated stromal Chol/Eth index relative to that of the whole cornea reflects the low PC plasmalogen content of the epithelium.

The remainder of the five indices in the phosphatide metabolite set (Table 3) address the relationships among the four phospholipids principally responsible for membrane asymmetry:[24] PC, SPH, PE, and PS. The index Outside is the sum of PC and SPH, the index Inside the sum of PE and PS, the index Leaflet the ratio of tissue. The indices PC/PE and SPH/PS are the most restrictive of the asymmetry indicators. Considering the Outside index, the sclera is unique; however, from the point of view of the Inside index, the whole cornea is unique and richer in inner leaflet phospholipids. Considering the ratio index, Leaflet, stroma (epithelium- and endothelium-denuded cornea) is significantly different from the whole cornea and the sclera in that the whole cornea and sclera index values are smaller than those of the stroma. The PC/PE index finds the whole cornea to be the significantly unique tissue in parallel with that of the Inside index. The SPH/PS index finds the stroma to be unique.

For most of the nine indices in the phosphatide metabolite set (Table 3), the stroma differs from whole cornea. Further, without exception, the direction of the differences is consistent with an interpretation that involves a stromal plasma membrane relatively enriched in PC and SPH and depleted in PE and PS.

The plasma-membrane-bound carbohydrates are exclusively a component of the plasma membrane outer leaflet. Enrichment of this carbohydrate component should lower the relative PC-SPH content and/or enrich the relative PE-PS content of a tissue. This occurs since these membrane phosphatide residues require space on the surface of the outer leaflet that must be provided for, or that must be compensated for by the addition of phospholipid to the inner leaflet, if the plasma membrane is to remain flat. Thus, a low leaflet index, as in the whole cornea and sclera, is interpreted to indicate a relative enrichment of membrane-bound carbohydrate, a consistent interpretation in light of the tissues studied.

An/Neut is the ratio of all anionic phospholipids to all neutral-ionic phospholipids, with the exception of the lyso derivatives. It is an index of membrane surface charge, since the greater the anionic phospholipid content, the greater will be the negative charge density and, with this property, the greater will be the capacity to electrostatically, or by complexation, bind metallic cations. As monitored by this index, the sclera is not different from the whole cornea, although it is different from the stroma. The An/Neut ratio is lowest for the stroma. These differences among the corneal tissues are attributable to the presence of an epithelium or endothelium or some other compensatory membrane system.

The remainder of the five indices of Table 3 relate to the presence of the lyso phospholipids, LPC and LPE Lyso A is the sum of LPC and LPE, and Lyso B is the sum of PC and PE. Lysprop is the ratio of Lyso A and Lyso B. Lyso A and the LPC/PC ratio are the two indices that do not vary significantly among the tissues studied, which indicates that the phospholipase A1 activity equilibrium position is constant among the ocular tissues examined. The differences

among the tissues seen in the indices Lyso B, Lysprop, and LPE/PE must reflect variation in the ethanolamine phospholipids. Again, the sclera is unique among the tissues examined. No significant variations were detected among any of the corneal tissues for any one of the five lyso-dependent indices, and this indicates a constant phospholipase equilibrium point among these tissues.

Regarding phospholipid metabolism, the sclera is different from corneal tissues and differs in the relative amount of most phospholipids detected and most metabolic indices that can be formulated with reasonable reference to known metabolic pathways. The contribution of phospholipids to the overall phospholipid profiles may be influenced by membranes of at least four different cell types in the sclera: pigmented melanocytes, macrophages, lymphtocytes, and fibroblasts. Considering the corneal tissues examined, the stroma is significantly different from the whole cornea, and this indicates a significant contribution of the corneal epithelium and endothelium to the phospholipid profile of the whole cornea

Because of the complexity of the total phospholipid systems, the types of insights into the operant biochemistries described above are not readily apparent from an examination of the phospholipid mole-percent profile data alone (Table 2), without the use of metabolic indices (Table 3). Thus, even without knowledge of the function a phospholipid has in a given living system or of its chemical identity, the relative mole-fraction of the metabolite can be used as a parameter, or index, into the operant metabolic activity.

Key words: cornea, sclera, phospholipids, ^{31}P nuclear magnetic resonance spectroscopy, ocular metabolism, intermediary metabolism, phosphorus.

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