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Abstract

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Keywords

Identification, phospholipids, human, meibum, nano, electrospray, ionisation, tandem, mass, spectrometry, CMMB

Disciplines

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Identification of Phospholipids in Human Meibum by Nano-Electrospray Ionisation Tandem Mass Spectrometry

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Abstract

Meibum is believed to be the major source of tear film lipids which are vital in the prevention of excess evaporation of the aqueous phase. The complete lipid composition of meibum has yet to be established. While earlier studies reported the presence of phospholipids in human meibum, recent mass spectrometric studies have not detected them. In this study we use electrospray ionisation tandem mass spectrometry to investigate the presence of phospholipids in meibum and provide comparison to the phospholipid profile of tears.

Lipids were extracted from human meibum and tear samples using standard biphasic methods and analysed by nano-electrospray ionisation tandem mass spectrometry using targeted ion scans. A total of 35 choline-containing phospholipids were identified in meibum and the profile of these was similar to that observed in tears, suggesting tear lipids are derived from meibum. The results shown here highlight the need for a combination of optimised techniques to enable the identification of the large range of lipid classes in meibum.

1. Introduction

Meibomian glands, located on the upper and lower tarsal plates of the eyelids, are believed to be the major source of tear film lipids (Bron and Tiffany 1998). Meibomian gland secretion - often termed meibum - is released through a holocrine mechanism, in which the cell ruptures and both the cell and its contents are released (Sirigu *et al.* 1992). The meibum is then spread across the outer surface of the tear film where it is vital in preventing excess evaporation of the aqueous phase (Ohashi *et al.* 2006).

Research into the lipid composition of meibum first began in 1897 (Pes 1897) where cholesterol and fatty acids were identified. Since then, a variety of techniques have been employed in an attempt to identify all lipid components. Despite this, the small sample size and low abundance of some of the lipid classes has hampered efforts and the complete lipidome has yet to be established. Recent studies by research groups such as Butovich and Green-Church and their respective co-workers (Butovich 2008, 2009; Butovich *et al.* 2007; Butovich *et al.* 2007; Butovich *et al.* 2009; Chen *et al.* 2010; Nichols *et al.* 2007) have gone a long way to completing the meibum lipidome with the confirmation of not only numerous classes of lipids but the detection and identification of individual molecules within these classes. Interestingly however, while earlier studies utilising chromatography (McCulley and Shine 1997; Shine and McCulley 2003; Tiffany 1978) and mass spectrometry (Sullivan *et al.* 2002; Sullivan *et al.* 2006) detected phospholipids in meibum they were not identified in these latest studies and therefore their presence has been questioned (Butovich 2009; Butovich *et al.* 2007). If this were true, it also raises the question as to source of phospholipids present in tears (Borchman

et al. 2007; Saville *et al.* 2010). Herein we investigate the presence of phospholipids in meibum using targeted electrospray ionisation tandem mass spectrometry and compare this with the phospholipid composition of tears.

2. Methods

All patients signed an informed consent form before enrollment in the study, which was conducted in compliance with the tenets of the Declaration of Helsinki and was approved by the Human Ethics Review Panel of the Brien Holden Vision Institute. Tears were collected (four men and four women; mean age, 42 years) as described previously (Sack *et al.* 1992). Briefly, basal tears were collected with a glass capillary at the lower lid margin without stimulating reflex tears. Meibum samples (three males; mean age, 47 years) were collected from volunteers by the methods described in Butovich *et al.* (Butovich *et al.* 2007) with the exception that chloroform was not added to the vial prior to collection. In brief, meibum was expressed using a cotton bud to squeeze the eyelid and collected with a metal spatula. Care was taken to avoid scraping the surface of the eyelid. Samples were then placed in glass vials and stored at -80°C until analysis.

Lipid extraction of tear samples was performed using a biphasic extraction as described in Saville *et al.* (2010) with the only exception being that 5 µL of methanolic (HPLC grade; Crown Scientific, NSW, Australia) phospholipid standard containing PC (19:0/19:0), 0.8 µM; and SM (d18:0/12:0), 0.8 µM (Avanti, Alabaster, USA), was added prior to extraction. Lipid extraction of meibum samples was performed using the same method with the only exception being that 2.5 µL of the phospholipid standard solution was added.

All samples were analysed by nanospray ESI (nanoESI) using a triple quadruple mass spectrometer (QuattroMicro; Waters, Manchester, UK) as described previously (Saville

et al. 2010). Phosphocholine-containing lipids were identified by precursor ion scan for the *m/z* 184 fragment ion (Brugger *et al.* 1997; Cole and Enke 1991) and spectra were obtained over a range of *m/z* 640-860. Approximately 100 acquisitions were combined, before background subtraction and smoothing using a Savitsky-Golay algorithm was performed. The limit of detection (LOD) and limit of quantification (LOQ) of the instrument were determined as previously described in Saville *et al.* (2010).

3. Results

The targeted analysis of phosphocholine-containing molecules in meibum lipid extracts revealed a number of sphingomyelin (SM) and phosphatidylcholine (PC) ions in all three samples (a typical mass spectrum is shown in Fig. 1A). The most abundant SM molecule observed was SM 16:0 (*m/z* 703), while the most abundant PCs were PC 34:1 (*m/z* 760) and PC 34:2 (*m/z* 758). A typical spectrum obtained from a tear lipid extract (Fig. 1B) shows a similar profile of molecules to that observed in meibum. It should also be noted that there was no difference in the tear phosphocholine lipid profile between males and females.

[FIGURE 1]

Based on these data, a total of 35 choline-containing phospholipids were identified in meibum, while 24 were observed in tears (Table 1). Of those observed exclusively in meibum, a number have been tentatively identified (by nominal mass) as PCs with an ether-linked fatty acyl chain. Direct confirmation of fatty acyl chains could not be obtained due to the low lipid concentration and sample volume. The total concentration of PC and SM in meibum was estimated at 18 ± 5 ng/mg of meibum (n = 3). In tears the concentration was calculated at 11 ± 2 ng/mg of tear (n = 8).

[TABLE 1]

4. Discussion

Meibum is secreted by the holocrine mechanism (Sirigu *et al.* 1992) and given the high phospholipid content of cell membranes the presence of phospholipids might be expected. In this study, the phospholipid content of meibum was analysed using electrospray ionisation tandem mass spectrometry. The results show that human meibum contains at least 35 distinct PC and SM molecules, and the most abundant of these was PC 36:1 at *m/z* 760. The observation of this nominal mass is consistent with previous reports of LC-MS analysis of meibum (Sullivan *et al.* 2002; Sullivan *et al.* 2006), with the use of tandem mass spectrometry in the present work providing unequivocal assignment of the structure as a phosphatidylcholine.

The results in Table 1 suggest that tear phospholipids are derived from meibum, with all but two tear lipids also being identified in meibum. In this study the total concentration of choline-containing phospholipid was calculated at 18 ± 5 ng/mg meibum. Given the lipid layer of the tear film has been estimated at 20 molecules thick (Butovich *et al.* 2008) and polar lipids account for only those closest to the aqueous phase, it is likely that the phospholipid component detected here does not have any significant surfactant effect on meibum spreading. It should be noted, however, that this concentration is for choline-containing phospholipids only and may not reflect the total phospholipid concentration in meibum. That is, other phospholipid classes such as phosphatidylethanolamines were not analysed in the present study but have been previously observed in meibum (Sullivan *et al.* 2002).

The work of Sullivan *et al.* using both direct infusion MS and prior fractionation (HPLC) MS, revealed a number of phospholipid molecules, while later studies (Butovich *et al.* 2007; Chen *et al.* 2010) did not. These discrepancies in the presence or absence of phospholipid in meibum may be explained by their low concentration and the complexity of the meibum sample, with sample preparation and instrument conditions likely to impact on the spectra obtained. Spectra shown in later studies (Butovich *et al.* 2007; Chen *et al.* 2010) revealed a number of high abundance ions identified as wax esters and cholesteryl esters. Depending on the total lipid concentration in the analyte solution, the presence of other abundant lipids may result in suppression of phospholipid ionisation during the ESI process (Han and Gross 2005). It is also possible that the observed ions are isobaric, i.e., the low abundance phospholipids (18 ng/mg meibum) are swamped by more abundant ions with the same *m/z*. In this study, we have overcome these potential problems by the use of targeted scans (precursor ion scans), where phospholipids with a particular structural motif can be extracted from the chemical noise and clearly observed. The use of such scans has the added advantage of aiding in structural confirmation due to their targeting of fragment ions particular to the class of phospholipid being observed.

A further reason for discrepancy could be due to the ionisation method used in some of the studies. Although Atmospheric Pressure Chemical Ionisation (APCI) is a powerful technique in lipid analysis, it is more suited to non-polar lipid classes (e.g., wax and cholesteryl esters), and is thus less sensitive than ESI for the detection of phospholipids (Byrdwell 2001). Indeed, APCI has been shown to suppress the ionisation of phospholipids, in particular PCs (Ismaiel *et al.* 2008).

The definitive identification of phospholipids in meibum shown here highlights the need for a combination of techniques to enable optimised detection of such a large range of structurally different classes of molecules in the complex milieu of meibum.

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Table 1: Phospholipids detected in tear and meibum extracts.

Lipid*	*m/z* $[M + H]^+$	Tears	Meibum
SM 14:0	675	✓	✓
SM 15:0	689	✓	✓
SM 16:0	703	✓	✓
DHSM 16:0	705	✓	✓
DHSM 17:0	719		✓
SM 18:1	729		✓
SM 18:0	731	✓	
SM 20:0	759	✓	✓
SM 22:1	785	✓	✓
SM 22:0	787	✓	✓
SM 24:2	811	✓	✓
SM 24:1	813	✓	✓
*DHSM 24:1	815	✓	✓
PC 26:0	650		✓
PC 30:1	704	✓	
PC 30:0	706	✓	✓
PC 32:0p	718		✓
PC 32:0e	720		✓
PC 32:1	732	✓	✓
PC 32:0	734	✓	✓
PC 34:1p	744		✓
PC 34:0p	746		✓
PC 34:0e	748		✓
PC 34:2	758	✓	✓
PC 34:1	760	✓	✓
PC 34:0	762		✓
PC 36:1p	772		✓
PC 36:0p	774		✓
PC 36:0e	776		✓
PC 36:4	782	✓	✓
PC 36:3	784	✓	✓
PC 36:2	786	✓	✓
PC 36:1	788	✓	✓
PC 36:0	790		✓
PC 38:4	810	✓	✓
PC 38:3	812	✓	✓
PC 38:2	814	✓	✓

Only lipids with ion counts above the calculated LOD are included.

* SM and DHSM cannot be rigorously differentiated in this experiment because of the small samples. Assignment is based on natural occurrence. DHSM 24:1 is assigned based on its prevalence as an ocular lipid (Deeley *et al.* 2008).

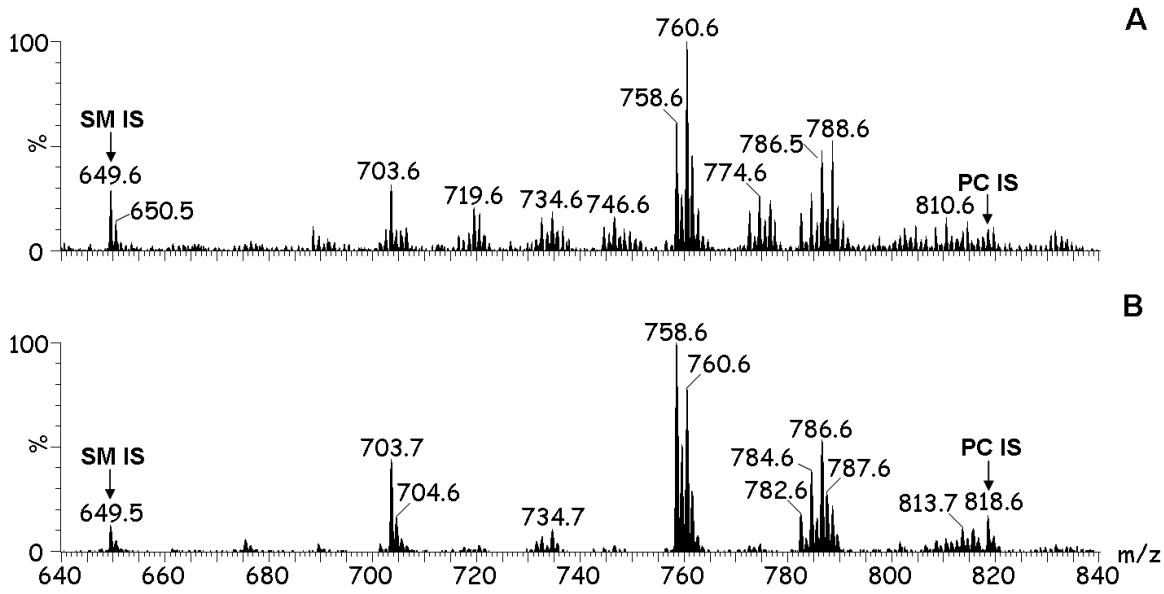

Figure 1: Comparison of targeted phosphocholine mass spectra from A) a meibum lipid extract, and B) a tear lipid extract.