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Lung Surfactant and Secretory Phospholipase A_2 in Inflammatory Lung Disorders

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

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LUNG SURFACTANT AND SECRETORY PHOSPHOLIPASE A_2 IN INFLAMMATORY LUNG DISORDERS

By Emma Louise Heeley

Lung surfactant is a complex mixture of phospholipids and apoproteins that lines the lungs of all air-breathing animals. Surfactant opposes surface tension forces within the lungs, and so prevents alveolar collapse on expiration. Inactivation of lung surfactant has been proposed as a role in the pathogenesis of lung diseases such as asthma and Acute Respiratory Distress Syndrome (ARDS), possibly by phospholipase mediated hydrolysis of surfactant phospholipids. Group IIa secretory phospholipase A_2 ($sPLA_2$) is secreted from many inflammatory cells, including mast cells and alveolar macrophages, and its activity is increased in bronchoalveolar lavage fluid (BALF) from asthmatic subjects after allergen challenge and from ARDS patients.

A rapid and sensitive method for the routine analysis of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) molecular species was developed using electrospray ionisation mass spectrometry (ESI-MS). This method permitted routine analysis of samples containing 25nmoles of phospholipid (typically 0.5-1ml BALF) on the mass spectrometer in less than 5 minutes. The use of ESI-MS for the analysis of lung surfactant phospholipids is a relatively new technique and has unrivalled detection limits (<0.5nmole of each phospholipid molecular species). Surfactant phospholipid composition was studied in the rat, rabbit, and guinea pig. Rabbit surfactant had the most similar composition to human surfactant and the rabbit should be used as an animal model to study diseases where alterations to the surfactant phospholipid composition are deemed to be crucial to the diseases process.

The acute asthmatic response was studied by local allergen challenge to mild asthmatics. BALF was obtained from controls and asthmatic subjects before and 24 hours after segmental allergen challenge. There were no differences in the PC or PG compositions between controls and asthmatic subjects before challenge. Allergen challenge in asthmatics but not control volunteers caused a significant increase in the PC to PG ratio because of increased concentrations of PC species containing linoleic acid (16:0/18:2PC, 18:0/18:2PC and 18:1/18:2PC). These molecular species were characteristic of plasma PC analysed from the same subjects, strongly suggesting that altered PC composition in BALF in asthmatic subjects after allergen challenge was due to infiltration of plasma lipoprotein, not to catabolism of surfactant phospholipid. Interactions between surfactant and lipoprotein infiltrate may contribute to surfactant dysfunction and potentiate disease severity in asthma.

In vitro studies using ESI-MS demonstrated that purified rabbit surfactant was susceptible to human group IIa $sPLA_2$ mediated hydrolysis, although this only occurred with high enzyme concentration and over a three-hour incubation. Preferential hydrolysis of the anionic phospholipid PG as opposed to PC was verified for this enzyme. Further studies of lung surfactant phospholipid compositions in ARDS patients and healthy human control subjects revealed the possible involvement of group IIa $sPLA_2$ in the pathogenesis of ARDS. However the changes observed during the acute asthmatic response are unlikely to have been caused by the action of $sPLA_2$.

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Abbreviations

γ_{min}	Minimal surface tension
ARDS	Acute Respiratory Distress Syndrome
BALF	Bronchoalveolar lavage fluid
CaLB	Calcium lipid-binding
CDP-DAG	Cytidine diphosphodiacylglycerol
$cPLA_2$	Cytosolic Phospholipase A_2
EGF	Epidermal growth factor
ESI-MS	Electrospray ionisation mass spectrometer
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL-2	Interleukin-2
$iPLA_2$	Intracellular Phospholipase A_2
LPC	Lysophosphatidylcholine
m/z	mass/charge ratio
MAP kinase	Mitogen-activated protein kinases
$nnPLA_2$	*Naja naja* Phospholipase A_2
PAF	Platelet activating factor
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGE_2	Prostaglandin E_2
PI	Phosphatidylinositol
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PP PLA_2	Porcine pancreatic Phospholipase A_2
PS	Phosphatidylserine
RDS	Respiratory distress syndrome
RSV	Respiratory syncytial virus
SP	Surfactant protein
Sph	Sphingomyelin
T_H	Helper T cells
TNFα	Tumour Necrosis Factor α
TNF-β	Tumour necrosis factor-β

Phospholipid molecular species were designated as n:a/m:b, where n and m are the number of carbon chains at sn-1 and sn-2 positions, respectively, and a and b are the number of double bonds.

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Chapter One

Introduction

1. Introduction

Lung surfactant is a complex mixture of phospholipids and apoproteins that lines the lungs at the air-liquid interface. It decreases the surface tension and thereby reduces the tendency of alveoli to collapse during expiration. An absence of adequate lung surfactant in the pre-term infant causes neonatal respiratory distress syndrome (RDS). Since the pioneering work by Avery and Mead in 1959 describing the association of surfactant deficiency and neonatal RDS (see section 1.3), lung surfactant has been widely studied [1]. Increasing evidence suggests that a surfactant dysfunction is involved in acute respiratory distress syndrome (ARDS) (see section 1.4) [2]. Elevated levels of PLA_2 in this disease may directly act on surfactant contributing to this dysfunction [3]. The increased levels of PLA_2 in the inflammatory response may lead to association with other inflammatory lung diseases, for example asthma [4,5]. The overall aim of this project is to identify if there is a connection between PLA_2 and lung surfactant in inflammatory lung diseases such as asthma and ARDS.

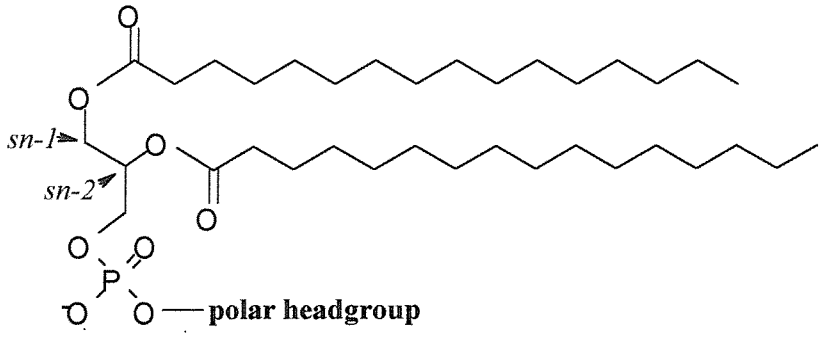
1.1 Phospholipids

The term phospholipid is given to a lipid structure having a phosphate ester group as the common constitutional feature. There are two main classes of phospholipids, glycerophospholipids containing glycerol, a three-carbon alcohol, and spingophospholipids containing sphingosine, a more complex alcohol.

Glycerophospholipids are structures based on phosphatidic acid; the polar headgroup attached to the phosphate defines the **class** of glycerophospholipid. The polar headgroups commonly found in animal tissue are shown in figure 1.1. The **molecular species** of phospholipid is determined by the combination of fatty acids attached at the *sn*-1 and *sn*-2 positions. The fatty acids usually contain an even number of carbon atoms, typically between 14 and 24, and may be saturated or unsaturated. The names of the most common naturally occurring fatty acids are given in table 1.1.

The systematic naming system for a phospholipid with a choline headgroup, and palmitic acid attached at the *sn*-1 position with oleic acid attached at the *sn*-2 position would be

*sn-1*palmityl *sn-2*oleoylphosphatidylcholine(POPC) or as I will classify phospholipids 16:0/18:1 PC.



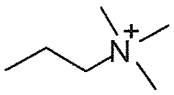
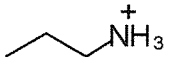
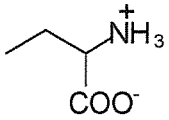
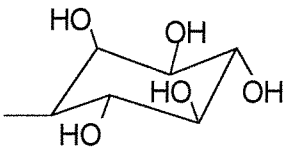
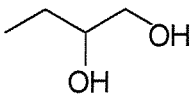
Phospholipid	***Headgroup***
Phosphatidylcholine (PC)	
Phosphatidylethanolamine (PE)	
Phosphatidylserine (PS)	
Phosphatidylinositol (PI)	
Phosphatidylglycerol	
Phosphatidic acid	

Figure 1.1 Molecular structures of phospholipids. The class of phospholipid is defined by the nature of the polar headgroup, and the species by the fatty acyl substitutes at the sn-1 and sn-2 positions. The dipalmitoyl species above would be designated 16:0/16:0PC, if the headgroup was choline. If arachidonic acid was esterified at sn-2, the molecule would be designated 16:0/20:4PC.

Phospholipid structures with only one fatty acyl attached at either the *sn*-1 or *sn*-2 position are described as lysophospholipids. These monoacyl compounds may be the result of phospholipase A_1 and A_2 action on a phospholipid molecule. Most cells contain only small concentrations of lysophospholipids owing to their strong detergent properties.

Table 1.1 Some of the naturally occurring fatty acids in eukaryotic cells.

No. carbons: double bonds	Common name	Structure
Saturated		
14:0	Myristic	$CH_3(CH_2)_{12}COOH$
16:0	Palmitic	$CH_3(CH_2)_{14}COOH$
18:0	Stearic	$CH_3(CH_2)_{16}COOH$
Unsaturated*		
16:1 (ω-7)	Palmitoleic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
18:1 (ω-9)	Oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
18:2 (ω-6)	Linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH-(CH_2)_7COOH$
20:4 (ω-6)	Arachidonic	$CH_3(CH_2)_4(CH=CHCH_2)_4CH_2-CH_2COOH$
22:6 (ω-3)	Docosahexaenoic	$CH_3CH_2(CH=CHCH_2)_6CH_2COOH$

** The nomenclature of the unsaturated fatty acids not only describes the number of double bonds but also gives the position of the first double bond from the methyl end of the fatty acid, for example (ω-3).*

1.2 Lung surfactant

In 1929 Kurt von Neergard postulated the existence of an essential surface-active material in the lungs, but unfortunately he published his pioneering ideas in German[6]. It was not until twenty-five years later that his work was confirmed by Radford who also demonstrated that the surface forces at the interface of moist alveolar walls caused a strong retractile force (even stronger than that of stretched elastic tissue)[7]. Pattle then published a paper in Nature suggesting the existence of an "insoluble protein layer that can abolish the tension of the alveolar surface" [8]. Pattle's results for surface tension disagreed with data from Radford and von Neergard that suggested a significant surface

tension in the airspaces. It was a year later that Clements had enough data from his experiments, measuring surface tension on a substance in lung oedema fluid and lung extracts, which provided results to account for these divergent observations. The results indicated that this material could stabilise alveoli against collapse by lowering surface tension dramatically to less than 10 dynes/cm[9]. The material consisted of a mixture of phospholipids and proteins; it was termed surfactant (**surface active agent**).

1.2.1. Anatomy of the alveoli

The airways of mammalian lungs form a system of branching tubes, whose diameter decreases from the trachea to periphery. The alveoli form the final branching of this system, they are small sac like structures closely surrounded by a network of capillaries that enable gas exchange. There are an estimated 300 million alveoli in the adult human lung whose surface area is approximately equal to the size of a tennis court [10,11]. The actual diameter of a fully inflated individual alveolus in the adult human lung is believed to be about 250μm - 145μm and this varies with age and body size. However in a given lung, there is very little variation in fully inflated alveolar size [12-14].

1.2.2. Alveolar surface tension

The concept of surface tension in the alveolus came from von Neergard's conclusion that alveoli normally have a wet lining and therefore the force of surface tension must add to their elastic recoil [6]. This was illustrated when lungs were isolated and inflated; less pressure was required to inflate lungs filled with saline than with air. This can be explained due to the removal of the air-liquid interface and therefore the surface tension [6,7].

Surface tension occurs at the interface between two different surfaces, e.g. at the air-liquid, liquid-solid interface; it is a force that acts tangentially to the surface and resists expansion of it. In the alveolus, surface tension is the force around the circumference resisting expansion, trying to make the alveolus smaller and therefore contributing to the collapsing pressure (figure 1.2). The collapsing pressure (P) is proportional to the surface tension (γ) and inversely proportional to the alveolar radius (r), as stated in LaPlace's law: $\mathbf{P=2\gamma/r}$. Lung surfactant lines the air-liquid interface and reduces the

surface tension thereby reducing the collapsing pressure. The surface pressure opposes the collapsing pressure.

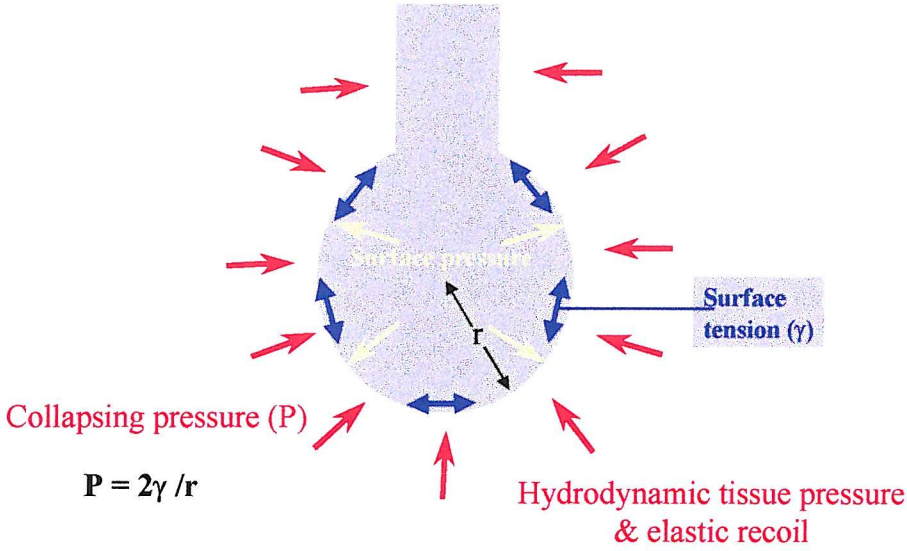


Figure 1.2 Pressures present in an alveolus in the absence of surfactant. The surface tension (γ) contributes to the collapsing pressure from the hydrodynamic tissue pressure and elastic recoil, and the surface pressure opposes these forces.

The alveoli are very small to enable efficient gas exchange and they change in size during the breathing cycle. Upon expiration the alveoli get smaller and the collapsing pressure increases in accordance with LaPlace's law. Surfactant is necessary to maintain constant alveolar pressure (P) throughout the ventilatory cycle and prevent alveolar collapse; this is thought to be achieved by differentially reducing the surface tension. During expiration the alveolar radius decreases along with the dynamic reduction of surface tension from $50mNm^{-1}$ till it levels of below $10mNm^{-1}$ and then rises again with the next inspiration.

16:0/16:0PC is thought to be the component in lung surfactant that is responsible for reducing the surface tension at the air-liquid interface. Almost pure 16:0/16:0PC is solid at 37°C and forms an incompressible solid layer at the air-liquid interface, preventing fluid seeping into the alveolar space and therefore collapse (figure 1.3). The exact mechanism by which surfactant achieves reduced surface tension in the alveoli is not

clear. A plausible explanation of how the variable surface tension effect is achieved is by the rapid recruitment of semihydrous lung surfactant from the type II cells (see section1.2.4.1) during inspiration. Surfactant does however play a crucial role in the lungs maintaining alveolar fluid balance, which keeps the alveolar space dry, as well as preventing alveolar collapse and atelectasis.

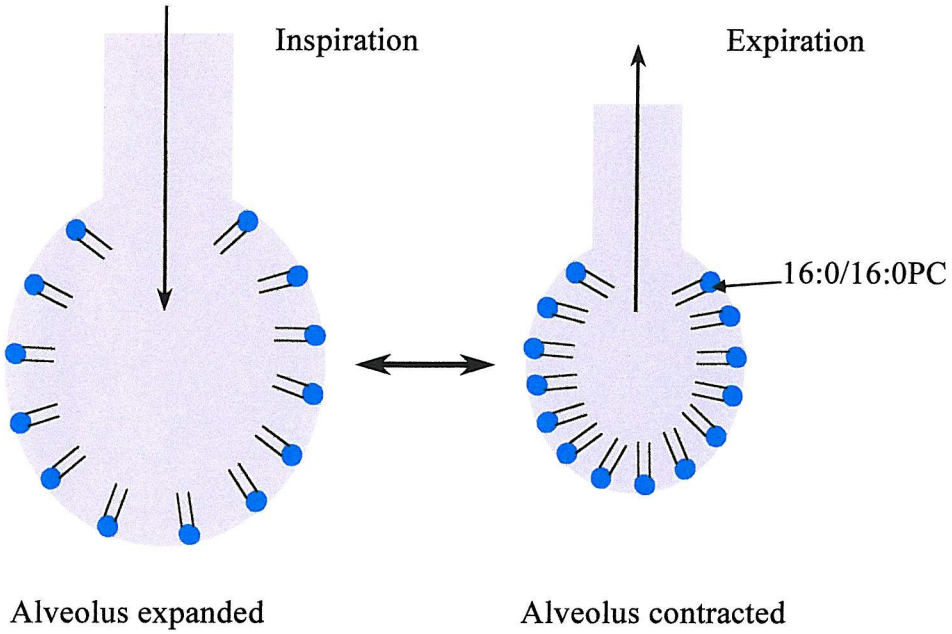


Figure 1.3 Schematic representation of an alveolus during the breathing cycle. The 16:0/16:0PC lines the air-liquid interface forming an incompressible layer preventing alveolar collapse upon expiration.

The absence of adequate surfactant in premature babies causes neonatal respiratory distress syndrome (RDS). Atelectasis is a result of neonatal RDS and is the failure of part of the lung to expand due to the absence of lung surfactant, the surface tension forces within the alveoli become too large to be overcome. This can lead to the smaller alveoli becoming unstable as the collapsing pressure increases along with surface tension (an absence of surfactant) and forming one larger alveolus where the pressures are not so great (figure 1.4).

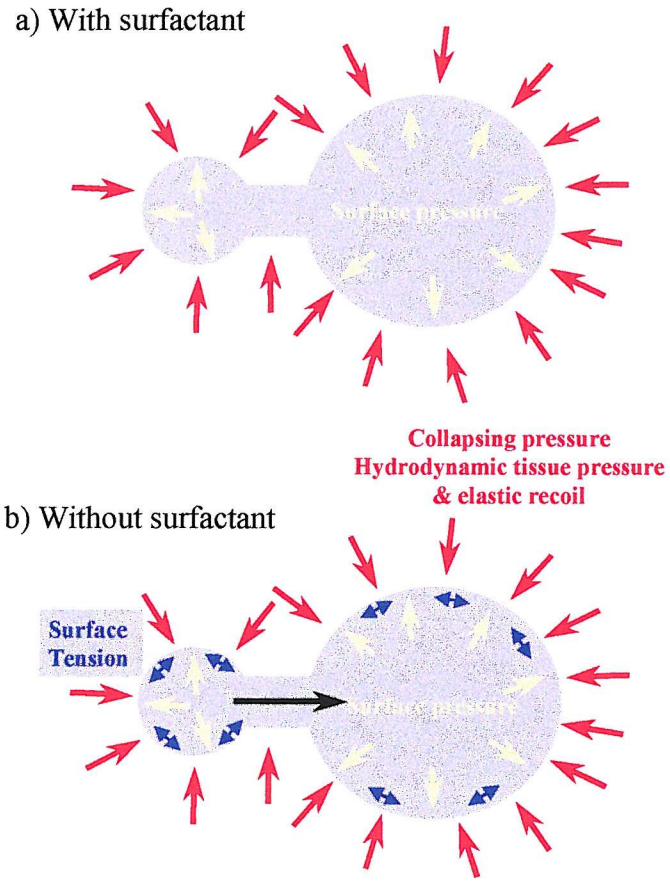


Figure 1.4 Schematic representation of how the presence of surfactant prevents atelectasis.

1.2.3. Surfactant composition

The composition of lung surfactant has been established from studying bronchoalveolar lavage fluid and isolating what is thought to be lung surfactant. Lipid is the major component of human surfactant by weight making up approximately 90% of total surfactant with the remaining 10% comprising of proteins. There are four surfactant associated proteins, surfactant protein (SP)-A, SP-B, SP-C and SP-D, as well as a large number of other serum derived proteins. Phospholipids account for 90% of the lipid fraction, the remaining 10% being neutral lipid, mainly cholesterol. The most abundant phospholipid, is phosphatidylcholine, however lung surfactant also contains an unusually high proportion of phosphatidylglycerol.

1.2.3.1 Neutral phospholipids

Phosphatidylcholine is the major class of phospholipid present in surfactant accounting for approximately 80% of total surfactant phospholipids and for about two-thirds of whole surfactant. Dipalmitoyl-phosphatidylcholine (16:0/16:0PC) is the major species present in surfactant and possibly the most important. 16:0/16:0PC is solid at room temperature and is thought to be the directly responsible for the reduction of alveolar surface tension (see section 1.2.2). Studies on synthetic surfactants used therapeutically in neonatal RDS led to the conclusion that 16:0/16:0PC and PG are the active components of lung surfactant. It is thought that PG is present to enable spreading of this solid material [15], as 16:0/16:0PC is inactive on its own but with PG present it seems to adequately substitute for the lack of natural surfactant in the pre-term infant. Phosphatidylethanolamine (PE) and phosphatidylserine (PS) are present in surfactant in very small quantities and appear to have no active role in the functioning of surfactant; they may be present in extracted surfactant as contaminants from cell membranes.

1.2.3.2. Acidic (anionic) phospholipids

The acidic phospholipid phosphatidylglycerol (PG) is thought to be responsible for adsorption of 16:0/16:0PC to the air-liquid interface. The role of phosphatidylinositol (PI) has yet to be clearly defined but it seems possible that surfactant PG may be largely replaced by PI without altering normal lung function [16-18]. In human lung surfactant the concentration of PI is much lower than PG. In rhesus monkeys the concentrations of the acidic phospholipids is reversed; PI is the major acidic phospholipid, whereas PG is only a minor component [19]. This is not the only animal species where PG is not a major component of surfactant, PG was virtually absent from chicken [20] and turtle surfactant [21].

Rustow (1988) found that similar molecular species patterns of PI and PG were synthesised by rat lung microsomes, suggesting they are synthesised from a common CDP-DG pool in the lungs [22](see section 1.2.4 for synthesis pathways). This could fit in with the observations that PG and PI show different developmental patterns; the proportion of PG starts to increase near term, while at the same time the PI decreases [23]. This may be due to a decrease in serum inositol [24] or a dramatic decrease in the availability of inositol to specific lung cells [25] leading to decreased PI synthesis. The

precursor CDP-DG is common in both the synthesis of PG and PI and the decrease in the PI synthesis would lead to more CDP-DG available for PG synthesis. Another potential explanation is a high CMP level produced by increasing PC synthesis in fetal lung near term [26] shifts the equilibrium of the CDP-DG: inositol phosphatidyltransferase towards CDP-DG, which then is available for PG synthesis [27].

1.2.3.3. Surfactant proteins

Proteins account for 8-10% of purified surfactant and four surfactant specific proteins have been identified. The first three Surfactant Protein A, B and C are named in descending rank of their molecular masses [28]. Since then another protein has been identified surfactant protein D. The two hydrophilic surfactant proteins are SP-A and SP-D, while SP-B and SP-C are hydrophobic.

Table 1.2 Main features of surfactant-associated proteins

Surfactant Protein	Molecular weight of monomer	Quaternary structure	Homologies	Phospholipid binding selectivity
SP-A	26-38 KDa	Hexadecamer	Collectins Mannose-binding protein C1q	16:0/16:0PC
SP-B	8.7 KDa	Dimer	Saposins Nk-lysin	Anionic phospholipids
SP-C	4.2 KDa	Monomer	Signal-peptides	Anionic phospholipids
SP-D	43 KDa	Dodecamer	Collectins conglutinin	PI

Adapted from [29]

SP-A, B and C are important in the dynamics and intra-alveolar metabolism of the surfactant system [30]. So far, there is no evidence to support a role for SP-D in the adsorption of surfactant lipids to the alveolar surface [31]. However SP-D along with SP-A

play an important role in the interactions of the pulmonary surfactant system with phagocytic cells and pathogens, by facilitating phagocytosis of opsonised particles [32]. SP-A is also reported to be involved in the inhibition of surfactant secretion [33,34].

1.2.4. Surfactant synthesis

Surfactant is continually being made throughout the lives of air breathing animals. In mammals the alveolar type II cell has been identified as the site of surfactant synthesis. It is assumed that all phospholipid components of surfactant are synthesised in alveolar type II cells, as there is no evidence to suggest otherwise. The pathways involved in the synthesis of PC, PG and PI are illustrated in figure 1.5.

16:0/16:0PC is the most abundant and the most studied surfactant phospholipid [35,36]. There are two main pathways for 16:0/16:0PC synthesis, *de novo*- synthesised from blood derived phospholipid precursors and acyl remodelling. Studies on rat lung microsomes and type II pneumocytes indicate that the *de novo* pathway supplies up to 50% of total 16:0/16:0PC directly, and the remainder is formed by remodelling mechanisms [37,38].

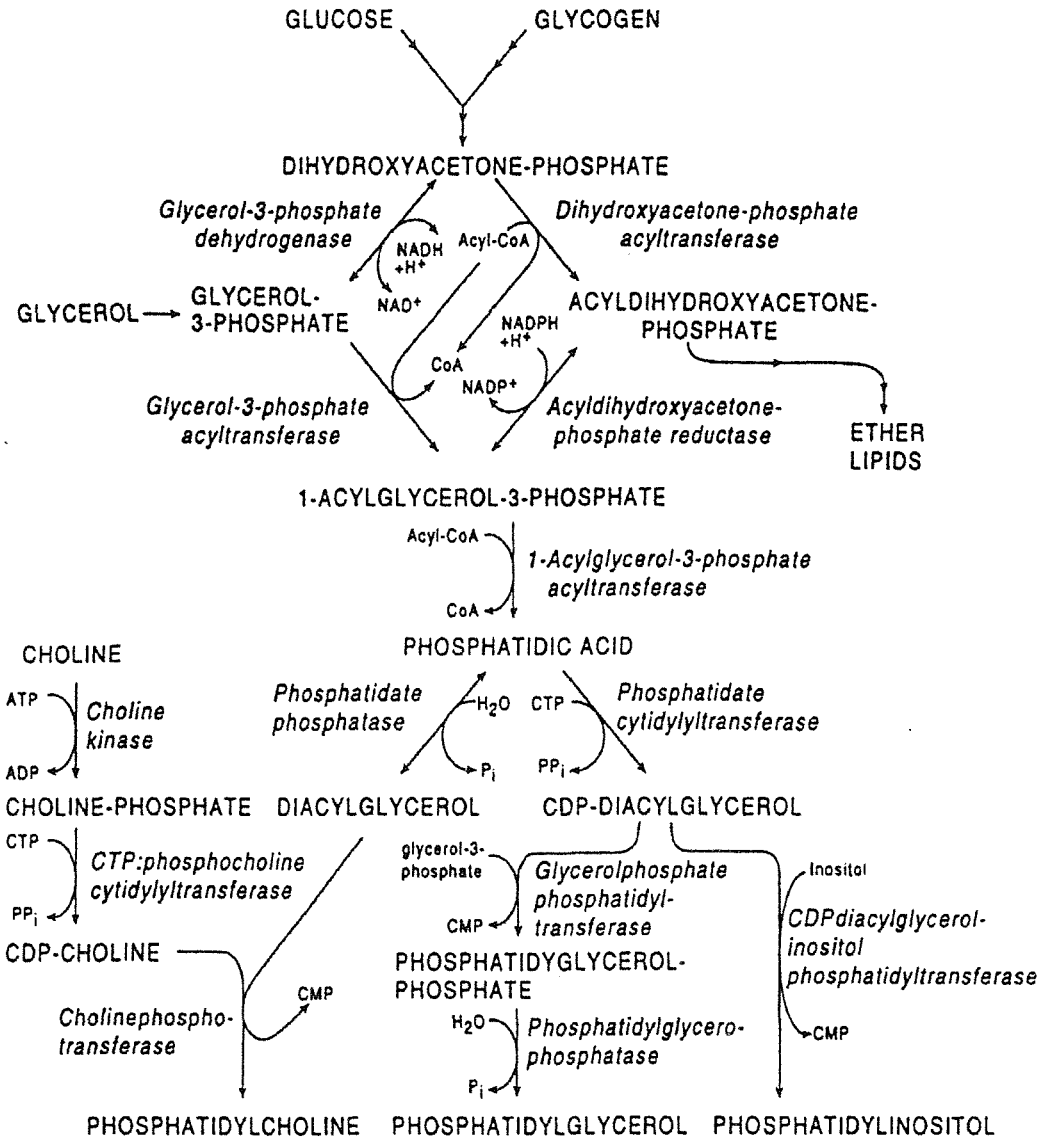


Figure 1.5 Pathways involved in the synthesis of PC, PG and PI [31]

Acyl remodelling involves changing the fatty acids attached to the glycerol backbone, in order to form 16:0/16:0PC the fatty acids must both be palmitoyl. Due to the unusual high proportion of 16:0/16:0PC in surfactant it is essential that acyl remodelling occurs in order to prevent crystallisation in the cells. It is not only recycled material that undergoes acyl remodelling but the composition of newly formed PC is subsequently modified. The process of acyl remodelling involves the sequential actions of phospholipase and acyltransferase enzymes [39]. Phospholipase A_2 has an important role in the remodelling process converting 1-palmitoyl-2-enoyl PC to 1-palmitoyl-sn-glycero-3-phosphorylcholine (lysoPC) [38]. Previous work performed in Child Health

demonstrated the activity of PC acyl remodelling in human fetal lung, before the initiation of surfactant production [40].

The major site of surfactant protein synthesis is in the type II cells. However the Clara cells of the peripheral airways have been shown to synthesis surfactant proteins A, B and D [41-43]. There is no evidence to suggest that the Clara cells synthesise surfactant phospholipids or secrete intact surfactant, as they do not contain lamellar bodies, characteristic of surfactant stored in the type II cells.

1.2.4.1 Secretion and turnover

Surfactant is packaged in the lamellar bodies of the alveolar type II cells as an anhydrous mixture of selected surfactant phospholipids and proteins [44]. The lamellar bodies are released by exocytosis from these cells and it is thought that the surfactant mixture is secreted into a liquid layer between the alveolar space and the epithelial cells. The released lamellar body material is rehydrated and converted to large vesicles (often termed large aggregates, LA). *In vitro* these large vesicles appear in many possible forms, however it is not known what form occurs *in vivo*. How surfactant arranges itself at the air-liquid interface is still subject to some debate. During the ventilatory cycle surfactant is adsorbed to the air-liquid interface. The surfactant is then thought to be refined at the air-liquid interface as the surface pressure increases the unsaturated phospholipids are squeezed out of the interface leaving what is thought to be a 16:0/16:0PC layer (single or multi-layer). A basic overview of surfactant synthesis can be seen in figure 1.6.

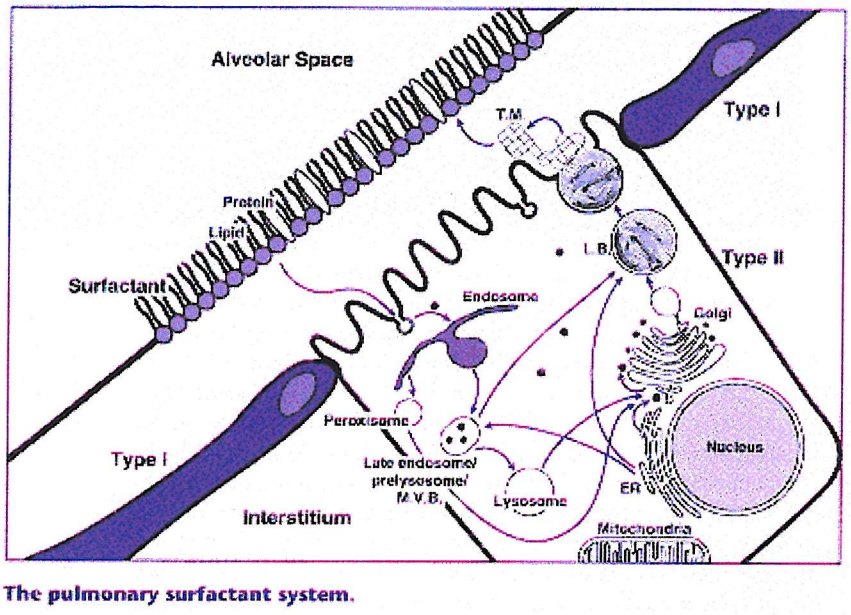


Figure 1.6 The pulmonary surfactant system [45].

Turnover studies with labelled surfactant phospholipids have demonstrated alveolar half lives of 15-30 hours in neonates and about 6 hours or less in adults [46,47]. The 4 mechanisms involved in surfactant metabolism are:

1. Recycling into the alveolar type II cell.

2. Phagocytosis and degradation by alveolar macrophages.

3. Intra-alveolar catabolism of phospholipids and proteins by phospholipases and convertases.

4. Removal from the alveolus up the bronchial tree by surface pressure and mucocillary transport (7%).

There is substantial evidence that surfactant phospholipid secretion and re-uptake is regulated at least in part by SP-A via a type II cell SP-A receptor [48].

1.2.5 Airway surfactant

Morphologically, a bronchial surfactant layer has been demonstrated by electron microscopy [49] between the sol and gel phase of the airway mucus [50]. Evidence suggests that the airways are supplied with alveolar surfactant components via the mucocillary escalator [51], it is thought that approximately 7% of alveolar surfactant reaches the bronchial tree by this mechanism. During expiration the surface pressure increases

expelling the surfactant film at the alveolar air/liquid interface into adjacent conducting airways during expiration [52,53].

Enhorning proposed that surfactant function is not only important in the alveoli, but also crucial in the small peripheral conducting airways [54]. These terminal bronchioles are not rigid structures and airway closure could occur by a number of means. Possible depletion of surfactant will lead to meniscus formation of the airway lining film and this is termed film collapse. This film collapse is thought to be accompanied by simultaneous collapse of the bronchiolar wall due to surfactant dependent increased surface tension leading to a reduction of inner wall pressure and an increase in collapsing pressure and is termed compliant collapse (figure 1.7). Kamm demonstrated that instability of the liquid film in the airways due to impaired surfactant function could result in airway closure [55,56]. However, local factors such as liquid volume and airway wall properties, will determine the predominant type of collapse.

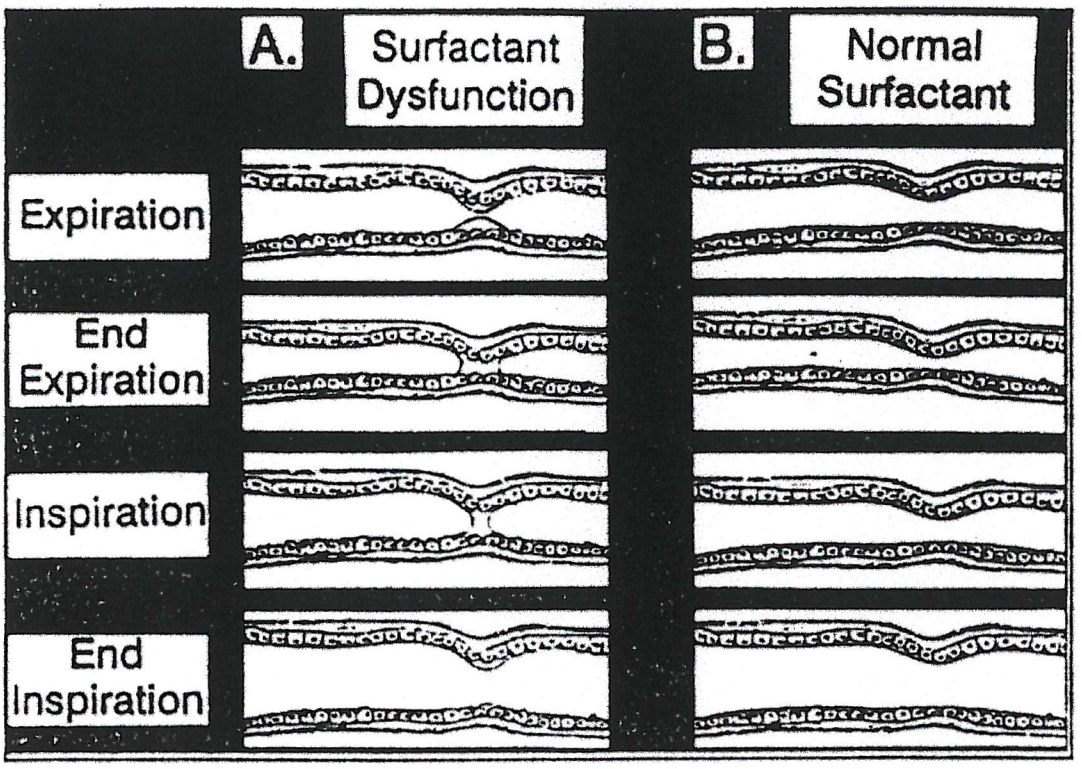


Figure 1.7 In the absence of adequate surfactant there is a risk that at least during part of the respiratory cycle a small airway will become blocked with liquid (A). With lung surfactant air-way patency is secured (B). [57]

In healthy subjects airway closure only occurs at very low lung volumes. Airway closure is important in asthma as it is characterised by a variable and reversible airway obstruction due to airway inflammation and bronchial hyperresponsiveness [58].

Theoretical analyses have been performed predicting the closure of small airways. They are modelled as thin elastic tubes, coated on the inside with a thin viscous liquid lining. Solutions show that there is a critical film thickness, dependent on fluid, wall and surfactant properties above which liquid bridges form [59]. Airway wall thickness and diameter are indirectly modulated due to airway surfactant improving bronchial clearance and regulating airway liquid balance [58]. Furthermore, the ability of surfactant to maintain free airflow through a narrow tube was lost with the addition of albumin or fibrinogen (two potent surfactant inhibitors); suggesting that oedema may lead to surfactant inhibition that in turn may lead to airway closure.

1.3 Neonatal Respiratory Distress Syndrome

Respiratory distress syndrome (RDS) occurs in very low birth weight babies. Studies by Avery and Mead published in 1959 clearly demonstrated that surfactant deficiency in the underdeveloped lungs of the pre-term infant is responsible for RDS [1]. About 20 years later, Fujiwara and co-workers were the first to use natural surfactant extract to successfully treat premature infants with RDS [60]. They studied a series of 10 premature infants with severe RDS requiring assisted ventilation. The infants improved dramatically following treatment with a modified bovine surfactant extract. This work led to the widespread study of natural and synthetic surfactants that are now available for the treatment of neonatal RDS. The use of artificial surfactant for the treatment of RDS in pre-term babies has reduced the mortality rates of this disease significantly over the past 15 years.

1.4. Acute (adult) Respiratory Distress Syndrome (ARDS)

ARDS is a condition where the respiratory distress is a secondary effect that can be initiated by various different mechanisms. It is a non-specific reaction of the lungs to a wide variety of insults; e.g. septic shock, sepsis, fat embolism, trauma, burns, pancreatitis, cardiopulmonary bypass, lung contusion, pneumonia and near drowning [61].

ARDS is characterised by severe dyspnea, hypoxemia, increased lung stiffness, and diffuse bilateral pulmonary infiltrates that lead to a need for artificial ventilation. The extended inflammatory processes in the lung microvascular, interstitial, and alveolar compartments cause fluid to seep into the alveolar space, requiring artificial removal by suctioning the airways [62,63].

Major alterations of surfactant phospholipid composition and surfactant function, along with reduced surfactant protein concentration in BALF, have been described in ARDS [2,62,64-67]. ARDS is a multifactorial disease where surfactant alteration is one of the factors. Four possible mechanisms for surfactant alteration in ARDS have been proposed:

1. Decreased formation of active components of surfactant (phospholipids and apoproteins) due to impaired alveolar type II cell function.
2. Increased permeability of the alveolar-capillary barrier leads to a massive accumulation of fluid and protein in the alveolar compartment [68]. This oedema fluid blocks the alveolar air space and impairs normal gas exchange. Evidence suggests that these plasma-derived proteins inactivate surfactant functions [69-71].
3. Incorporation of surfactant phospholipids into polymerising fibrin clots [69].
4. Phospholipid degradation by phospholipases [3,62,72-74].

Persistent atelectasis of surfactant deficient and in particular fibrin-loaded alveoli may represent a key event to trigger fibroblast proliferation and fibrosis in late phase ARDS. Initial trials to treat ARDS with surfactant supplementation therapy were disappointing [75,76]. This may be due to the low dose used, the phospholipid and protein composition and the severity of ARDS at the time of administration. Trials are currently underway to treat ARDS with bovine or porcine surfactant in higher effective doses. However other factors of surfactant therapy need to be addressed, administration techniques, ideal timing, role of BAL before administration to remove surfactant inhibitors, and the beneficial effects of administrating phospholipase inhibitors along with the surfactant preparation. Even with the advancement of treatment of RDS a significant mortality will still remain since the cause of death of ARDS is not always related to respiratory failure.

1.4.2 Phospholipid content and profile in ARDS

All studies, which at present have investigated the surfactant phospholipid composition in ARDS, have only looked at phospholipid classes. The major changes in the phospholipid composition are a decrease in PC and a dramatic decrease in PG [64,67,77], along with an increase in PI, PE and sphingomyelin (Sph)[78]. The increase in PI, PE and Sph could be associated with cell contamination in the samples as these classes of phospholipids are predominantly found in cell membranes especially PE and Sph which are virtually absent in normal lung surfactant. This is not surprising considering ARDS is a disease that involves infiltration of fluid and neutrophils into the lungs.

The dramatic decrease of PG in the BALF of ARDS patients could be due to hydrolysis of lung surfactant by group IIa $sPLA_2$. Group IIa $sPLA_2$ has been reported to increase in ARDS (see section 1.7.4.8) and has also been reported to demonstrate preferential hydrolysis of PG instead of PC. It still has to be established whether the $sPLA_2$ acts directly on the surfactant and is the cause of this decrease in the surfactant PG.

1.5 Asthma

Asthma is a chronic and debilitating disease, causing swollen and inflamed airways that are prone to constrict suddenly and violently [288]. Asthma affects 3 million people in the U.K with 110,000 admissions to hospital excluding those dealt with in accident and emergency. It has been defined as a disorder characterised by variable airflow obstruction; symptoms of wheeze, cough, dyspnoea, and chest tightness; reversibility to bronchodilators and corticosteroids; increased airway responsiveness to a variety of stimuli; and evidence of inflammation in which eosinophils, mast cells, and lymphocytes together with a multitude of cytokines have an important role [288](NHLBI/WHO 1993). Asthmatic airway inflammation is initiated by immunoglobulin E (IgE) - mediated allergy to allergens (e.g. house dust mites).

Over the past 20 years the prevalence of asthma has increased in both developed and developing countries [79]. Its is unlikely that the genetic background of a stable population can change significantly over this period, so the probable cause of this epidemic must lie in the environment. Interestingly the higher rates of prevalence occur in westernised

countries and developing countries that are adopting the westernised lifestyle[80]. Possible reasons for the increase in occurrence of asthma and accompanying allergies (allergic rhinitis and eczema [81,82]), may include changes in housing allowing greater proliferation of house dust mites, therefore increasing both sensitisation and exposure [83]. Other environmental factors include both outdoor (e.g. diesel particulate, ozone and nitric oxide [84,85])and indoor pollutants (e.g. passive smoking [86]). The effect of changes in diet and the impact of early childhood infections and their treatment have also been suggested and will be discussed in more detail.

The increase in the prevalence of asthma has been paralleled by a fall in the consumption of saturated fat and an increase in the amount of polyunsaturated fat in the diet [87]. The reduction in the consumption of animal fat and an increase in the use of margarines (that contain ω-6 polyunsaturated fatty acids (PUFAs)) have reduced the occurrence of coronary heart disease. However over this time period the prevalence of asthma has increased and has been proposed to be due to the accompaning decrease in the consumption of oily fish that contain ω-3 PUFAs, such as eicosapentaenoic acid [88]. ω-3 PUFAs are metabolised into less bronchoconstricting inflammatory mediators than ω-6 PUFAs. Dietary supplementation of ω-3 fatty acids to asthmatic subjects has yielded variable results that may be accounted for by the different doses of fatty acids given over varying time periods with the effect on asthma being measured by various parameters[89,90]. An encouraging report looked at the asthmatic response to inhaled allergen after 10 weeks on a double blind fish oil supplementation study and found that increased dietary fish oil intake attenuated the late phase asthmatic response [91]. This along with other studies supports the use of ω-3 fatty acids in the treatment of clinical asthma [92,93], however further studies are required to sort out the discrepancies. As yet the effect diet has on the surfactant phospholipid composition has not been investigated.

It has been suggested over recent years that the first year of life is crucial when the infants immune system hovers between the T_H1 and T_H2 type of response to allergens. Helper T cells (T_H) modulate inflammation and can be classified into T_H1 (non-atopic) and T_H2 (atopic) types depending on their cytokine production [94]. T_H1 cells secrete interferon-γ (IFN- γ), interleukin-2 (IL-2) and tumour necrosis factor-β (TNF-β), whereas

T_H2 cells secrete IL-3, IL-4, IL-5, IL-9 and IL-13 and up regulate the production of IgE. The absence of infections and some viruses in the early years of life may tip the balance towards the T_H2 phenotype and predispose children to developing allergic conditions such as asthma. This has been supported by studies showing the youngest child in large families is least likely to get asthma, it is thought that this is due the child having a high exposure to viruses in early years of life[95]. Other studies in Venezuela and Africa have shown in populations where their is a high parasite load it is protective against asthma, despite up-regulation of the T_H2 response [96,97]. Parasite infection produces high levels of IgE that, possibly by saturating the number of binding sites for the IgE on mast cells and other effector cells of allergy, prevent activation of these cells by the relatively trivial exposures to allergens [98].

As already highlighted asthma is a very complex disease and the exact cause is probably a combination of all these factors in different proportions depending upon the individual, however the resulting symptom of airway inflammation is the same. Asthmatic airway inflammation typically due to exposure to allergens, leads to accumulation of liquid and mucus within distal bronchioles. The role of lung surfactant in the asthmatic disease process has been rarely studied. It is possible that surfactant dysfunction is involved in this accumulation of liquid in the airways, since normal functioning surfactant prevents liquid filling. A paper by Liu 1995 describes the inflammatory response to aerosolized allergen in ovalbumin-sensitized guinea pigs leading to increased endothelial and epithelial permeability[99]. It has been postulated that the inactivation of surfactant is involved in the acute asthmatic response although the exact mechanism of this surfactant inactivation is unknown.

Initial results from a double blind, placebo controlled pilot study in Japan [100] showed that inhalation of surfactant during acute asthmatic attacks led to an improvement of lung function in the surfactant treated subjects. However in another study on asthmatic children with mild airflow limitation, nebulization of surfactant did not alter airflow obstruction and bronchial responsiveness to histamine [101]. Therefore further studies are required to ascertain the usefulness of surfactant therapy in the acute asthmatic response.

Surfactant therapy is expensive and the correct formulation could be very effective, but first the mechanism of surfactant inactivation must be established.

1.6 Phospholipases

Phospholipases are a group of enzymes that hydrolyse specific bonds of phospholipids. The site of hydrolysis determines the name of the phospholipase, as shown in figure 1.8.

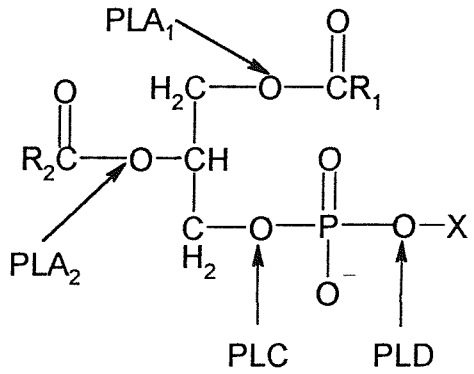


Figure 1.8 Sites of action of phospholipases on a phospholipid molecule. R_1 and R_2 denote the fatty acids and X the headgroup.

Two general types of phospholipases exist, the acyl hydrolases (PLA_1, PLA_2), and the phosphodiesterases (PLC, PLD). The phospholipases are a very diverse group of enzymes that differ both in structure and function. The functions ranging from digesting phospholipids, membrane repair and remodelling to signal transduction. Phospholipase A_2 (PLA_2) describes a group of enzymes that have a variety of functions but in particular are thought to be involved in the inflammatory response. The PLA_2s are discussed in more detail.

1.7 Phospholipase A_2

PLA_2 enzymes play an essential part in diverse cellular responses, including phospholipid digestion and metabolism, host defence and signal transduction. The PLA_2 enzymes are characterised by their ability to hydrolyse fatty acids from the *sn*-2 position of phospholipids, releasing lysophospholipids and free fatty acids [102,103]. The products formed after hydrolysis by these enzymes can have very important biological roles depending upon the phospholipid hydrolysed. Two important products of PLA_2 catalysed hydrolysis are (1) arachidonic acid and (2) platelet activating factor (PAF).

Arachidonic acid is the precursor of a large family of compounds known as the eicosanoids, such as prostaglandins and leukotrienes [104]. The eicosanoids possess a wide spectrum of biological activities, among which is their ability to mediate a number of signs and symptoms associated with inflammatory reactions [104]. PAF is another potent inflammatory mediator that is formed when the *sn*-1 position of the PC contains an alkyl ether linkage[105]. The recent discovery of cell surface receptors for secretory PLA_2 suggests that the protein itself may also have a role in signal transduction [106].

There are many PLA_2 enzymes and this family is rapidly expanding with the discovery and identification of new members. The PLA_2 family can be divided into three main types: the secretory PLA_2 ($sPLA_2$) [107], the cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$) [103], and the intracellular Ca^{2+}-independent PLA_2 ($iPLA_2$) [108]. The newly identified $iPLA_2$ is classified as the group VI enzyme in Table 1.3. There is also a class of PLA_2s called PAF acetyl hydrolases (classified as group VIIB in Table 2), which appears to act on PAF and oxidised lipids [109-111]but these will not be discussed further. Table 1.3 is modified from an updated classification of the PLA_2s, based on the comparison of nucleotide gene sequences [112].

Table 1.3 Characteristics of the major groups of phospholipase A_2 s

Group		Sources	Location	Size (kDa)	Ca^{2+} requirement
I	a	Cobras, kraits	Secreted	13-15	mM
	b	Porcine/human pancreas	Secreted	13-15	mM
II	a	Rattlesnakes, vipers, human synovial fluid/platelets	Secreted	13-15	mM
	b	Gaboon viper	Secreted	13-15	mM
	c	Rat/mouse testes	Secreted	15	mM
III		Bees, lizards	Secreted	16-18	mM
IV	a	Raw 264.7/ rat kidney, human U937 cells/platelets	Cytosolic	85	< μM
	b	Human brain	Cytosolic	100	< μM
	c	Human heart/skeletal muscle	Cytosolic	65	None
V		Human/rat/mouse heart/lung, $P388D_1$ macrophages	Secreted	14	mM
VI		$P338D_1$ macrophages, CHO cells	Cytosolic	80-85	None
VII	a	Human plasma	Secreted	45	None
	b	Bovine brain	Cytosolic	42	None
VIII		Bovine brain	Cytosolic	29	None
IX		Marine snail	Secreted	14	< mM
X		Human leukocytes	Secreted	14	mM

Modified from[112]

Each class of PLA_2 will be discussed with regard to its size, location and a suggested role. The group IIa $sPLA_2$ will be considered in greater detail as it has been proposed to be involved in inflammation and lung diseases such as asthma and ARDS [113,114].

1.7.1 Secretory PLA_2s

All the $sPLA_2$s have a molecular mass of approximately 14kDa and require millimolar calcium for effective hydrolysis of phospholipids at an optimum pH of 8 to 9 [103,115,116].

The $sPLA_2$s are found in extracellular fluids and this is consistent with the enzyme requiring mM calcium for full activity. They have a very rigid tertiary structure that enables them to retain their activity in such hostile surroundings. This ability of the enzyme to resist denaturation and its established robustness to proteolysis is thought to be due to the enzymes stable structure that arises from the presence of 5-8 disulfide bonds [117]. There have been no reports to suggest that these enzymes exhibit significant fatty acid selectivity *in vitro* [118-120].

1.7.2 Cytosolic PLA_2

Cytosolic PLA_2 ($cPLA_2$ or group IV PLA_2) is a high molecular mass (85 kDa) enzyme, found in the cytosolic fraction of practically all cell types that have been studied [121,122]. The enzyme is apparently one of the most important isoenzymes of PLA_2 involved in regulating the lipid mediator generation resulting from cell activation [103,116,123]. The activation of $cPLA_2$ is regulated by several post-receptor signal transduction events, such as Ca^{2+} mobilization, phosphorylation and gene induction. The enzyme responds to increases in intracellular Ca^{2+} by translocating to membranes via a calcium lipid-binding (CaLB or C-2) domain within the protein [121]. Phosphorylation of the enzyme instigates a modest increase in its specific activity and is caused by kinases of the mitogen-activated protein kinase (MAP kinases) cascades [112]. $cPLA_2$ is constitutively expressed in most cell types but the presence of proinflammatory cytokines (such as IL-1) [124]and Tumour Necrosis Factor α (TNFα) [125]) and some growth factors (e.g. Epidermal growth factor (EGF)[126]) increased the expression of the protein . Finally $cPLA_2$ preferentially hydrolyses phospholipids containing arachidonic acid at an optimum pH of 7 to 9 [123,127]. Transgenic experiments in mice have clearly established a crucial role for this enzyme in the inflammatory response [128 129].

1.7.3 Intracellular Ca^{2+}-independent PLA_2s

The $iPLA_2$s are the most recently identified members of the PLA_2 family. So far only one $iPLA_2$ has been sequenced and characterised in detail, as the group VI enzyme from macrophages [130]. Similarly to $cPLA_2$ this PLA_2 has a molecular weight of approximately 80kDa and is located in the cytosol. However $iPLA_2$ does behave like $sPLA_2$ by demonstrating no apparent substrate specificity for arachidonic acid containing

phospholipids. A unique feature of the $iPLA_2$, in addition to the absence of a Ca^{2+} requirement, is that it contains eight ankyrin motifs at the N-terminal half of the molecule and suggests it may bind both tubulin and integral membrane proteins, including several proteins that regulate ionic fluxes across membranes [131-133]. Other Ca^{2+} independent PLA_2 activities have been reported in many tissues and cell homogenates with distinct substrate specificities at an optimum pH range 6 to 9 but as yet they have not been characterised in detail [108].

1.7.4 Group IIa $sPLA_2$

Many physiological and pathological roles have been proposed for group IIa $sPLA_2$ these include inflammation [112,134], eicosanoid generation [104,135], antimicrobial activity [115,136], ischemia and tissue injury [115,137,138], anticoagulation [139]and degranulation [140]. The involvement of group IIa $sPLA_2$ in inflammation is of particular interest as it may be involved in the inactivation of lung surfactant in inflammatory lung diseases such as asthma and ARDS [114]. Therefore the focus of this section will be on the human group IIa $sPLA_2$: the sources of this enzyme, what leads to its expression, its inhibitors as well as the structure, mode of catalytic activity and contribution to inflammation.

Group IIa $sPLA_2$ is synthesised in a precursor form containing a signal sequence and is then processed to a mature enzyme during translocation from the cytosolic to luminal side of the endoplasmic reticulum [141-143]. Group IIa $sPLA_2$ is not synthesised as a zymogen, unlike group I PLA_2, indicating that it is present intracellularly in a form inactivated by some unknown mechanism so that it does not hydrolyse intracellular membrane phospholipids. After secretion a notable feature is that group IIa $sPLA_2$ appears incapable of eliciting hydrolysis of phospholipids in the plasma membrane of intact cells [144-147]. In contrast the enzyme expresses high activity using anionic bacterial cell membranes and this dramatic difference must reflect the physiological role of the enzyme [148-151].

1.7.4.1 Sources of group IIa PLA_2

The proposed involvement of group IIa $sPLA_2$ in inflammation has been confirmed by the finding of variable expression of group IIa $sPLA_2$ in several tissues associated with

organs related to inflammatory responses, such as the spleen, thymus, intestine, tonsil, liver and bone marrow[133,141,142,152-158]. The constitutive expression of group IIa $sPLA_2$ has also been identified in inflammatory effector cells, such as neutrophils [159-163], macrophages[164,165] and mast cells [166-170]. These findings strongly suggest that group IIa $sPLA_2$ is involved in inflammatory responses and host defence. The association of group IIa $sPLA_2$ with inflammation has been validated with reports that have detected large amounts of the enzyme at various inflamed sites and in the plasma of patients with arthritis and septic shock, as well as in experimental models of inflammation where the plasma level of the enzyme may rise over 50-fold compared to controls [134,171-175].

1.7.4.2 Induction of gene expression of group IIa $sPLA_2$

The major inducers of group IIa $sPLA_2$ expression are the pro-inflammatory cytokines, such as IL-1α and -β and TNFα, which induce transcription and secretion of group IIa $sPLA_2$ [176-178]. IL-6 is another cytokine that has been reported to induce group IIa $sPLA_2$ expression, and is thought to occur due to the presence of IL-6-responsive elements at two locations in the promoter region of the group IIa $sPLA_2$ gene [115]. The expression of group IIa $sPLA_2$ is suppressed by anti-inflammatory glucocorticoids [165 179-181],and the inhibition of TNFα secretion probably elicited by the release of prostaglandin E_2 (PGE_2)[182].

1.7.4.3 Pharmacological inhibitors of $sPLA_2$

The use of specific chemical inhibitors allows an assessment of the involvement of a specific PLA_2 in a given process and these inhibitors may prove to be crucial drugs for the treatment of PLA_2 related diseases. Although many $sPLA_2$ inhibitors have recently been described, only one is a relatively specific and effective $sPLA_2$ inhibitor called 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy) propane sulphonic acid (LY311727). LY311727 is an indole derivative that is an excellent example of rational drug design. The chemical structure was refined as a result of screening the X-ray structure of the lead inhibitor and its derivatives when complexed with human group IIa $sPLA_2$ [183]. This compound binds in the low nanomolar range and is selective for group IIa $sPLA_2$ over group IIb $sPLA_2$; however, it does not necessarily distinguish among other $sPLA_2$ groups as it also inhibits the more recently discovered group V $sPLA_2$ [184].

1.7.4.4 Structure of $sPLA_2$s

The $sPLA_2$ enzymes are water soluble, compact globular proteins that have about 120 amino acid residues. The amino acid sequences and high disuphide bond content (seven) are conserved among all Group I and II species [103]. All contain 50% α-helix and share a small region of anti-parallel pleated sheet (termed 'beta wing'). The group II $sPLA_2$ requires a Ca^{2+} ion that binds in the Ca^{2+} binding loop, which is conserved in the $sPLA_2$s [117,185,186]. The presence of the Ca^{2+} ion is required for binding of the substrate and it is also thought to be essential for electrophillic catalysis in the chemical step of hydrolysis [187]. The key catalytic residues are His-48 and Asp-99, which form a catalytic dyad responsible for the generation of an OH^- species during the hydrolysis [188].

1.7.4.5 Interfacial binding and catalytic action of $sPLA_2$

The $sPLA_2$ enzymes act on natural phospholipid with very low critical micelle concentration where the monomer concentration of phospholipid in water is extremely low. Therefore the $sPLA_2$s will normally see the phospholipid substrate as part of an interface such as a micelle, vesicle or membrane. The binding to the interface results in considerable enzyme activation, the enzyme is far more active in this state than in free solution [189]. It is proposed that the effect of interfacial binding is to seal the enzyme to the membrane, in order to facilitate phospholipid transfer into the catalytic site slot. The interfacial binding properties are different for various mammalian $sPLA_2$s and are likely to account for some of the variation in substrate specificity demonstrated by these enzymes [144,190-192]. If the enzyme cannot bind productively to the interface, it cannot perform catalysis. Other differences in substrate specificity may be due to the active site selectivity of the enzyme resulting in classical enzyme specificity.

The binding of $sPLA_2$ to the interface is a process separate from the binding of a single phospholipid substrate molecule into the catalytic site of the enzyme (figure 1.9) as the enzymes catalytic site and interfacial recognition surface are topologically distinct [193,194]. The catalytic site slot starts at the surface of the enzyme where it contacts with the interface and runs all the way through the enzyme [187,195,196]. During hydrolysis a phospholipid molecule occupies the catalytic slot, with the end of the polar head group

protruding through the face of the enzyme that is opposite to the interface. The *sn*-2 fatty acid is then cleaved when the His and Asp residues at the catalytic site polarise a bound water molecule, which then attacks the carbonyl group [103]. Crystallographic studies suggest that no conformational change of the enzyme occurs when a phospholipid analog occupies the active site.

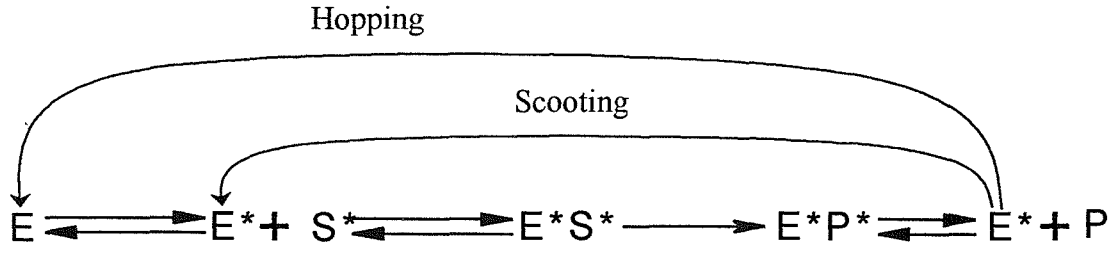


Figure 1.9 Interaction of enzyme and substrate. The enzyme in the aqueous phase (E) binds to the interface (E) where it carries out binding and hydrolysis of a substrate molecule (S*) to release the products (P*) at the interface. If the enzyme dissociates from the interface after each catalytic event then the enzyme is said to be in "hopping mode". In contrast, " scooting mode" occurs when the enzyme stays associated to the interface after each catalytic event and goes on to hydrolyse successive phospholipid molecules.*

Once bound to the substrate there has been uncertainty as to whether the PLA_2 stays associated with the interface while it hydrolyses successive phospholipid molecules ("scooting mode") or whether it dissociates from the interface after each catalytic event ("hopping mode") (see figure 1.9)[197]. Work by Jain and co-workers has demonstrated that the scooting mode of PLA_2 hydrolysis occurs not only with pure anionic vesicles [198], but it was also observed with vesicles of zwitterionic lipids so long as a critical amount of anionic lipid was present [199].

1.7.4.6 Preferential hydrolysis

Group IIa $sPLA_2$s preferentially hydrolyse negatively charged phospholipids. Work done by Kinkaid and Wilton has shown that Group IIa $sPLA_2$ demonstrates a substrate specificity, showing almost zero activity of $sPLA_2$ with egg PC or synthetic 18:1/18:1PC, but high activity with 18:1/18:1PG [200]. The presence of membrane bound anions, such as PG and PS enhances the hydrolysis of PC by the human $sPLA_2$. In particular, cholesterol sulphate, which is not a substrate, dramatically enhances the hydrolysis of PC. These

results suggest that the enzyme first requires to bind to an anionic surface for interfacial binding before hydrolysis of phospholipid molecules can occur[200]. A combination of interfacial binding and substrate specificity of $sPLA_2$ may explain why cells are not hydrolysed under normal conditions, as PC is the major phospholipid in the outer monolayer of plasma membrane and also in plasma lipoproteins. Neither of these interfaces normally contains anionic phospholipid in the outer monolayer.

Human group IIa $sPLA_2$s requirement for a negative interface is a characteristic that is utilised as part of the antimicrobial arsenal mobilised by the host in response to invading micro organisms [115]. Bacteria have a high content of PG in their outer membrane [201]and are therefore more susceptible to $sPLA_2$ hydrolysis. Phagocytosis of Gram-negative bacteria by neutrophils is an essential first line defence against invading bacteria [159]. The subsequent bacterial envelope phospholipid degradation has been proposed to be due to PLA_2 hydrolysis as the extent of intracellular destruction of these ingested bacteria is closely linked to the magnitude of the PLA_2 action [136]. It is probable that a major physiological role of human group IIa $sPLA_2$ is part of the innate defence system of the body.

1.7.4.7 $sPLA_2$ in inflammation

The detection of $sPLA_2$ in a wide variety of inflammatory conditions and sites, such as human rheumatoid arthritis [141,143,202], serum and colonic mucosa of patients with Crohn's disease and ulcerative colitis [203-205] led to the proposal that it plays a critical role in the process of inflammation. Indeed, the severity of rheumatoid arthritis [206,207], Crohns disease and ulcerative colitis [203-205] was found to parallel the serum levels of group IIa $sPLA_2$. It has been suggested that the group IIa $sPLA_2$ at inflamed sites is probably secreted from leukocytes and resident cells that are activated by pro-inflammatory stimuli, such as pro-inflammatory cytokines. However the mechanisms whereby group IIa $sPLA_2$ potentiate the inflammatory processes *in vivo* have not been clarified.

1.7.4.8 Involvement of PLA_2 in inflammatory lung diseases

PLA_2 action has been established in diseases associated with inflammation. However the effect $sPLA_2$ exerts on the lung has only recently been investigated. Lung surfactant has

an unusually high proportion of PG and has been proposed to be a substrate for $sPLA_2$-mediated hydrolysis. There have been a few studies suggesting the involvement of PLA_2s in the pathogenesis of inflammatory lung disorders such as asthma [4,208] and ARDS [3,114]. For instance in asthma there has been a reported antigen induced generation of lysophospholipids and an increase in $sPLA_2$ in airways of allergic patients when compared to saline challenged controls [4]. Bowton (1997) also reported an increase in $sPLA_2$ along with arachidonate in bronchoalveolar lavage fluid (BALF) from antigen challenged asthmatics when compared with control subjects [208]. Further studies *in vitro* have shown that glucocorticoid treatment diminishes the inflammatory stimuli induced increased expression of $sPLA_2$ by tracheobronchial smooth muscle cells and alveolar macrophages[209].

The association of increased PLA_2 levels with lung diseases has also been demonstrated in ARDS; a multifactorial disease with poor prognosis (see section 1.4). Reported elevated levels of serum PLA_2 in acute pancreatitis and sepsis correlated to poor clinical outcome, frequently leading to ARDS [134,175,206,210,211]. A recent study on BALF from patients with ARDS showed an increase in PLA_2 activity that correlated positively with lung injury score. Two iso-forms of PLA_2 were identified: a group II PLA_2, and one that was biochemically and immunochemically distinct from group I, II, and cytosolic PLA_2 [3]. This will be discussed further in chapter 7.

1.8 Mass Spectrometry

Mass spectrometry is a rapidly developing field; it can be used to analyse molecules (i.e. proteins, phospholipids, and fatty acids) according to their mass/charge ratio (*m/z*). It is advantageous over previous methods of phospholipid analysis as it has unequalled levels of detection and sensitivity. The fundamental basics of mass spectrometry with particular reference to the electrospray ionisation mass spectrometer (ESI-MS) will be described as all the mass spectrometry analysis of phospholipids in this thesis was performed on a triple quadrupole ESI-MS.

A simple mass spectrometer contains the following elements:

1. Ionising source; to produce ions from the sample to be analysed.

2. Analyser; to separate these ions according to their mass (there can be more than one of these).
3. Detector; to detect the ions and measure their mass/charge (*m/z*) ratio and abundance.
4. Computer; to process the data and control the instrument.

To appreciate the variety of possibilities some of the ion sources, mass analysers and detectors available are listed below:

Ion sources: Electron impact source, chemical ionisation source, Fast Ion or Atom Bombardment Ionisation (FAB), Field Desorption (FD), Laser Desorption (LD), Plasma Desorption (PD), Thermospray (TSP), Electrospray (ESI), Inductively Coupled Plasma (ICP) and Atmospheric Pressure Ionisation (API).

Mass analysers: Quadrupolar analyser, Time-of-Flight analyser, Magnetic and Electromagnetic analysers and Ion Cyclotron Resonance and Fourier Transform Mass Spectrometry.

Detectors: Photographic plates, Faraday cylinders, Electron multipliers, Array detectors and Photon multipliers.

The mass spectrometer used in this thesis was a Fisons VG quattro II that has an electrospray ionisation source coupled to two quadrupole analysers separated by hexapole collision cell. The detector is a Dynolite™ detector system, which is a low noise photomultiplier.

1.8.1 Electrospray ionisation

Electrospray ionisation allows soft ionisation of a sample in solution to produce a spray of fine droplets. The molecules are ionised by applying a strong electric field, under atmospheric pressure, to the sample solution passing through a capillary tube. The electric field is obtained by applying a potential difference of 3-6kV between this capillary and counter electrode (figure 1.10). The high potential and small radius of curvature at the end of the capillary tube creates a strong electric field that causes the liquid to break and form highly charged droplets. The solvent evaporates from the sample ions as they pass through into the analyser region of the mass spectrometer. The characteristics of the molecule determine whether it is detected in positive or negative ionisation. PC is detected in positive ionisation and also in negative ionisation because

of its zwitterionic nature. PG and PI are anionic phospholipids and are detected in negative ionisation. If the molecule has several ionisable sites then multiply charged ions could be formed. Phospholipids have only one ionisable site so the *m/z* ratio is equal to the mass.

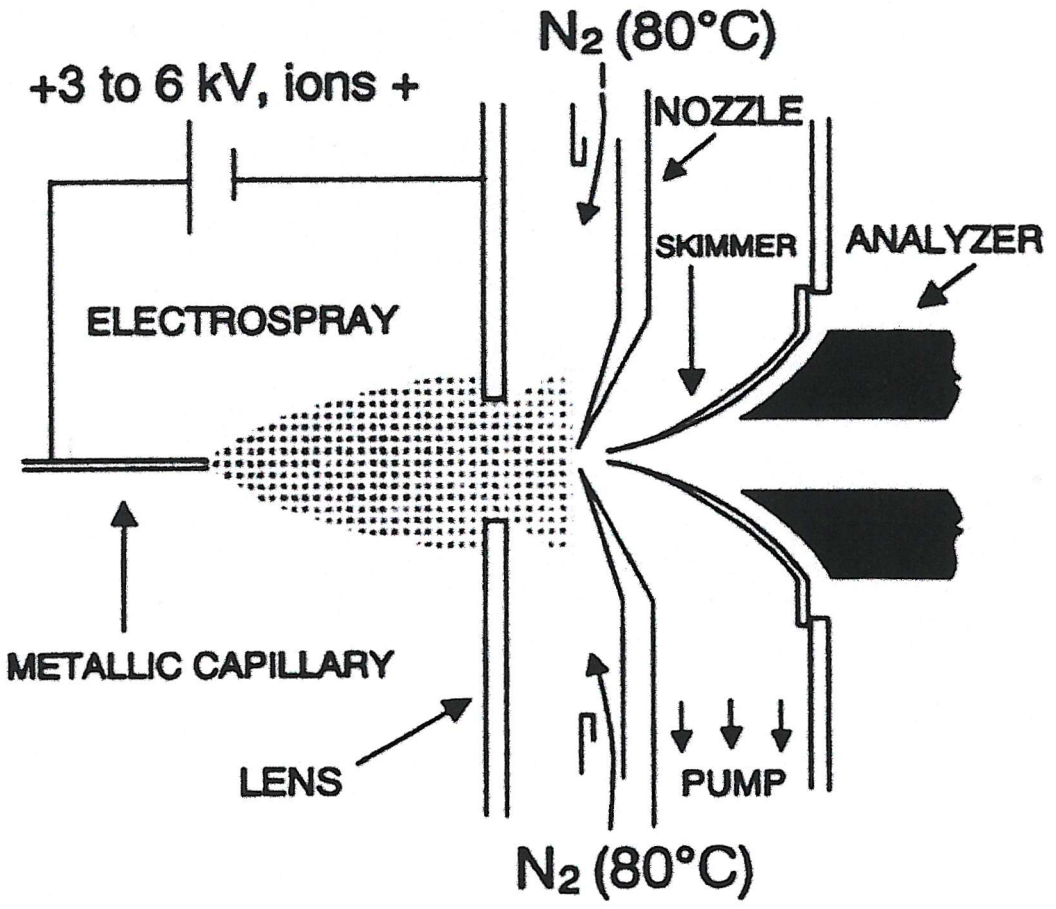


Figure 1.10 Diagram of an electrospray source[212]

1.8.2 Quadrupole analyser

The quadrupole is a mass filter that uses the stability of the trajectories to separate ions according to their *m/z* ratio. A quadrupolar field is achieved by four rods with circular or ideally hyperbolic section creating a DC compartment and an AC compartment such that only ions with a given *m/z* pass through. The relative intensities of these ions are then detected at the detector and a mass spectrum can be produced. A mass spectrum has the *m/z* on the x axis and the relative intensities on the y axis (figure 1.11).

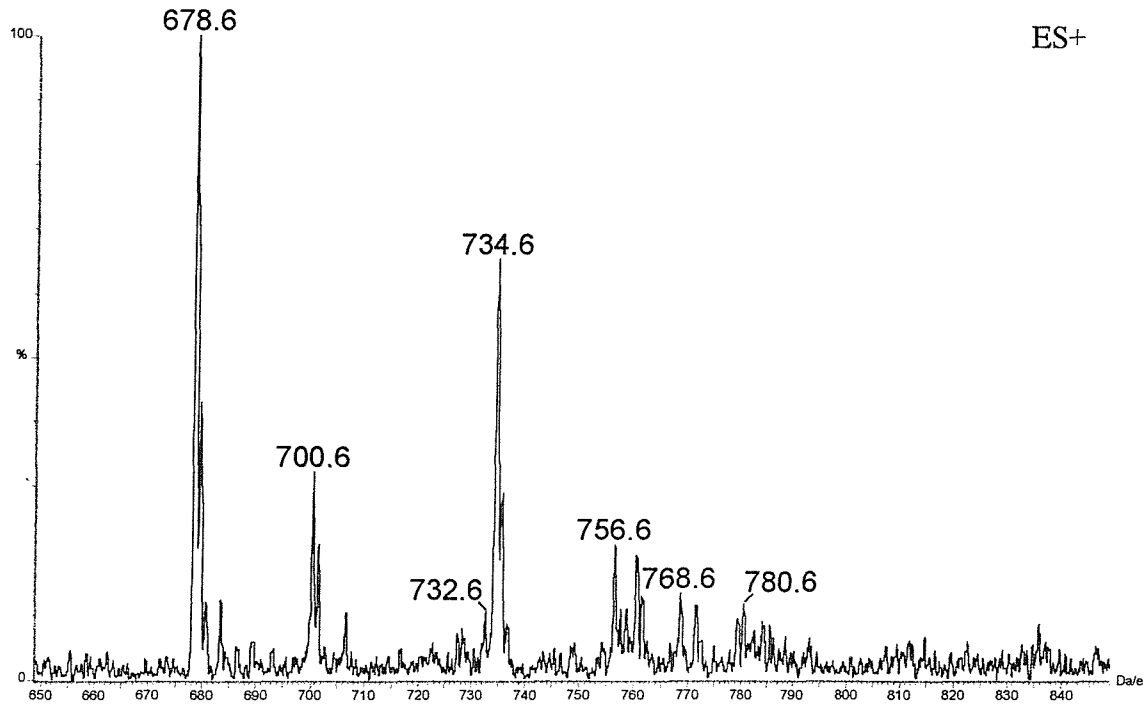


Figure 1.11 A positive ionisation mass spectrum of a lipid extract of human bronchoalveolar lavage fluid (BALF)

1.8.3 Tandem mass spectrometry

Tandem MS-MS can only be performed on mass spectrometers that have 2 mass analysers and a collision cell (figure 1.12). The triple quadrupole ESI-MS that was used had this facility, however for the routine analysis of phospholipids only the first mass spectrometer (MS1) was used. Tandem MS-MS aids in identifying the structural conformation of compounds. There are many different modes of data acquisition that can be performed to help gain such structural information and only two of these modes will be described, namely daughter ion analysis and the parent ion scan.

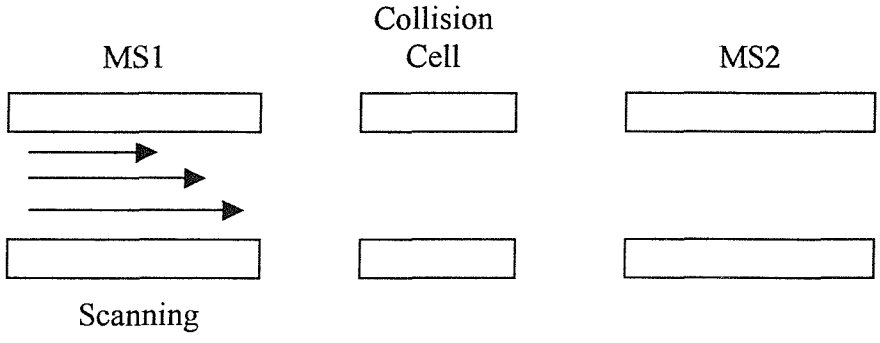


Figure 1.12 Diagrammatic representation of a triple quadrupole mass spectrometer using only MS1 for the routine analysis of phospholipids.

1.8.3.1 Daughter ion analysis

Daughter ion analysis was used for structural elucidation of the various mass ions (figure 1.13). The main use of daughter ion analysis was to determine the fatty acids attached and there position on the glycerol phosphate backbone. In this form of analysis a single mass ion, produced at the source was selected for transmission through to MS1. The selected ion was then accelerated into the collision cell by means of a collision energy voltage. Fragmentation was induced in the cell using pressurised argon gas colliding with the selected mass ion. MS2 was scanned to record all of the ions exiting the collision cell. The ions passing through MS1 are termed parent or precursor ions and the ions produced by fragmentation in the collision cell are termed daughter, fragment or product ions.

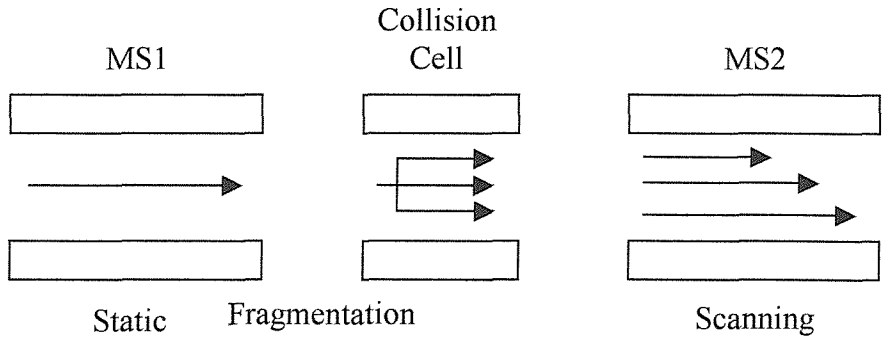


Figure 1.13 Diagrammatic representation of a daughter ion scan. MS1 was fixed to let only a selected mass ion (parent ion) through, fragmentation was then induced in the collision cell and the resultant daughter ions were scanned in MS2.

1.8.3.2 Parent ion scan

Parent ion analyses were used to identify and confirm known groups of compounds that produce a common daughter ion on fragmentation. MS2 was set to monitor only the mass of the specified daughter ion. MS1 was set to scan a mass range, which included all the possible parent ion masses. Each time, as MS1 was scanning an ion which was transmitted and subsequently fragmented to give a daughter ion of the selected mass the parent ion was identified. MS1 produced a mass spectrum of all the parent ions in the given mass range.

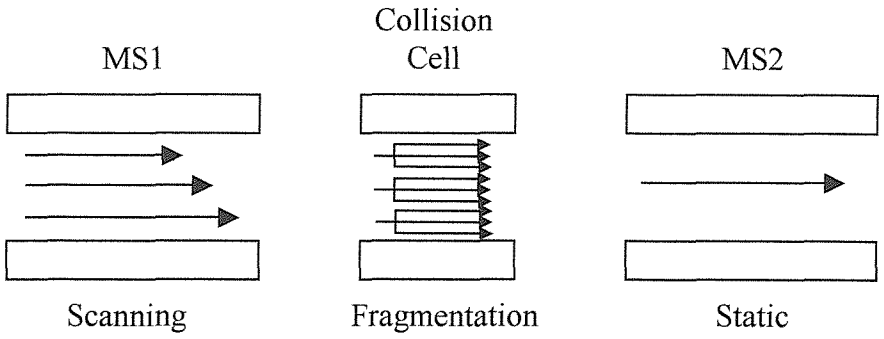


Figure 1.14 Diagrammatic representation of a parent ion scan. MS2 was fixed to a particular daughter ion whilst MS1 scanned the given mass range for all the parent ions that produce the daughter ion upon fragmentation.

1.9 Aims

The aim of this project was to develop the analysis of individual phospholipid molecular species using ESI-MS and to use this method to study lung surfactant phospholipids. The surfactant phospholipid compositions were studied in healthy animals and humans as well as in acute asthma and ARDS. The hypothesis investigated in this thesis was that the action of the pro-inflammatory enzyme $sPLA_2$ might account for some of the changes observed in inflammatory lung diseases.

Chapter Two

Methods

2.1 Materials

All materials were obtained from Sigma-Aldrich (Poole,UK) unless otherwise stated. Rats and rabbits were obtained from the animal house at Southampton General Hospital. Fatty acid binding protein (FABP) was provided by Andy Buckland and Jo Davies and was expressed in *E.coli* [219].

2.2 Sample processing

Bronchoalveolar lavage fluid (BALF) was collected from a variety of sources and these will be discussed in the relevant chapters. BALF was all processed by a common protocol. It was first filtered through a 100μm filter to remove mucus and large particles, followed by centrifugation at 400xg for 10 minutes to pellet the cellular components. The supernatant was then aliquotted and stored at -20°C.

2.2.1 Preparation of purified human lung surfactant

Dr P.Hockey collected BALF from control subjects; by wedging the fiberoptic bronchoscope into the appropriate bronchus and lavage was done with warm 0.9% (w/v) NaCl solution in six aliquots of 20ml and processed as in section 2.2. Purification of human lung surfactant from small volumes of BALF was achieved by sodium bromide discontinuous density centrifugation. 1ml of BALF was added to 1.5ml of 26.7% (w/v) NaBr in 0.9% (w/v) NaCl to give a concentration of 16% NaBr in the bottom of a 14ml ultracentrifuge tube. This mixture was overlaid with 7.5ml of 13% (w/v) NaBr in 0.9% (w/v) NaCl followed by 3ml of 0.9% (w/v) NaCl. The density gradients were then centrifuged at 110,000xg (30,000rpm, 6x14ml swing out rotor) for 100 minutes. The surfactant band was pipetted from the interface between the 13% NaBr and the 0.9% NaCl layers, resuspended in 0.9% (w/v) NaCl and centrifuged at 80,000xg (32,000rpm, 8 x 50ml fixed angle rotor) for 1 hour to pellet the surfactant and remove the NaBr. The pelleted surfactant was resuspended in 0.9% (w/v) NaCl and stored at -20°C until analysis.

2.3 Preparation of purified rat and rabbit surfactant

Two female New Zealand white rabbits (3 kg) and four female Wistar rats (250g) were given lethal injections of phenobarbitone. Trachae were cannulated with a polyethylene

tube connected to a three-way tap. An aliquot (10ml for the rats and 50ml for the rabbit) of 0.9% (w/v) NaCl was introduced into the lungs and the lavage effluents were then collected. This procedure was repeated five times. The pooled lavage was then centrifuged at 200xg (900rpm, fixed angle Sorvell rotor) for 10 minutes at 4°C to remove any cells. The cell pellet was resuspended in 0.9% (w/v) NaCl and then stored at -20°C. The surfactant was pelleted at 65,000xg (27,000 rpm 8x50ml fixed angle rotor) for 1 hour, then resuspended in 0.9% (w/v) NaCl and purified by density gradient centrifugation over 0.75M sucrose in 0.9% (w/v) NaCl at 85,000xg (27,000rpm, 6x14ml swing out rotor) for 1 hour. The interfacial pellet was then washed in saline, centrifuged at 30,000xg (20,000rpm, 10x10ml fixed angle rotor) for 30 minutes, and then resuspended in 0.9% (w/v) NaCl [44].

2.4 Preparation of rat and rabbit lamellar bodies

The lungs were removed from the animals after the lavage procedure, weighed and homogenised in 10mM Tris in 0.9% (w/v) NaCl. The crude homogenate contained 4ml of the buffer per g of lung. The homogenate was then centrifuged at 65,000xg (27,000rpm, 8x50ml fixed angle rotor) for 1 hour, the pellet was resuspended in 0.9% (w/v) NaCl and layered on 0.75M sucrose in 0.9% (w/v) NaCl density gradient for 1 hour at 85,000xg (27,000 rpm, 6x14ml swing out rotor). The interfacial band containing lamellar bodies was then diluted 1 in 3 with 0.9% (w/v) NaCl and centrifuged at 30,000xg (20,000rpm, 10x10ml fixed rotor) for 30 minutes, the pellet was then resuspended in 0.9% (w/v) NaCl [44].

2.5 Phospholipid phosphorous determination

Phospholipid phosphorous was determined on all collected supernatant according to the method described by Bartlett in 1959 [213]. Total lipid extract was performed on 800µl BALF, 300µl of tracheal aspirate, 20µl of purified surfactant and 20µl of purified lamellar body material, all samples were diluted with 0.9% (w/v) NaCl to give a volume of 800µl. The samples were mixed with 2ml chloroform/ 2ml methanol / 1ml distilled water and centrifuged at 1000xg for 5 minutes [214]. The lower phase was removed and dried under nitrogen at 40°C. 60µl distilled water and 150µl perchloric acid (60%) were added and heated at 180°C for 40 minutes, followed by 5µl hydrogen peroxide (30%) for

a further 30 minutes. Samples were cooled, 710μl distilled water, 40μl ammonium molybate and 150μl Fiske and Subbarow reducer were added and the mixture heated at 100°C for 10 minutes for colour development. The absorbance at 830nm was determined, and phospholipid concentration calculated against a standard curve of dimyristoylphosphatidylcholine (14:0/14:0PC)(Avanti Polar Lipids, Alabaster, USA) 0-60nmoles.

2.6 Preparation of samples for mass spectrometry

Aliquots of BALF, tracheal aspirates, purified surfactant and lamellar bodies containing 25nmoles of phospholipid phosphorous were extracted with chloroform and methanol according to Bligh and Dyer [214]. Internal standards of dimyristoylphosphatidylglycerol (14:0/14:0PG, 1nmole) and 14:0/14:0PC (5nmoles) were added before extraction. Aliquots of serum (50μl) were extracted by the same method, after adding 15nmoles of 14:0/14:0PC as internal standard. All lipid extracts were dried under a stream of nitrogen gas and stored at -20°C until analysis.

2.6.1 Separation of phospholipid classes using solid phase extraction

The individual phospholipid classes were separated prior to ESI-MS analysis of the purified rabbit surfactant both before and after incubation with PLA_2 enzymes and rabbit and rat purified lamellar bodies. The separation of the classes was essential in the lamellar bodies due to interferences in the negative ionisation mass spectra from the PE species. Rabbit surfactant phospholipid classes were also separated after incubation with the PLA_2 enzymes due to the presence of contaminating non-phospholipid components that were removed by solid phase extraction prior to ESI-MS analysis.

The dried lipid extract containing 50nmoles of phospholipid phosphorous (prepared as in section 2.5) was dissolved in 1ml of chloroform and loaded onto a preconditioned (1ml chloroform) NH_2 solid phase extraction column (Varian SPP, USA). The sample was allowed to drip through to ensure all the phospholipid had bound to the column. The PC fraction was removed from the column by applying 1ml chloroform: methanol (6:4, v/v), the fraction was collected and dried under nitrogen. 1ml of methanol was added to the column and the fraction containing the PE species was collected. The anionic

phospholipids were isolated from the column by applying 3mls of methanol: water: phosphoric acid (96:4:1, v/v) containing 40mM choline chloride. This fraction was dried down under nitrogen and then re-extracted according to Bligh and Dyer[214]. All dried fractions were then stored at -20°C prior to mass spectrometry analysis.

2.7 Mass Spectrometry

All the mass spectrometry was performed using a triple quadrupole instrument (Fisons VG Quattro II, Micromass UK) fitted with either an electrospray or a nanoflow interface. The electrospray was used for the quantitative analysis of phospholipids where PC molecular species were detected using positive ionisation conditions, while PG and PI species were detected under negative ionisation conditions. Confirmation of the identity of the various PC and PG mass ions was performed by tandem MS-MS using the nanoflow probe. The original method by Han and Gross in 1994[215] used a syringe pump to inject the sample, but this is time consuming, labour intensive and not suitable for the routine analysis of a large number of samples on a study. Therefore the method described by Han and Gross was refined to use rheodyne valve sample injection into a flow of mobile phase, thus enabling the routine analysis of small volume of samples.

2.7.1 Electrospray ionisation mass spectrometry (ESI-MS) of human BALF phospholipids

Extensive method development was undertaken to improve the analysis of phospholipids by ESI-MS and a brief description of the method development is described in section 3.2. The method described in this section was used to analyse the allergen challenged human BALF (chapter 6), tracheal aspirates from ARDS patients (chapter 7), guinea pig BALF (chapter 4) and PC and PG vesicles incubated with PLA_2 (chapter 5). The method used for the analysis of the other samples was very similar but different solvents were used to overcome problems with partial sodiation (see section 3.2.2). The dried lipid extracts (25nmoles) were dissolved in 25µl of the injection solvent, methanol: chloroform: water (7:2:1v/v) containing 1% (w/v) NH_4OH. A sample aliquot (5µl) was introduced into the capillary of the electrospray interface by rheodyne valve injection into a flow of mobile phase (methanol: chloroform: water (7:2:1 v/v) containing 1% NH_4OH), pumped at a flow rate of 50µl/minute. Spectra were acquired in both positive

and negative ionisation on alternating scans between *m/z* 400 and 900 with a scan time of 2 seconds and a resolution of 0.1 mass units. The various voltages applied to the sample in the mass spectrometer are shown in table 2.1. The voltages presented for tandem MS-MS are only typical values as the instrument was optimally tuned for each MS-MS analysis.

Table 2.1 Typical tune page settings used in the mass spectrometer

Settings	ESI-MS (MS-1)		Tandem MS-MS (MS-2)	
Ionisation mode	**-ve**	**+ve**	**-ve**	**+ve**
Capillary (kV)	2.5	3.0	1.35	1.2
HV lens	0.23	0.14	*	*
Cone (V)	77	48	40	30
Skimmer (V)	1.5	1.5	1.5	1.5
RF lens (V)	0.2	0.3	0.2	0.2
Source temperature (°C)	100	100	30	30

* The HV lens was removed when in nano-flow mode

2.7.2 Electrospray ionisation mass spectrometry (ESI-MS) of surfactant phospholipids

The method described in section 2.7.1 had problems with the PC species being partially sodiated. However, this was not a problem for the analysis of human BALF, tracheal aspirates from ARDS patients and PC and PG vesicles as they contained negligible 16:0/20:4PC (see section 3.2.2 for more details). The method in section 2.7.1 was modified to analyse PC species from the other surfactant samples as the sodium adducts. This method was more time consuming. The samples were prepared as in section 2.5 but each sample was divided in two and placed in two insert vials, this was so the negative and positive ionisation could be run using different solvents, all the other conditions remain the same. The PC molecular species were run under positive ionisation as the sodium adducts, this was achieved by the addition of 20mM sodium acetate to the injection solvent of chloroform: methanol (1:2 v/v). The dried lipid extract was dissolved in 10μl of the injection solvent and then injected into a mobile phase of

chloroform: methanol: water (7:2:1 v/v) at a flow rate of 50µl/minute. The PG molecular species were collected under negative ionisation using the same conditions as described in section 2.7.1, but only dissolving the lipid extract in 10µl of injection solvent (methanol: chloroform: water (7:2:1 v/v) containing 1% (w/v) NH_4OH). The voltages applied to the sample in the mass spectrometer remained the same (table 2.1)

2.7.3 Electrospray ionisation mass spectrometry (ESI-MS) of serum phosphatidylcholine

Serum samples were dissolved in 75µl of the injection solvent, methanol: chloroform: water (7:2:1 v/v) containing 20mM sodium acetate, and an aliquot (2.5µl) was introduced into the ESI-MS by injection into a flow of methanol: chloroform: water (7:2:1 v/v) pumped at a flow rate of 50µl/minute. Spectra were collected only in the positive ionisation mode, using the conditions described in section 2.7.1.

2.7.4 Tandem mass spectrometry (MS-MS)

Lipid extracts of samples were prepared as in section 2.5 and then dissolved in an appropriate solution typically methanol: chloroform (1:2 v/v) containing 1% NH_4OH to make a sample solution of 1nmole/µl. An aliquot (10µl) of sample solution was loaded into a borosilicate tip (Micromass, UK) and then attached to the nanoflow probe. The sample was gently ionised using conditions described in table 2.1. Fragmentation was induced using argon gas typically at $2.7x10^{-3}$mbar with collision energy of 30eV. Spectra were acquired in either positive or negative ionisation using the Multiple Channel Acquisition (MCA) mode with a scan time of 3 seconds and a resolution of 0.1.

2.7.5 Processing the mass spectra data

The mass spectrometer generates an enormous amount of data, which can be visualised as a spectrum with the mass/charge ratio (*m/z*) being plotted against intensity (see figure 2.1a). The individual molecular species were identified according to their *m/z* ratio or molecular weight as phospholipids only have one charge (therefore $z=1$). The concentration of each molecular species in the sample was calculated by comparing the peak size to the internal standard peak size. For instance in figure 2.1 the internal standard 14:0/14:0PC (*m/z* 700) is equivalent to 5nmoles and the main PC species in

BALF is 16:0/16:0PC (*m/z* 756) which therefore must be present at approximately 10nmoles in the aliquot of BALF taken.

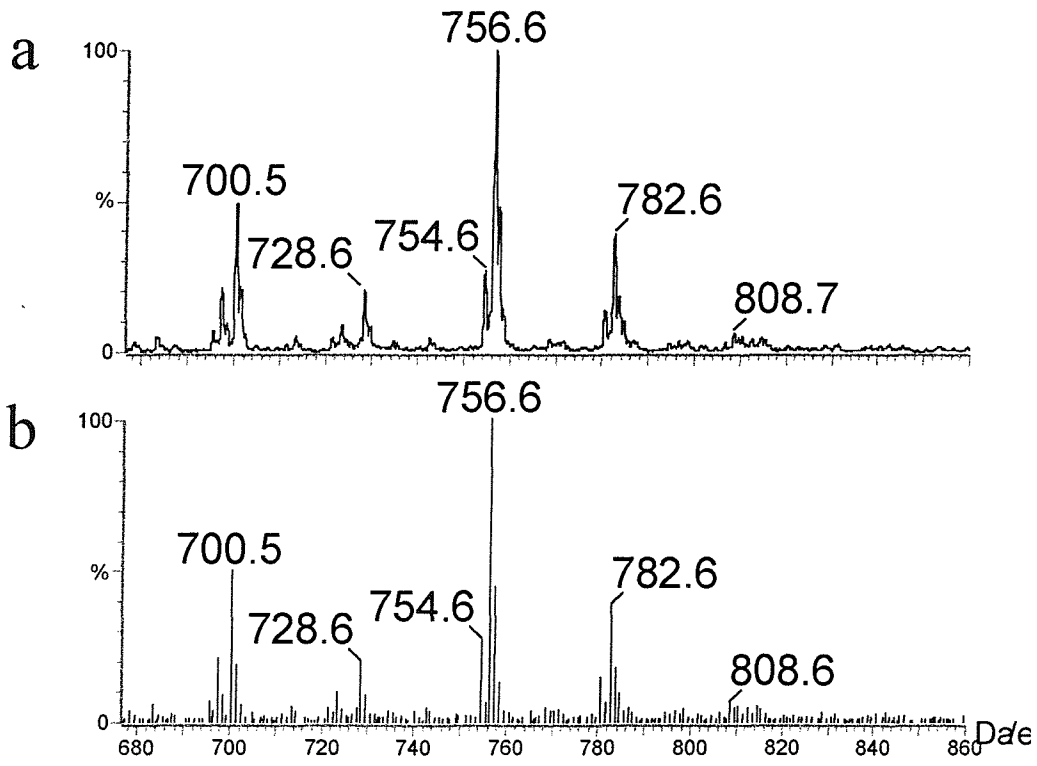


Figure 2.1 Mass spectra of human BALF under positive ionisation, (a) typical spectrum (b) centered spectrum

Spectral data was averaged over a scan period of one minute and then the background noise was subtracted followed by smoothing using the moving mean method. In order to analyse the data a numerical value must be assigned to each mass ion. The mass spectrometer collects 16 data points per dalton so each mass ion has about 16 intensity values. To assign one numerical value for each mass ion the spectrum must be centered, whereby one peak height value is given for each mass. The centered spectrum looks like a series of vertical lines or sticks as they are sometimes referred to (see figure 2.1b). The data was then displayed as a list of peak intensities as opposed to a picture. These numerical values were then copied onto the computer's clipboard and retrieved into an excel spreadsheet.

I have written a macro that identifies selected mass ions and their relative intensities. The macro finds the selected mass ions and copies the corresponding relative intensities

into a separate results sheet with the filename of the sample at the top of the column. The macro was written in visual basic and the first page of the macro used to analyse PG species from surfactant is shown in appendix 1. Separate macros were written to obtain data from positive and negative ionisation spectra. The advantages of the macro over the previous method of manually selecting the peaks and noting them down, is it is much quicker, allowing for faster generation of results. There are limitations to the macro, for example if a value for a selected mass ion is missing the program will stop working. However, the macro has revolutionised the analysis of mass spectra, but it does now require greater user editing when acquiring the data in order not to analyse noisy traces. When the data was in the results sheets it was then possible to calculate the isotope effect followed by conversion of values to nmoles/ml and relative percentage compositions.

2.7.5.1 Calculation of theoretical isotope effect

Most of the elements appear in nature as isotope mixtures, table 2.2 shows the elements in a phospholipid molecule e.g. 14:0/14:0PC ($C_{36}H_{72}O_8NPNa$) and their relative abundance. Thus, natural carbon is a mixture of 98.9% isotope ^{12}C and 1.1% ^{13}C. The mass of a phospholipid molecule will vary depending on how many isotopes are present. The most abundant isotope after ^{12}C in a phospholipid molecule is ^{13}C, the effect these isotopes exert on the mass of a phospholipid molecule is referred to as the ^{13}C effect. Consequently a phospholipid molecule will not only produce a mass (m) peak but also an m +1, m +2, m +3 etc (figure 2.2). Using the isotope-modelling tool in the masslynx program (Micromass, UK) the percentage of the parent peak that contributes to the m+1 and m+2 peaks for all the molecular species was theoretically calculated. For example the m +1 peak for 14:0/14:0PC is theoretically 41.79% of the parent peak and m+2 is 10.11% parent peak. Comparing the theoretical values of the ^{13}C effect with some actual values found them to be very similar in cases where there was no peak interference.

Table 2.2 Relative abundances of naturally occurring isotopes

Element	Relative abundance	Mass
Carbon	98.9000	12.00000000
	1.1000	13.00335484
Hydrogen	99.9850	1.00782504
	0.0150	2.01410179
Oxygen	99.7620	15.99491464
	0.0380	16.99913060
	0.2000	17.99915939
Nitrogen	99.6340	14.00307401
	0.3660	15.00010898
Sodium	100.0000	22.98976970
Phosphorous	100.0000	30.97376340

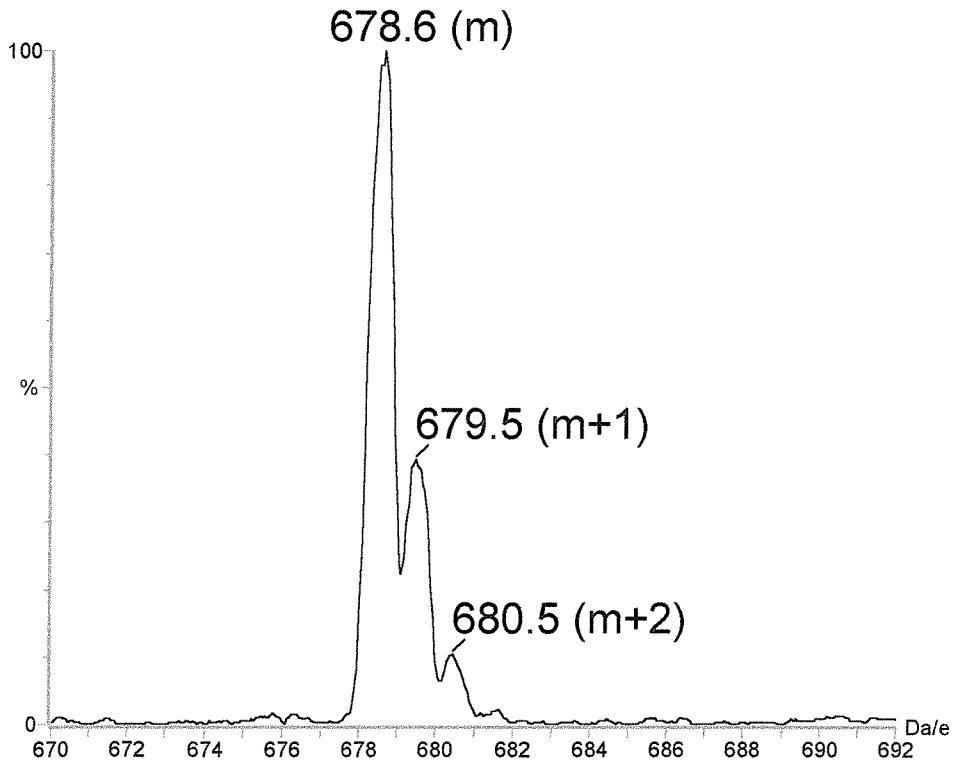


Figure 2.2 Isotope effect on the internal standard 14:0/14:0PC (m/z 678).

When accurately measuring the proportions of each molecular species of phospholipid in a complex mixture of phospholipid molecular species it is important to take into account the isotope effect. In particular the case where phospholipids have a mass difference of

the isotope effect. In particular the case where phospholipids have a mass difference of 2 for example the presence of a double bond in a fatty acid would cause the mass of the phospholipid to be reduced by 2 mass units. For instance the m+2 peak of 16:0/18:2PC (*m/z* 780) would have the same mass as 16:0/18:1PC (*m/z* 782). This problem of the m+2 peak interfering with the analysis was overcome by removing the theoretically calculated m+2 peak from the peak where this phenomenon occurs. Table 2.3 illustrates an example of why it is necessary to remove the theoretically calculated m+2 peak. Three PC species with consecutive *m/z* values that differ by 2 mass units are listed accompanied by the intensity values obtained at the corresponding *m/z* values, the next column is the result of removing the m+2 peak where applicable and then finally the corrected intensity taking into account the m+1 peak in order to give a more accurate analysis.

Table 2.3 An example of calculating the ^{13}C effect

Molecular species	***m/z***	**Intensity**	**Intensity – (m +2 peak)**	**Corrected intensity**
16:0/18:2 PC	780	5.0×10^5	5.0×10^5	7.43×10^5
16:0/18:1 PC	782	8.0×10^4	1.43×10^4	2.12×10^4
16:0/18:0 PC	784	1.5×10^5	1.48×10^5	2.23×10^5

In the example shown in table 2.3, a proportion of the ion peak at *m/z* = 782 could be due to 16:0/18:2 PC m +2 peak (containing two ^{13}C atoms), failure to correct for the ^{13}C effect in this case would have led to a misleadingly high concentration for 16:0/18:1PC. The theoretical m +2 peak was removed where applicable and then using this new intensity value for the PC species the theoretical m + 1 peak was accounted for. These corrected intensities are shown in the last column of the table above and are then used to calculate nmoles and % composition.

2.7.5.2 Calculation of PI correction factor

Due to the lack of an available internal standard for PI and the inherent difficulty in making one a correction factor had to be used to quantify the PI molecular species using the PG internal standard (14:0/14:0PG). The PI response on the mass spectrometer

varied from day to day therefore an external standard was used to calculate the PI response in relation to the PG response prior to each batch of samples being analysed. The external standard used was an equimolar mixture of 14:0/14:0PG and a commercially available soya bean PI. The composition of the soya bean PI was established on the mass spectrometer (n=19) as listed in table 2.4. Knowing the composition of the soya bean PI it was possible to work out a correction factor for the PI response relative to 14:0/14:0PG. Figure 2.3 is a typical negative ionisation mass spectrum of the PG and PI mix, the spectrum has been centered so the PI correction factor can then be calculated.

Table 2.4 Percentage composition of soya bean phosphatidylinositol

PI species	***m/z***	**% Composition**
16:0/18:2	833	60.5
16:0/18:1	835	15.3
18:1/18:2	859	5.2
18:0/18:2	861	14.8
18:0/18:1	863	4.2

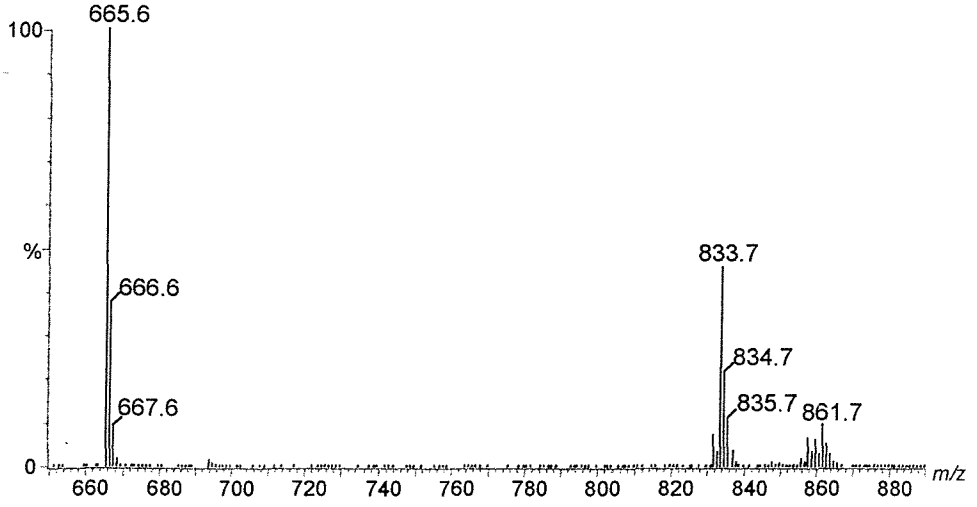


Figure 2.3 A negative ionisation mass spectrum of PG and PI mix that has been centered to enable the calculation of the PI correction factor

By analysing the centered mass spectrum of an equimolar mix of 14:0/14:0PG and the soya bean PI (figure 2.3) it was then possible to calculate a PI correction factor by comparing the relative intensities of 14:0/14:0PG(*m/z* 665) to 16:0/18:2PI(*m/z* 833) using the formula below.

$$\frac{60}{\%\ 16{:}0/18{:}2\ \text{PI peak relative to } 14{:}0/14{:}0\text{PG}}$$

For instance if PI and PG gave equal responses on the mass spectrometer then 16:0/18:2PI (*m/z* 833) would be 60% of the 14:0/14:0PG (*m/z* 665) and the correction factor would be 1. If the 16:0/18:2PI peak is greater than 60% of the 14:0/14:0PG peak then the correction factor is less than 1. In figure 2.3 the peak at *m/z* 833 is 46% of *m/z* 665, therefore the correction factor would be 1.3 (60÷46). The intensity values of the PI species were multiplied by 1.3 before nmoles/ml were calculated using the internal standard 14:0/14:0PG.

2.8 Preparation of recombinant human group IIa $sPLA_2$

2.8.1 Expression of human group IIa $sPLA_2$

Luria-Bertani (LB)-agar plates containing 10g/l Tryptone, 5g/l yeast extract, 5g/l sodium chloride, 15g/l agar were autoclaved for 20 minutes then supplemented with 100μg/ml ampicillin (sterilised through a disposable 0.45μM filter) and poured into a sterile petri dish to form a solid growth media. The plates were then used to grow *Escherichia coli (E.coli)* (B121 DE3) cells transformed with pET11a containing the synthetic PLA_2 gene [340] overnight at 37^{0}C. Single colonies were picked off and grown overnight in 10 ml LB containing 100μg/ml ampicillin at 37^{0}C and termed overnight cultures (ONCs). Two ONCs were added to a fluted flask containing 1litre of LB and 50μg/ml ampicillin and incubated at 37^{0}C on a shaker until the Optical density at 600nm ($O.D._{600}$) was between 0.6 -1.0. The cells were induced with 0.4mM Isopropyl-β-thiogalactopyranoside (IPTG) and grown for a further 5 hours before being harvested by centrifugation at 5,600 x g at 4^{0}C for 20 minutes.

2.8.2 Isolation of Inclusion Bodies.

The pelleted cells were resuspended in 50ml of resuspension buffer (50mM Tris.HCl pH 8.0, 50mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 0.5mM phenylmethylsulfonyl fluoride (PMSF)) containing 0.4% (v/v) Triton-X100 and 0.4% (w/v) sodium deoxycholate and washed for 20 minutes at 4^0C. The cells were sonicated for 15 seconds on, 15 seconds off for 15 cycles using an MSE-Soniprep 150 at medium amplitude, followed by centrifugation at 12,000 x g for 10 minutes at 4^0C to collect the cells. The washing of the pellet was repeated as earlier, but with 0.8% (v/v) Triton-X100 and 0.8% (w/v) sodium deoxycholate followed by sonication and centrifugation as described previously. The washing procedure was repeated at room temperature with 1% (v/v) Triton-X100, the pellet was collected by centrifugation and washed in resuspension buffer alone. The inclusion bodies were collected as the pellet after centrifugation[216].

2.8.3 Solubilisation of Inclusion Bodies.

The inclusion bodies were solubilised overnight at 4^0C in 25ml resuspension buffer containing 6M Guanidine.HCl and 5% β-mercaptoethanol. The solubilised protein was collected as the supernatant after centrifugation at 27,200 x g for 15 minutes at 4^0C.

2.8.4 Protein Refolding.

The protein was refolded by dialysing at a concentration of 0.1mg/ml (dialysing tubing had a molecular weight cut off at 8000, Spectra/Por 7, Medicell International Ltd, UK) against 4 litres of refolding buffer (25mM Tris.HCl pH 8.0, 5mM $CaCl_2$, 5mM cysteine, 900mM Guanidine.HCl) at 4^0C for 72 hours, with the buffer changed every 12 hours[217]. Prior to column purification of the refolded protein the excess Guanidine.HCl was removed by dialysing against 4 litres of dialysis buffer (20mM Tris.HCL pH 8.0, 2.5mM KCl) overnight at 4^0C. Any precipitated protein was removed by centrifugation.

2.8.5 SP-Sepharose Chromatography Column Purification.

The refolded protein solution was loaded onto a 5ml HiTrap SP-Sepharose column (Pharmacia Biotech, UK.) at 1ml/min then washed with Buffer A (10mM Sodium acetate pH 6.0) until the absorbance at 280 nm was zero. The protein was eluted with

Buffer B (Buffer A + 2 M KCl) over a linear gradient from 0 - 100%. Fractions (1.5 ml) were collected throughout the elution. The PLA_2 content of the fractions was established by measuring the absorbance at 280 nm and by using the continuous fluorescence displacement assay (see section 2.9) to determine PLA_2 activity. The fractions containing PLA_2 that eluted between 0.4-0.5M KCl were pooled.

2.8.6 Heparin-Sepharose Chromatography Column Purification.

The pooled fractions from the SP-Sepharose column were diluted 3 times with Buffer C (20mM Tris.HCl pH 7.4) to reduce the concentration of KCl before being applied to a 5 ml Hitrap Heparin -Sepharose column (Pharmacia Biotech, UK). The column was washed with Buffer C until the absorbance at 280 nm was zero. A gradient of Buffer D (Buffer C + 1 M KCl) was then run to achieve 40% KCl at 20 ml and 100% at 80 ml. The fractions were collected and assayed for PLA_2 content as before, with the fractions containing PLA_2 being pooled. The active enzyme eluted at 1M KCl. A final yield of 4.35mg of protein was formed from 2 litres of bacterial culture.

2.9. Fluorescence displacement assay

PLA_2 activity was measured using a continuous fluorescence displacement assay [218] in which the released fatty acid displaces the fluorescent fatty acid analogue DAUDA (11-(dansylamino)undecanoic acid), from recombinant rat liver fatty acid binding protein (FABP). The initial rate of loss of fluorescence was monitored (excitation was at 350nm and emission was measured at 500nm) with a Hitachi F2000 fluorimeter and all data were recorded with a microcomputer. A decrease in fluorescence indicates that DAUDA is being displaced from the FABP by fatty acids (figure 2.4). This decrease in fluorescence can be related to enzymatic activity, as fatty acids are products of PLA_2 hydrolysis of phospholipid substrates.

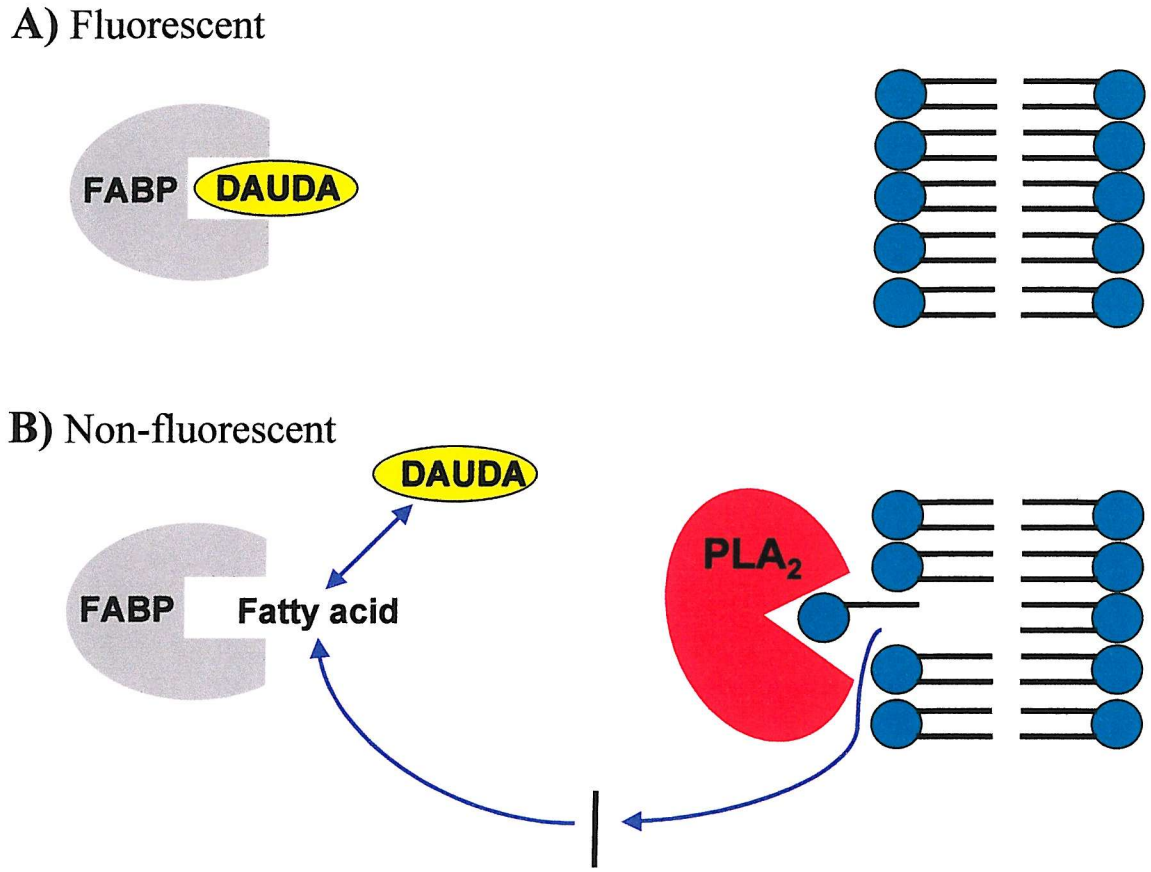


Figure 2.4 A diagram showing the basis of the fluorescence displacement assay. (A) Interaction of DAUDA and FABP in the absence of enzyme to produce a highly fluorescent complex. (B) Displacement of DAUDA from FABP by fatty acids released as a result of PLA_2 activity on phospholipid substrate with a resulting loss of fluorescence.

The assay cocktails were prepared in 20ml sterilin tubes so an aliquot could be taken into a cuvette and give the appropriate concentrations in 1ml. The final composition of the assay cocktail was 0.1M Tris/HCL, pH 8.0, 0.1M NaCl containing 1μM DAUDA (from a 1mM methanol stock solution), 2.5mM calcium and a varying phospholipid substrate concentration, typically 10μg/ml. 10μg of FABP (40μl of a 0.25mg/ml solution) was added to each individual assay immediately prior to measurements being taken.

Assays were performed at 37°C and the buffer was always equilibrated to this temperature prior to the addition of the phospholipid and DAUDA. The cocktail was gently mixed, and returned to the water bath before commencing the experiment. Figure 2.5 shows a typical trace obtained from the fluorescent displacement assay using a lipid extract of rabbit surfactant as a substrate. Firstly a blank rate was measured with no

extract of rabbit surfactant as a substrate. Firstly a blank rate was measured with no enzyme present (figure 2.5A) and secondly with 2μg of recombinant human group IIa $sPLA_2$ (figure 2.5B). The initial rate of hydrolysis was measured by calculating the drop in fluorescence relative to the calibration curve. The assay mixtures were calibrated in the absence of PLA_2 using 0, 2, 4, 6, 8 and 10nmoles of oleic acid (0.2mM stock) to displace the DAUDA and produce a calibration curve; an example trace is shown in figure 2.6. .

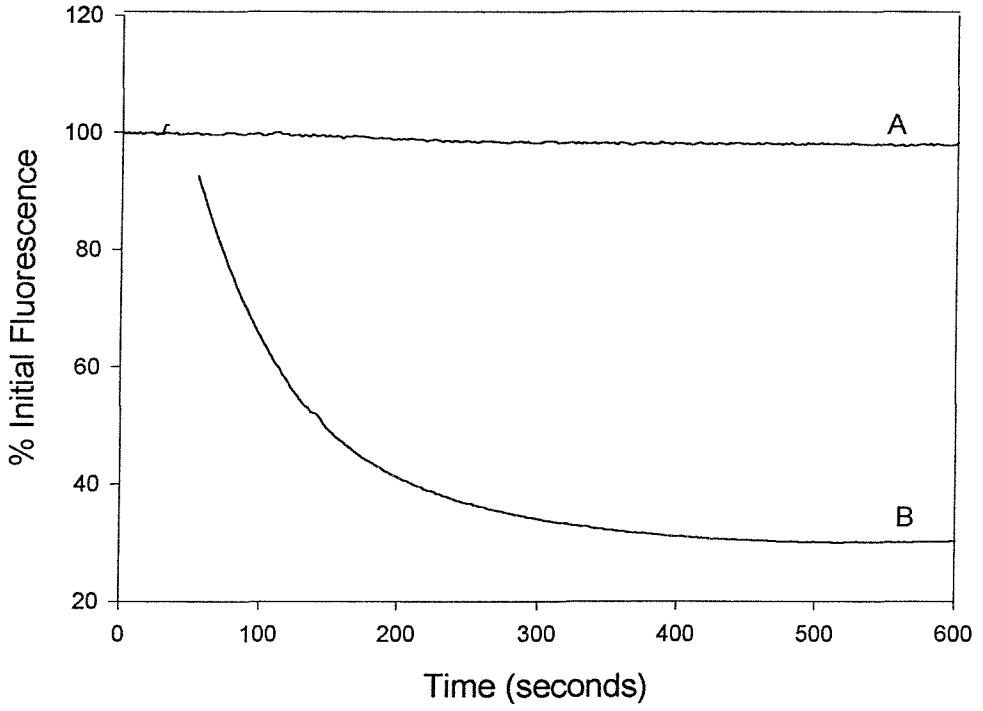


Figure 2.5 A fluorescent displacement assay trace of a lipid extract of rabbit surfactant with no enzyme (A) and with 2μg of recombinant human group IIa $sPLA_2$ (B).

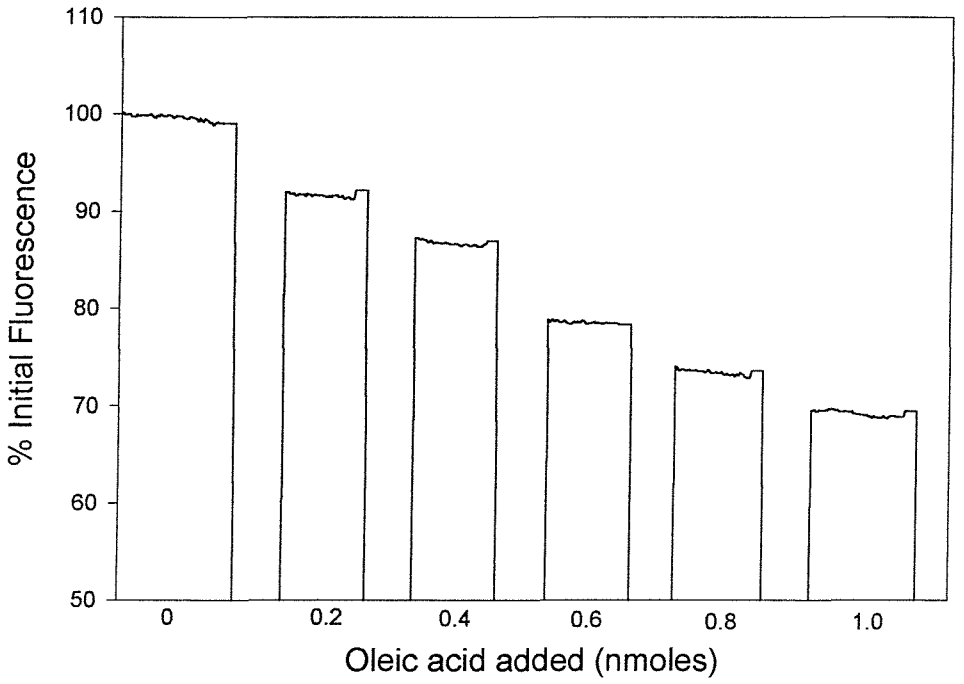


Figure 2.6 A calibration curve showing the decrease in fluorescence after the addition of increasing amounts of oleic acid.

2.9.1 Preparation of mixed small unilamellar vesicles (SUVs)

Stock solutions of the phospholipids were dried under nitrogen and resuspended in methanol to give the appropriate ratios and a concentration of 10mg/ml. An aliquot of this stock solution was rapidly injected into the assay cocktail using a Hamilton micro syringe. Typically for the fluorescent displacement assay 100µl was injected into 20ml to give a final substrate concentration 10µg/ml. The rapid injection of a methanol solution into an aqueous medium produces evenly distributed SUVs [220].

2.9.2 Preparation of rabbit surfactant vesicles for the fluorescent displacement assay

An aliquot (0.5ml) of purified rabbit surfactant was sonicated at 40W for 5 minutes to produce a clear solution. The solution was used in the fluorescence displacement assay to determine rates of hydrolysis with different PLA_2 enzymes. The sonication procedure was then repeated and the experiment performed again so that duplicates involved the use of two separate preparations of surfactant.

2.9.3 Preparation of methanol solution of rabbit surfactant

The purified rabbit surfactant was extracted according to Bligh and Dyer in chloroform and methanol [214]. The lower layer was removed, dried under nitrogen and resuspended in methanol to produce a solution of 1.4nmoles of phospholipid phosphorous/μl. To ensure the dried lipid extract dissolved, the methanol solution was then heated to 50°C prior to use in the fluorescence displacement assay.

2.10 PLA_2 hydrolysis of phospholipid vesicles as measured by ESI-MS

Assay cocktails were at a final concentration of 0.1M Tris/HCl, pH 8.0, 0.1M NaCl and 2.5mM calcium. This solution was allowed to equilibrate to 37°C prior to the addition of the selected phospholipid via a Hamilton syringe (41.6μl from a 10mg/ml methanol stock) to form SUVs (see section 2.9.2) to give a final concentration of 41.6μg/ml. 760μl of cocktail was added into a glass tube followed by the addition of 100ng of group IIa $sPLA_2$ (10μl of 10μg/ml dilution) giving a final concentration of 132ng/ml. This mixture was then placed on a shaker in an oven at 37°C for a pre-set time period of either 30secs, 60 secs, 5 min, 10 min or 30 min. The control samples were incubated for 30 minutes with 10μl of water instead of enzyme. When the incubation was complete 8μl of EGTA (from 500mM stock) was added to the cocktail giving a final concentration of 5.2mM, to prevent any further hydrolysis occurring. The solution was vortexed followed by the addition of 2ml methanol. The internal standards 14:0/14:0PC and 14:0/14:0PG were added in appropriate proportions depending on the percentage of PG present in the cocktail and vortexed. The lipid was extracted according to Bligh and Dyer by the addition of 1ml of chloroform followed by vortexing and the further addition of 1ml chloroform and 1ml analar water, vortexing and placed at -20°C for 1 hour [214]. The lower chloroform phase was removed and dried under nitrogen, redissolved in 150μl chloroform and transferred into a sample vial and dried under nitrogen. The dried sample was stored at -20°C until ESI-MS analysis was performed (see section 2.7).

2.11 Mass spectrometry to study the incubation of lung surfactant with PLA_2s

Purified rabbit surfactant was used as a substrate to study the effect of PLA_2s on lung surfactant. Rabbit surfactant was prepared as in section 2.3 and had a concentration of 1.19nmoles phospholipid phosphorous/μl. The substrate was either used in its native

purified form or sonicated. Sonication of the rabbit surfactant was performed using an MSE soniprep 150, 1.0ml of neat rabbit surfactant was sonicated for 5 minutes on ice, 1 minute on 1 minute off at 14 microns. The surfactant went from a cloudy to a clear solution.

84µl of substrate (100nmoles of phospholipid phosphorous) was added to 10µl 0.2M Tris/HCl, pH 8.0, 0.2M NaCl, 5µl CaCl (final concentration 2.5mM) followed by the addition of the enzyme. The cocktail was incubated in an oven at 37°C for a set time (typically 3 hours) whilst being continually shaken. The cocktail was removed and 8µl of EGTA (50mM stock) added followed by 700µl of analar water. This solution was mixed then added into a glass tube containing 2ml methanol and vortexed. The internal standards (14:0/14:0PC-20nmoles and 14:0/14:0PG-2nmoles) were added and the solution vortexed. 1ml of chloroform was added and vortexed followed by the addition of 1ml chloroform and 1ml methanol this was vortexed again and placed at -20°C for 1 hour. The lower chloroform layer was removed and dried under nitrogen, the individual phospholipid classes were separated using solid phase extraction (see section 2.6.1).

2.12 Western blot of ARDS lung fluid samples for group IIa $sPLA_2$

The lung fluid samples from the ARDS patients, human group IIa $sPLA_2$ and low molecular weight markers were run on a 15% SDS-PAGE gel. The resolving gel was made up of 5mls 30% bis-acyrlamide (National diagnostics), 0.45g Tris and 10mg SDS in 5mls analar water (pH 8.8), 5µl TEMED (N,N,N',N'-Tetramethyl-ethylenediamine) and a few crystals of ammonium persulphate, the stacking gel contained 0.11g Tris, 7.5mg SDS, 6.5mls analar water (pH 6.8), 1ml 30% bis-acrylamide 5µl TEMED and a few crystals of ammonium persulphate. Two concentrations of the lung fluid samples from patients suffering from ARDS were loaded onto the gel, either 10µl or 20µl of the samples made up to 20µl with analar water and mixed with 5µl of sample buffer (1.89g/50mls Tris, 10% SDS, 10% sucrose pH 6.8 (HCl), 0.0025% bromophenol blue, 5% β-mercaptoethanol). Low molecular weight markers (Enhanced ChemiLuminescence (ECL)) were run at either end of the gel, 1µl of markers mixed with 9µl of sample buffer. Four concentrations of human group IIa $sPLA_2$ (2,10,20 and

50ng) were run as standards these were also mixed with 5µl of sample buffer. All of the samples were boiled for 10 minutes allowed to cool prior to being loaded onto the gel.

The proteins were transferred from the gel onto a nitrocellulose sheet using a semi-dry transferring kit (Semi-Phor™, Hoefer scientific instruments, San Francisco, U.S.A.). The system was run at 45mA for 1 hour with a small amount of transfer buffer (5.8g Tris, 2.9g glycine, 0.37g SDS in 1litre of 20% methanol), as this was deemed to be the optimal conditions for the protein transfer. The nitrocellulose was removed and soaked in blocking solution (10% marvel milk in Phosphate Buffered Saline (PBS, 11.5g/l Di-sodium hydrogen orthophosphate, 2.96g/l sodium di-hydrogen orthophosphate, 5.84g/l sodium chloride, pH 7.5) containing 0.1% Tween 20) for 2 hours at 25°C. The nitrocellulose was washed in PBS Tween prior to the overnight incubation at 25°C with the primary antibody (1:500 dilution of anti human group IIa $sPLA_2$ from rabbit (batch R244) kindly donated by Dr. A. Kinkaid). The membrane was again washed and then incubated with the secondary antibody (1:2000 dilution of Horse radish peroxidase (HRP) anti-rabbit IgG from donkey) and streptavidin peroxidase (10µg/20ml PBS Tween) for 2 hours. The membrane was washed again before the blot was developed in the dark room.

ECL developing reagents (1ml of each) were applied to the nitrocellulose sheet and allowed to react for 1 minute. Then a piece of photographic film (Kodak Biomax MR film, Koadak, U.K.) was placed over the nitrocelluose in the developing cassette. The cassette was shut and the film was exposed for 1-5 minutes prior to removal and being placed in developer followed by stopper and then fixer, finally the film was washed in distilled water and then the film was analysed.

Chapter Three

Mass spectrometry of phospholipids

3.1 Introduction

The use of mass spectrometry for the analysis of individual phospholipid molecular species in clinical samples is a novel technique. The previous methods used for phospholipid analysis were multistep procedures, often involving hydrolysis or derivitization prior to analysis by either HPLC or TLC [221-223]. Unfortunately, these methods are time consuming and due to a lack in sensitivity require large amounts of phospholipid, which is not always available.

Recent advances in the study of phospholipids via electrospray ionisation mass spectrometry (ESI-MS) have enabled the development of a rapid and sensitive method for the analysis of phospholipids. The majority of the early work on the mass spectrometry of phospholipids was performed using fast atom bombardment mass spectrometry (FAB-MS), this involved the phospholipids being mixed into a matrix (i.e. triethanolamine) and then bombarded with high energy atoms (e.g. Xe or Cs^+)[224]. FAB-MS has been very useful in providing structural information on phospholipids, it cannot however be used to quantitatively analyse phospholipids. This is because it is a high-energy ionisation technique that results in substantial and differential fragmentation rates of molecular ions from individual phospholipid classes and molecular species [225,226].

The development of ESI-MS represented a major breakthrough in biological mass spectrometry, its low energy ionisation produces phospholipid molecular ions without fragmentation in an event that is largely independent of the surface properties of individual phospholipid classes and molecular species [227]. Although tandem MS via fast atom bombardment (FAB-MS/MS) provides complete structural information of phospholipids, ESI-MS offers several advantages over this technique, including lower background signals because of the absence of matrix ions, longer lasting and stable ion currents, ease of sampling, compatibility with liquid chromatographs and quantitative analysis of phospholipids.

At present ESI-MS has been used in very few studies with clinical implications. The majority of the work on the mass spectrometry of phospholipids has tended to be mainly method development using standards [228], and more recently on biological membranes [229-

[232]. Most of the work performed on the ESI-MS has used constant infusion via a syringe pump; however, this is not a suitable technique for the routine analysis of large numbers of samples. The method developed in this thesis uses rheodyne valve injection into a flow of mobile phase, thus enabling rapid analysis of large numbers of small volume samples. An attempt was made to try and automate the analysis using an autosampler but to no avail, the negatively charged phospholipids failed to emerge out of the autosampler. This may have been due to the sticking of the sample to large amount of metal tubing inside. The use of ESI-MS to study lung surfactant phospholipids is unique and provides an enormous amount of detailed information on the phospholipid composition.

3.2 Method development

The method for analysing phospholipids by ESI-MS has already been described in section 2.7. However, some of the method development and the reasons behind it are described in this section.

3.2.1 Mobile phase

The original mobile phase used was 100% methanol, pumped at a flow rate of 10μl/minute. It was thought that this mobile phase might have been the cause of problems such as samples sticking in the tubing and slowly bleeding off. Therefore, the mobile phase was changed to include 10% chloroform that was later increased to 20%, followed by an increase in flow rate to 20μl/min and finally 50μl/min. These changes were found to greatly reduce the amount of sticking and decrease run times. The addition of 10% water was found to enhance the PI response, an observation that may be due to the water increasing the solubility of the PI species. The water probably interacts with the five-hydroxyl groups on the PI headgroup and would therefore increase ionisation.

3.2.2 Analysis of phosphatidylcholine molecular species using ESI-MS

There was a difficulty in analysing phosphatidylcholine (PC) molecular species due to the tendency of PC to form cation adducts. The main adduct formed was the sodium adduct, this was probably due to sodium ions associating to the PC species when

incubated with phosphate-buffered saline and also from the glassware used to extract the samples. The PC species were present as both the molecular ion $[M+H]^+$ and the sodium adduct $[M+Na]^+$. However, the proportion of the PC species present as the sodium adduct varied considerably. For example the *m/z* 734 peak in figure 3.1 is the 16:0/16:0PC molecular ion and *m/z* 756 is the corresponding sodium adduct. A list of the *m/z* values of both the PC molecular ions and the sodium adducts is shown in table 3.1. The presence of the sodium adducts is a potential problem due to the crossover between the sodium adduct and another molecular ion; for instance *m/z* 782 is the mass of both 16:0/18:1PC (sodium ion) and 16:0/20:4PC (molecular ion). This is not a problem when analysing human BALF as it contains negligible 16:0/20:4PC.

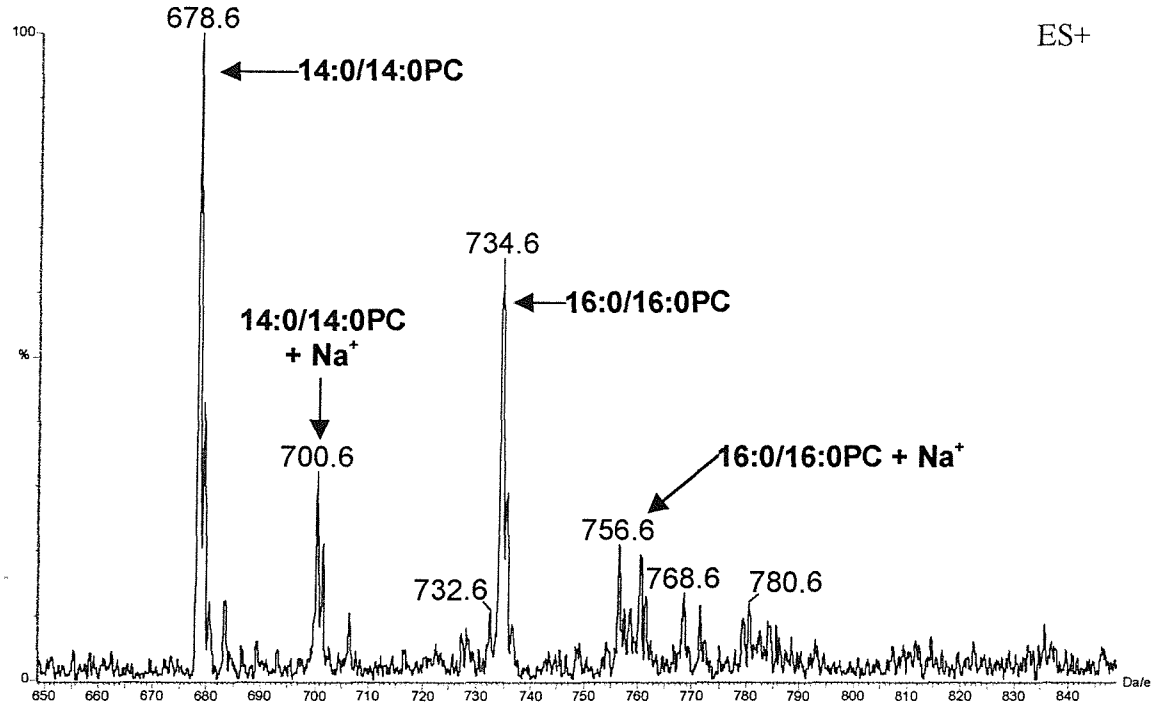


Figure 3.1 Mass spectrum of a lipid extract from BALF under positive ionisation showing both molecular ions and sodium adducts of the PC molecular species.

Table 3.1 The m/z values of the PC molecular ions and the sodium adducts.

PC Species	Molecular ion m/z values	Na^+ adducts m/z values
14:0/14:0	678	700
14:0/16:0	706	728
16:0/16:1	732	754
16:0/16:0	734	756
16:0/18:2	758	780
16:0/18:1	760	782
16:0/20:4	782	804
18:1/18:2	784	806
18:0/18:2 & 18:1/18:1	786	808
18:0/18:1	788	810
18:0/18:0	790	812
16:0/22:6	806	828
18:1/20:4	808	830
18:0/20:4	810	832
18:0/20:3	812	834

Various chemicals were added to the injection solvent and the mobile phase to try and encourage the formation of only the sodium adducts or the molecular ion. The addition of assorted bases to both the injection solvent and the mobile phase were an attempt to displace the sodium ions and run the PC molecular species as only the molecular ion. The addition of 2% triethylamine was quite effective as mainly the molecular ion was formed. The disadvantage of using triethylamine was the formation of adducts at $[M+101]^+$, as well as retention in the MS and causing problems for other users. Ammonia was also found to have variable effect on displacing the sodium ions, but it did however enhance the response of the negative ions. 2% NH_4OH was used in the mobile phase and injection solvent to analyse some of the human BALF samples (see chapter 6). However partial sodiation is a problem when trying to analyse other animal species that contain both 16:0/18:1PC and 16:0/20:4PC.

Sodium salts were added to the injection solvent to encourage the formation of the sodium adducts. The initial method involved the use of 5mM sodium hydroxide in the injection solvent, and this concentration was increased to 50mM in an attempt to gain only the sodium adducts. However, at that time there were also problems with blockages in the mass spectrometer, this may have been due to the formation of salts in the fine tubing. The addition of 20mM sodium acetate to the injection solvent was found to be very successful at forming sodium adducts and did not cause any blockages. However the disadvantage was that it did not enhance the negative response. Therefore, to be able to analyse the negative spectrum of a sample, it had to be run separately using NH_4OH in the injection solvent.

3.3 Results

A method for the routine analysis of surfactant phospholipids using the electrospray ionisation mass spectrometer was developed. It is a rapid method (<5minute per sample) requiring small volumes of BALF, typically between 0.5-1ml. This is very useful in the clinical setting where there is limited availability of samples. The molecular species identity of the various mass ions were confirmed using tandem MS-MS. There are a variety of tandem MS-MS scans that can be performed and these were discussed in section 1.8.

3.3.1 Electrospray ionisation mass spectrometry of surfactant phospholipids

A typical positive and negative ionisation mass spectrum of human lung surfactant is shown in figure 3.2. The PC species were analysed as the sodium adducts, this was achieved by using an injection solvent containing 20mM sodium acetate (figure 3.2a). The negative ionisation mass spectrum was collected after the sample was dissolved in the injection solvent containing 2% NH_4OH (figure 3.2b). The *m/z* values of both the PG and the PI molecular species are listed in table 3.2.

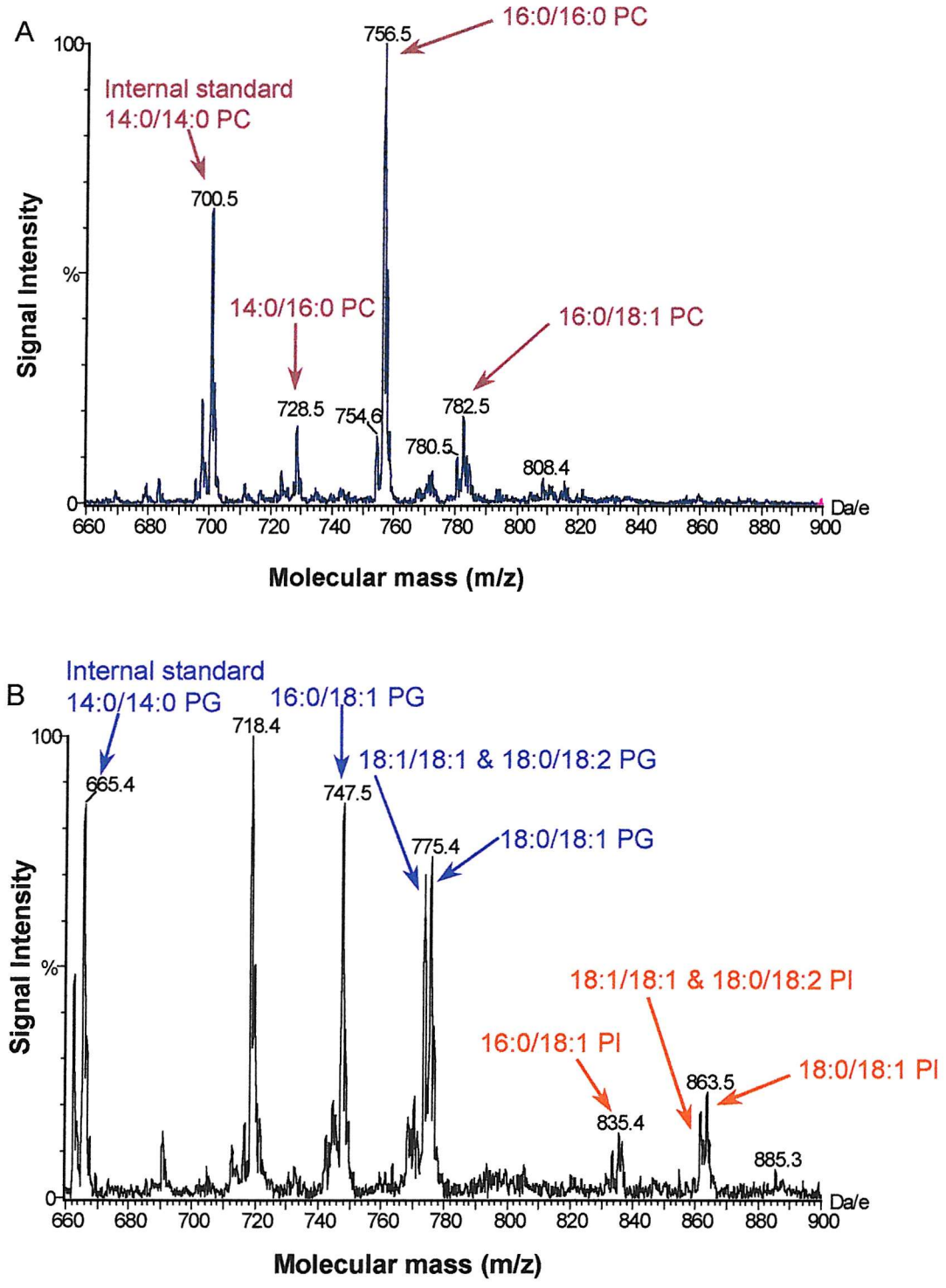


Figure 3.2 Typical mass spectra of a lipid extract from human lung surfactant under (A) positive ionisation and (B) negative ionisation.

Table 3.2 The m/z values of PG and PI molecular species

Molecular Species	PG m/z values	PI m/z values
14:0/14:0	665	753
16:0/16:1	719	807
16:0/16:0	721	809
16:0/18:2	745	833
16:0/18:1	747	835
16:0/20:4	769	857
18:1/18:2	771	859
18:0/18:2 & 18:1/18:1	773	861
18:0/18:1	775	863
18:0/18:0	777	865
16:0/22:6	793	881
18:1/20:4	795	883
18:0/20:4	797	885
18:0/20:3	799	887

3.3.2 Tandem MS-MS of phosphatidylglycerol molecular species

Tandem MS-MS was performed to confirm the molecular species identities of the various mass ions, firstly using authentic standards and then on samples. Tandem MS-MS analysis of phospholipids under negative ionisation enables the determination of the fatty acid positions on the glycerol-phosphate backbone. Fragmentation of PG molecular species under negative ionisation generated ion products corresponding to the fatty acid anions and dehydrated glycerophosphate (*m/z* 153). Using authentic standards, it was shown that such fragmentation can provide unambiguous structural information, as the intensity of fatty acid anions produced from the *sn*-2 position was double that from the *sn*-1 position, this agrees with reports from Han & Gross 1995 [233]. For instance figure 3.3 shows the daughter (product) ions of *m/z* 747 (16:0/18:1PG) after fragmentation. As the intensity of the 18:1 fatty acid anion at *m/z* 281 was twice that of the 16:0 fatty acid anion at *m/z* 255, this confirmed the identity of the *m/z* 747 ion as *sn*-1 16:0/*sn*-2 18:1 PG.

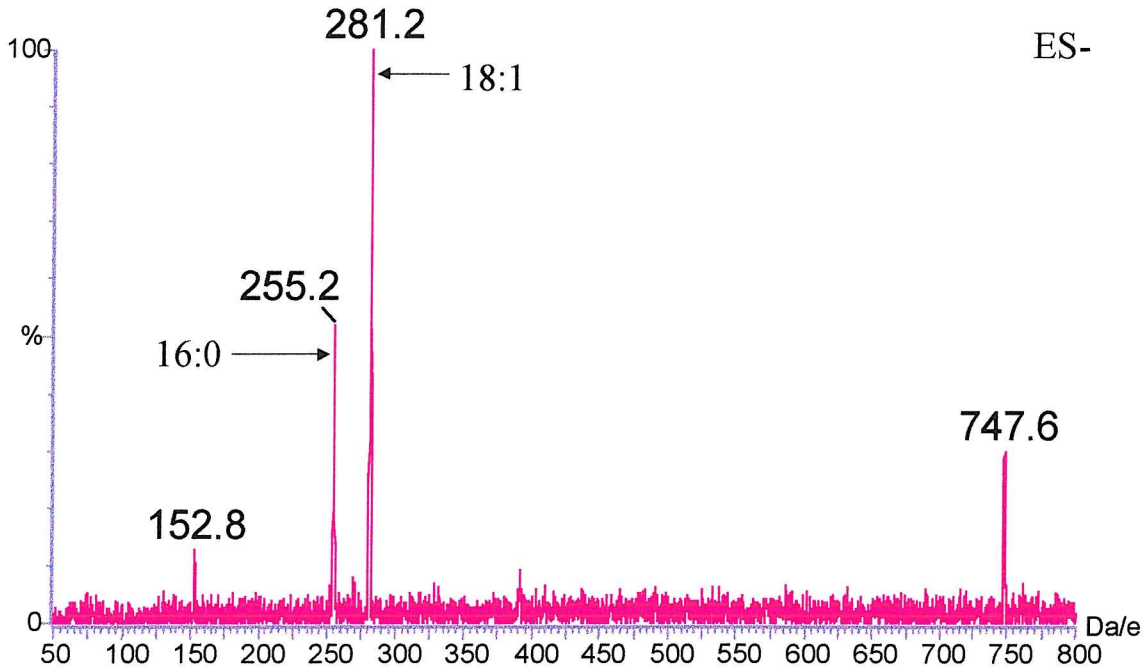


Figure 3.3 Daughter ions of the m/z 747 ion confirm the identity as sn-1 16:0/ sn-2 18:1PG. The 18:1 fatty acid anion at m/z 281 is twice the intensity of the 16:0 fatty acid anion at m/z 255. The dehydrated glycerol phosphate backbone is seen at m/z 152.8.

Tandem MS/MS was particularly useful for the analysis of molecular species of the same mass. The product ions of the isobaric components at *m/z* 773, 18:1/18:1PG and 18:0/18:2PG are illustrated in figure 3.4. The predominant product was the fatty acid anion at *m/z* 281, with smaller amounts of 18:2 at *m/z* 279 and 18:0 at *m/z* 283. Consequently, the major molecular species present at peak *m/z* 773 was 18:1/18:1PG, with about 20% 18:0/18:2PG.

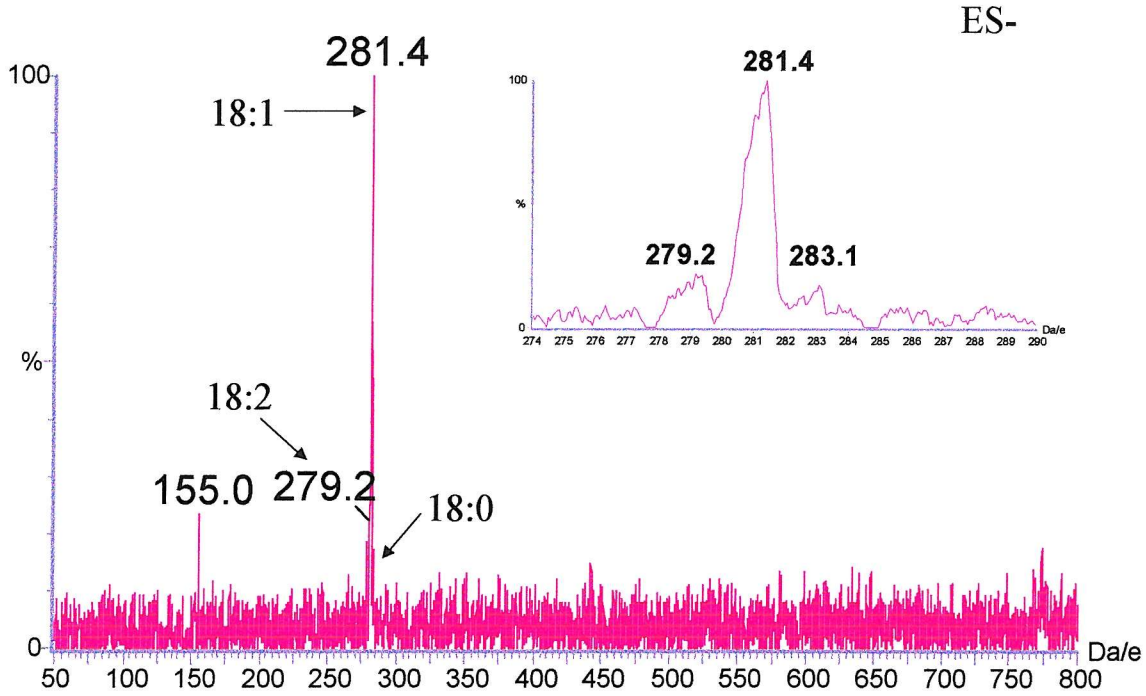


Figure 3.4 Fragmentation of the isobaric components at m/z 773 identified the mass ion as predominantly 18:1/18:1PG with about 20% 18:0/18:2PG. The major product formed was the fatty acid anion 18:1 (m/z 281), with smaller amounts of 18:2 (m/z 279) and 18:0 (m/z 283).

In one report the production of fatty acid anions from the *sn*-1 and *sn*-2 position was proposed to be dependent on the collision energy, the phospholipid class and the fatty acids attached to the *sn*-2 position [47]. This was in contrast to the results obtained on the triple quadrupole ESI-MS used in this thesis and Han and Gross 1995 [233]. The results from an investigation in to the effect of collision energy on the production of the fatty acid anions, performed on the ESI-MS are listed in table 3.3. A syringe pump was used to introduce the samples into the ESI-MS and then fragmentation was induced with increasing collision energy. The ratio of the *sn*-2 to *sn*-1 fatty acid anion produced was then calculated. The effect of increasing collision energy from 25 to 65 eV was studied on both 16:0/18:1 PC and PG (table 3.3). The results were similar for both the PC and PG molecular species. At low collision energy a ratio of the *sn*-2 to *sn*-1 fatty acid anions produced was observed to be approximately 2:1, this ratio decreased to nearly a 1:1 ratio at higher collision energies. The conditions used for all of the tandem MS-MS scans were optimised to obtain a 2:1 ratio.

Table 3.3 The effect of increasing collision energy upon the ratio of sn-2 to sn-1 fatty acid produced upon fragmentation for both 16:0/18:1 PG and PC.

Collision energy (eV)	Ratio of 18:1 to 16:0 after fragmentation of 16:0/18:1PG	Ratio of 18:1 to 16:0 after fragmentation of 16:0/18:1PC
25	2.02	2.09
30	2.3	1.99
35	2.25	1.77
40	1.84	1.64
45	1.84	1.32
50	1.79	1.88
55	1.52	1.71
60	1.36	1.65
65	1.17	*

** No data was available as complete fragmentation of the PC species occurred when the collision energy was 65eV*

3.3.2 Tandem MS-MS of phosphatidylcholine

Molecular identity of the PC species can only be confirmed under negative ionisation, as the fatty acid anions are negatively charged and can only be seen under these conditions. PC species are zwitterionic and can form negative ion adducts under certain conditions, the fragmentation of such adducts enables molecular species confirmation. Using PC standards the negative adducts formed and the conditions required to generate them were identified. The three major adducts formed were $[m-16]^-$, $[m-61]^-$, and $[m+33]^-$, representing the loss of a methyl group, a methyl plus dimethylamine from the choline headgroup and the gain of a chloride ion respectively. The negative adducts were formed more readily at high cone voltages (greater than 80kV). This technique was used to confirm the molecular species identity of the PC species in various samples. Figure 3.5 shows the daughter ions of the chloride adduct of 16:0/16:0PC in human lung surfactant.

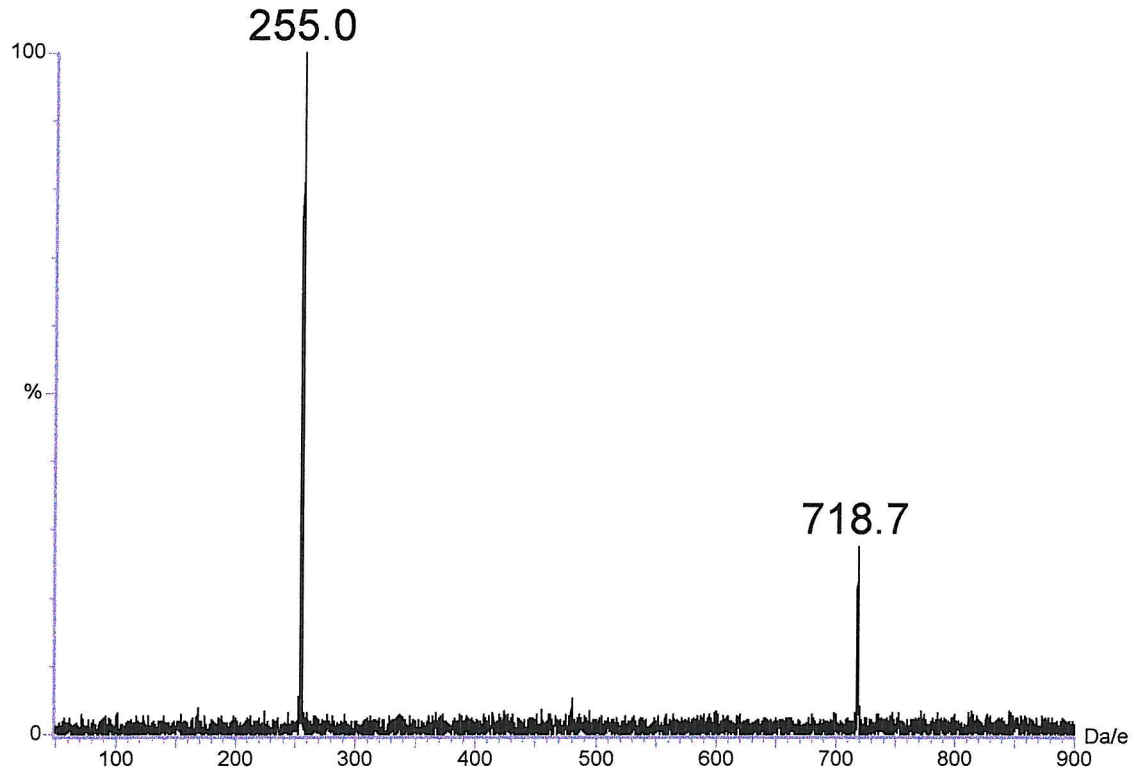


Figure 3.5 Daughter ions of the chloride adduct of 16:0/16:0PC (m/z 769) under negative ionisation. The 16:0 fatty acid anion produced at m/z 255 confirms this as 16:0/16:0PC, the m/z 718 is [M-16]⁻ ion representing loss of a methyl group.

Fragmentation of PC species under positive ionisation produces many fragments that are related to the phosphocholine headgroup. The presence of sodium ions was necessary to generate many of the fragment ions. This can be seen when comparing the daughter ions of the molecular ion 16:0/16:0PC (*m/z* 734) (figure 3.6b) with the sodium adduct (*m/z* 756) (figure 3.6a).

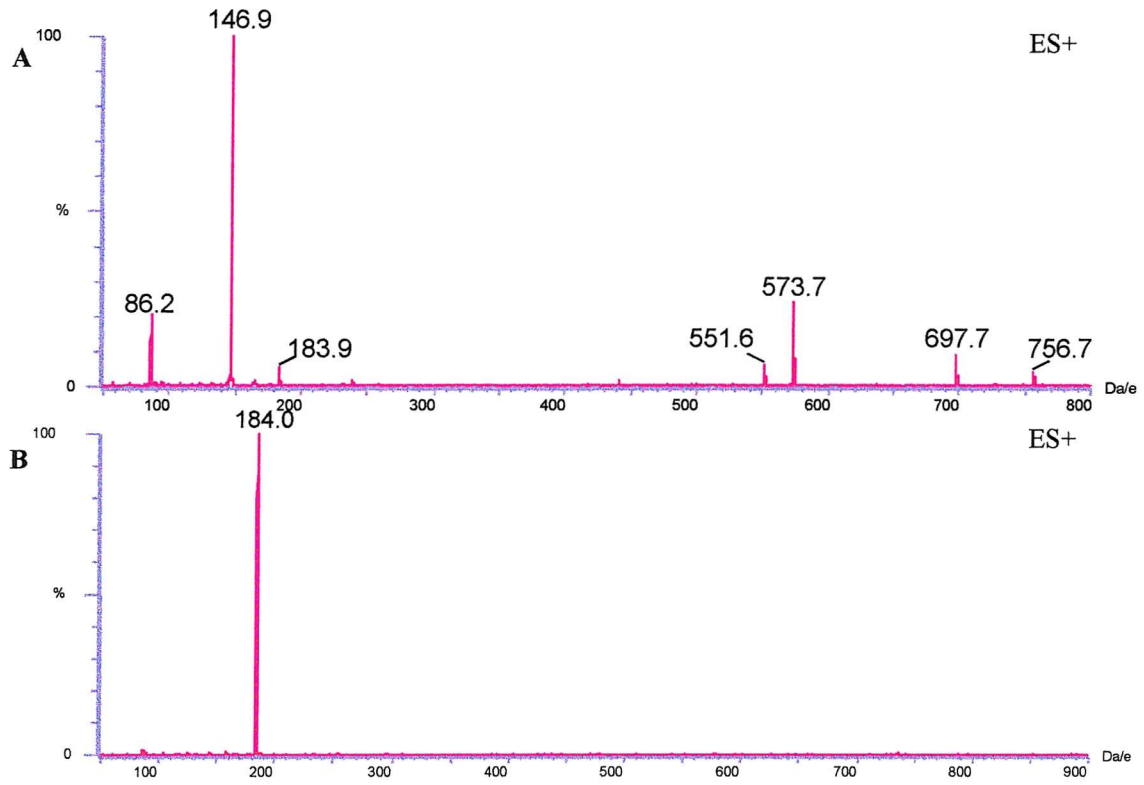


Figure 3.6 Fragmentation of 16:0/16:0PC under positive ionisation. The sodium adduct (m/z 756) (A) generates more daughter ions than the molecular ion (m/z 734)(B).

Many of the daughter ions produced from the fragmentation of the PC molecular species under positive ionisation were related to the phosphocholine headgroup, for instance *m/z* 86 (choline), 147 (sodiated five member cyclophosphane) and 184 (phosphocholine cation). Figure 3.7 illustrates the origins of these fragments from a 16:0/16:0PC molecule. The fragments that are generated with the presence of sodium ions are shown in red, while those that are generated from the molecular ion in green. The proposed pathway for the collision-induced formation of the sodiated five membered cyclophosphane (*m/z* 147) is shown in figure 3.8.

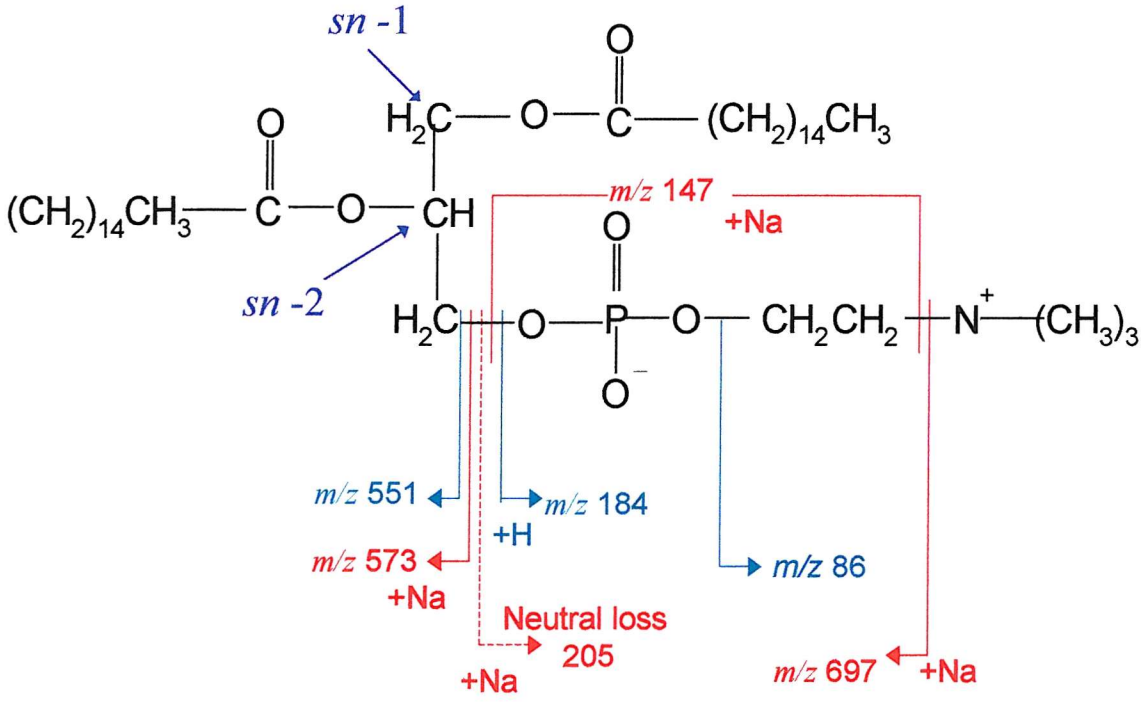


Figure 3.7 Proposed fragmentation of 16:0/16:0PC in positive ionisation mode. The fragments generated in the presence of sodium ions are shown in red and those in blue are generated from fragmentation of the molecular ion.

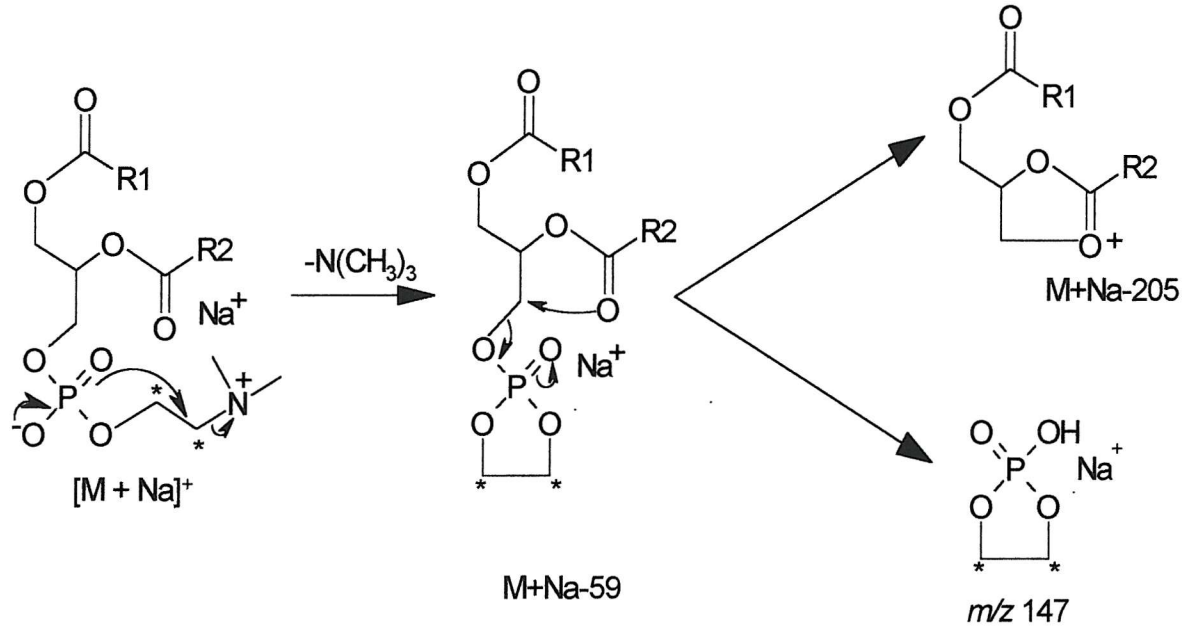


Figure 3.8 Proposed collision-induced fragmentation pathways for sodium adducts of PC molecular species in the ESI-MS. The first step involves the loss of trimethylamine generating the $[M + Na - 59]^+$ ion. The loss of the neutral fragment (205) produces the diglyceride-like cation $[M + Na - 205]^+$ ion. A sodiated five-member cyclophosphane (m/z 147) can also be generated.

A variety of tandem MS-MS scans (see section 1.8.3) were performed on the headgroup related fragments to identify only the PC molecular species. Parent scans of the charged fragments for instance *m/z* 147, or scanning for the loss of a neutral fragment (e.g. 205), generate a spectrum of only the PC molecular species. This was very useful when trying to distinguish between the molecular ions and the sodium adducts when both were present in a sample. This technique was used on adult human BALF in chapter 6, as both molecular ions and sodium adducts were there in these samples. Molecular ions were confirmed by a parent scan of the phosphocholine cation (*m/z*184) (figure 3.9a), while the sodium adducts were analysed by a parent scan of the sodiated five member cyclophosphane (*m/z* 147) (figure 3.9b). In the case of adult human BALF, the presence of both the molecular and sodium PC ions did not interfere with the analysis as the concentrations of 16:0/20:4PC (molecular ion *m/z* 782, sodium adduct *m/z* 804) were below detection limits in these samples.

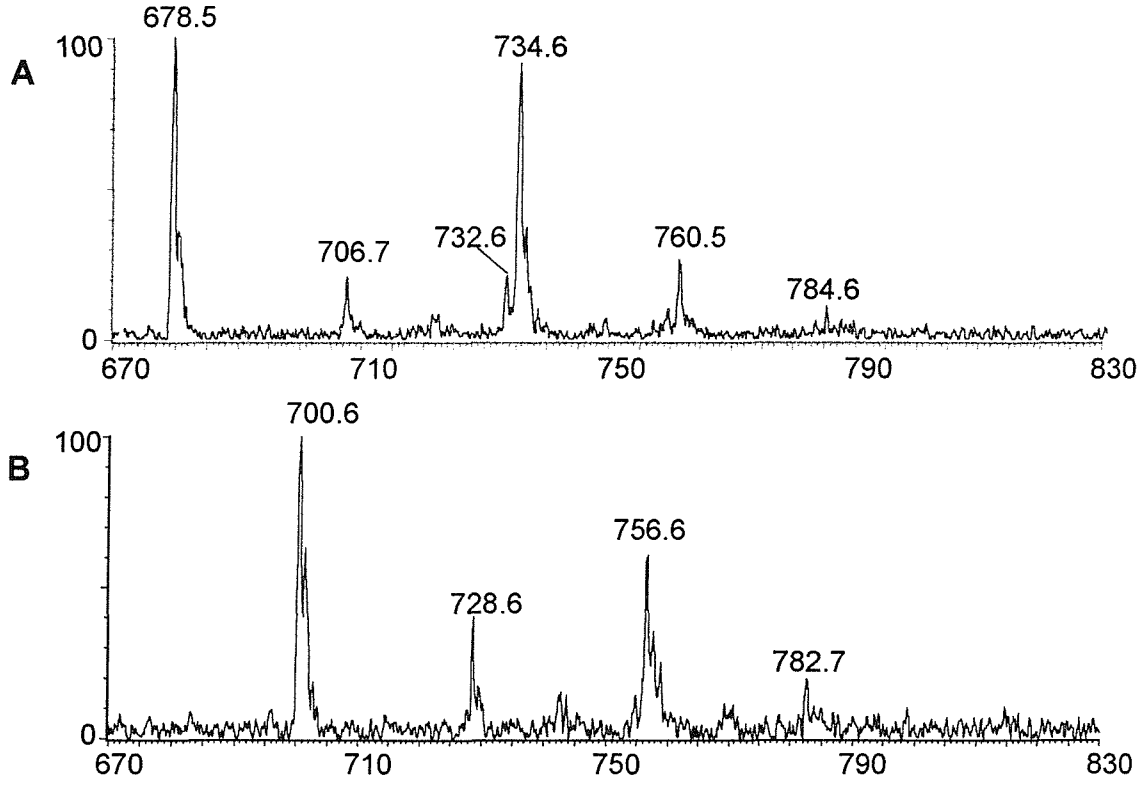


Figure 3.9 Tandem MS-MS was used to distinguish between the molecular ions and the sodium adducts. (A) Molecular ions were confirmed by a parent scan of the phosphocholine cation (m/z 184). (B) The sodium adducts were identified by a parent scan of the sodiated five-membered cyclophosphane (m/z 147).

Scanning for the loss of the neutral fragment 205, which is the phosphocholine headgroup produces a spectrum that only identifies the sodium adducts of the PC molecular species (figure 3.10).

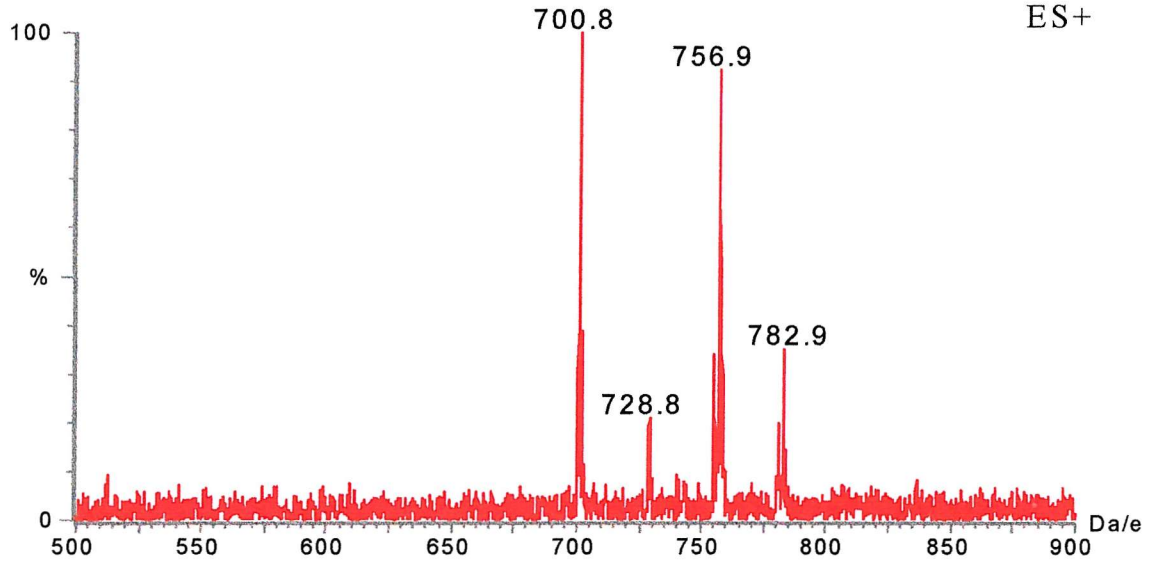


Figure 3.10 Neutral loss scan of 205 (phosphocholine headgroup) identifies all the sodium adducts of PC molecular species in a lipid extract from rabbit lung surfactant.

3.4 Discussion

The routine analysis of surfactant phospholipids has been performed using ESI-MS. The individual phospholipid molecular species were determined from their molecular masses. PG species were identified as the molecular mass minus one ion $[M-H]^-$ in the negative ionization mode, while PC species were identified as either the $[M+H]^+$ or $[M+Na]^+$ ion. Six PG species were identified in human BALF namely 16:0/16:0PG (*m/z* 721), 16:0/18:2PG (*m/z* 745), 16:0/18:1PG (*m/z* 747), 18:1/18:2PG (*m/z* 771), 18:1/18:1PG and18:0/18:2PG (*m/z* 773) and 18:1/18:2PG (*m/z* 775). While the PC molecular species analyzed were 14:0/16:0PC (*m/z* 706), 16:0/16:0PC (*m/z* 734), 16:0/16:1PC (*m/z* 732), 16:0/18:2 PC (*m/z* 758), 16:0/18:1PC (*m/z* 760), 18:1/18:2PC (*m/z* 784) and 18:1/18:1PC and 18:0/18:2PC (*m/z* 786). More PG, PC and PI species were identified in the rabbit, rat and human purified surfactant and guinea pig BALF and these are discussed in chapter 4.

Tandem MS-MS has proved to be a very useful tool for the identification of phospholipid classes and the structural confirmation of the PC and PG mass ions.

Unfortunately it was not possible to perform MS-MS on the PI species due to the low concentrations of PI in surfactant and difficulties in running PI on the triple quadrupole ESI-MS. However, fragmentation under negative ionisation mode provides information as to the fatty acids present and their positions on the carbon backbone. The fatty acid positional information generated was very useful when considering the actions of phospholipase A_2 and the release of free fatty acids from the *sn*-2 position. In general, saturated fatty acids are esterified at the *sn*-1 position, while unsaturated fatty acids are commonly found at the *sn*-2 position. Fragmentation of the PC species under positive ionisation aids in the identification of PC molecular ions and sodium adducts. However, tandem MS-MS was very time consuming and therefore it was not possible to perform on every sample.

Chapter Four

Comparison of mammalian lung surfactant phospholipid compositions

4.1 Introduction

Pulmonary surfactant is a mixture of lipids and proteins that lines the air-liquid interface of the lungs of all vertebrates, despite the wide variation in the lung structure and function of these animals [234]. There have been reported differences in the composition and proposed function of pulmonary surfactant in mammals and non-mammals. In mammals, its main function is to reduce surface tension in the alveoli preventing collapse and alveolar oedema [235]. In non-mammals surfactant does to a small extent maintain lung fluid balance and thereby prevent oedema but this is not thought to be its most important function [236]. The main function of surfactant in non-mammalian lungs may be to act as an antiglue, preventing adhesion of adjacent epithelial surfaces at low lung volumes when respiratory units fold on each other during expiration (e.g. during diving or swallowing of prey) [234].

The morphological differences between the mammals and other vertebrates are likely to account for such diverse roles of surfactant. The lungs of all vertebrates are essentially internal, fluid lined structures that cycle air by changing volume. However, the mammalian bronchoalveolar lung is very different to the basic saccular "bag" of nearly all of the other vertebrates. The non-mammalian lung (excluding birds) consists of much larger respiratory units (the alveoli can be up to 1000-fold larger[237]), and can be up to 100-fold more compliant than mammalian lungs (thereby reducing the work of inspiration). Therefore, non-mammalian surfactant may not be required to increase compliance or maintain inter-unit stability in the same manner as it does in mammals. For instance in the mammalian lung the small respiratory units (alveoli) would be subject to larger collapsing pressures than respiratory units with larger radii under conditions of constant surface tension according to the law of La Place (see section 1.2.2). Hence a major role of surfactant lining the small alveoli of the mammalian lung is to reduce the surface tension and prevent alveolar collapse, whereas surfactant lining other vertebrate lungs with large respiratory units does not need to fulfil this role.

16:0/16:0PC has been shown to be the active component in mammalian lung surfactant that reduces the surface tension at the air-liquid interface. The surface tension reducing property of surfactant has been attributed to 16:0/16:0PC being in the gel phase at end compression [236]. The proportion of 16:0/16:0PC in surfactant varies between mammals

and non-mammals. Mammalian lung surfactant has a relatively high 16:0/16:0PC content that accounts for about 40% of the total phospholipid in surfactant. Whereas non-mammals, for instance amphibians and most reptiles, have a much lower content of the surface active 16:0/16:0PC [238]. Differences between the body temperatures of mammals (37°C) and non-mammals with low body temperatures of 20-30°C (i.e. reptiles and hibernating animals) probably account for the differences in the proportion of 16:0/16:0PC in their surfactant. Pure 16:0/16:0PC has a relatively high transition temperature of 41°C, below which it enters the gel phase and is poorly spreadable. However, adding unsaturated phospholipids and/or cholesterol to pure 16:0/16:0PC will decrease the phase transition temperature of the mixture, improve spreadability of the surface film and promote the adsorption of surfactant to the air:liquid interface. Therefore a high proportion of 16:0/16:0PC is likely to be of limited use in the lungs of animals with low body temperatures of 20-30°C. These animals utilize other lipids, such as unsaturated phospholipids or cholesterol, to assist in the fluidising and adsorption of the surfactant [21,239,240].

PG and PI are the second and third most abundant phospholipids after PC in mammalian lung surfactant. However the role of PG and PI in lung surfactant has not been clarified. It has been proposed that these minor anionic phospholipids in particular PG may assist in adsorption of the surface active component 16:0/16:0PC to the air:liquid interface [241]. PG is also thought to stabilise surfactant PC at low surface tension and prevent collapse of a 16:0/16:0PC film [242]. Mammals and anurans (frogs and toads) are the only animal species to contain a significant (>10%) amount of PG in their lung surfactant [24,243]. Other animals such as turtles and chickens have a reported absence of PG in their lung surfactant [20,21] but higher proportions of PI than in mammalian surfactant. It has been proposed that since PI and PG both have negatively charged headgroups, and are formed by analagous synthetic pathways (see figure 1.5) they may be capable of fulfilling the same role [21,244] (see section 1.2.3.2).

In addition to phospholipid comparisons between mammals and non-mammals, differences in the phospholipid composition between mammalian species have also been reported [245,246]. Therefore, one of the aims of this chapter was to study the surfactant phospholipid composition of four mammalian species namely the rabbit, rat, guinea pig

and human using ESI-MS. To date there has not been a study that has directly compared the PC, PG and PI phospholipid molecular species composition of surfactant from different mammalian species. The compositions of the lung surfactant of the three typical laboratory animals were compared to human lung surfactant to determine any differences in the molecular species phospholipid composition. The purpose of these comparisons was to establish any differences in these mammalian species and to try and identify an animal that represents the surfactant phospholipid composition of humans. The use of animal models to study human diseases often has many drawbacks due to the animal being different to humans. By identifying an animal with a surfactant phospholipid composition most like humans, allows the identification of an animal that could be used to study diseases where alterations in the surfactant phospholipid composition are thought to be crucial to the disease process.

The study of animal surfactant involved terminal lavages of the animals, which not only generated large volumes of BALF (50ml rat and 250ml rabbit) but also allowed the lungs to be obtained from the same animals. Surfactant was then purified from most of the BALF for analysis and to provide a substrate for the *in vitro* studies of PLA_2 action on lung surfactant (see chapter 5). Lamellar bodies were also purified from the isolated lungs of both the rabbit and the rat and their phospholipid compositions were compared to that of the purified lung surfactant. Lamellar bodies are found in the type II cells in the lung and lung surfactant is packaged into the lamellar bodies prior to secretion into the alveoli.

The detailed analysis of the PC, PG and PI molecular species composition of BALF and purified surfactant from the same animals has not previously been reported. This was an important study as BALF has been used to determine surfactant composition but as yet no detailed analysis of PC, PG and PI molecular species composition has shown that BALF phospholipid composition is directly comparable to purified surfactant. Previous techniques for the analysis of surfactant phospholipids have required relatively large volumes of BALF that has a low phospholipid concentration. However the use of ESI-MS allows the analysis of small volumes of BALF (typically <1ml) at low concentrations (usually BALF containing 25nmoles of total phospholipid phosphorous). The ability to analyse small volumes of BALF is very useful in the clinical situation as it is not always

possible to obtain large volumes required for the purification of surfactant. For instance, in patients with severe lung diseases such as ARDS and acute asthma it would be more ethical and less intrusive to patients to use smaller volumes of lavage fluid.

4.2 Methods

Four rats and two rabbits were given lethal injections of phenobarbitone prior to bronchoalveolar lavage with saline followed by the removal of the lungs (section 2.3). Surfactant was purified from BALF by density gradient centrifugation as described in section 2.3. Lamellar bodies were isolated from lungs of the rats and rabbits by density centrifugation as described in section 2.4. Surfactant was purified from BALF of 10 healthy human volunteers by density gradient centrifugation as described in section 2.2.1. Dr Peter Hockey at Southampton General Hospital collected the human BALF. Aliquots of guinea pig BALF from 10 terminally lavaged control animals were kindly donated by Bayer (UK).

4.3 Results

There are two parts to this chapter and they will be considered separately. Firstly the phospholipid composition of BALF, purified surfactant and purified lamellar bodies from rabbits and rats will be discussed. Secondly the surfactant phospholipid composition of three typical laboratory animals, namely rats, rabbits and guinea pigs will be compared to human surfactant.

4.3.1 Comparison of BALF, purified surfactant and purified lamellar bodies phospholipid molecular species

The phospholipid compositions of BALF, purified surfactant and purified lamellar bodies have been compared for both the rat and the rabbit. The aim of this was to see if the phospholipid composition of the BALF represents that of the purified lung surfactant and to compare this with the composition of the purified lamellar bodies. No statistical analyses have been performed on this data as only 2 rabbits and 4 rats were used.

4.3.1.1 Phosphatidylcholine molecular species

The phosphatidylcholine (PC) molecular species composition of BALF, purified surfactant and purified lamellar bodies for both the rabbit and the rat are shown in table 4.1. For all three sample types, the same PC species were present in both the rat and the rabbit. The major molecular species in all fractions was 16:0/16:0PC for both animals, a result that is consistent with all previous studies. There were, however, some differences between the rat and rabbit in the distribution of the remaining PC species. Compared with rabbit BALF PC, the rat was enriched in 16:0/14:0PC, 16:0/16:1PC and 20:4-containing PC species. Conversely, the proportions of 16:0/18:1, 16:0/18:2, 18:0/18:1 and 18:1/18:1 were all greater in rabbit BALF PC than in rat. The differences in the PC molecular species distribution between the rat and rabbit will be discussed in greater detail in section 4.3.2.1.

The PC composition of the BALF appears to be very similar to the purified lung surfactant for both the rabbit and the rat. However, minor differences can be seen, notably purification of the BALF does not enhance the proportion of 16:0/16:0PC in the purified surfactant of both animals. Although, in rat purified surfactant there was a higher proportion of 16:0/18:2PC than in BALF.

Table 4.1 Phosphatidylcholine molecular species of BALF, purified surfactant and purified lamellar bodies for both the rat and the rabbit

mole %	Rat (n=4)			Rabbit (n=2)		
PC Species	**BALF**	**Surfactant**	**Lamellar bodies**	**BALF**	**Surfactant**	**Lamellar bodies**
14:0/16:0	11.1 ± 0.9	11.0 ± 1.5	8.2 ± 0.5	6.4 (6.4,6.5)	7.6 (6.9,8.4)	6.5 (6.3,6.6)
16:0/16:1	21.4 ± 1.6	19.7 ± 1.6	12.9 ± 1.6	12.1 (12.5,11.7)	11.3 (11.6,11.1)	12.0 (12.7,11.4)
16:0/16:0	43.2 ± 2.8	42.3 ± 1.7	35.0 ± 4.0	38.4 (38.1,38.8)	35.6 (36.5,34.7)	33.6 (32.4,34.8)
16:0/18:2	7.7 ± 0.5	9.0 ± 1.4	10.6 ± 1.3	14.1 (14.1,14.1)	13.8 (14.4,13.2)	15.5 (16.3,14.7)
16:0/18:1	6.5 ± 0.7	5.6 ±0.4	11.2 ± 1.3	18.7 (20.3,17.1)	17.7 (19.9,15.6)	17.6 (18.8,16.3)
16:0/18:0	4.3 ± 0.7	3.8 ± 2.2	3.5 ± 1.3	2.0 (1.5,2.5)	4.1 (2.3,6.0)	2.5 (1.8,3.1)
16:0/20:4	2.9 ± 0.6	5.0 ± 0.6	7.9 ± 0.8	1.9 (1.4,2.4)	2.5 (2.0,3.0)	3.4 (3.1,3.8)
18:1/18:2	0.5 ± 0.2	0.9 ± 0.3	1.7 ± 0.3	2.5 (2.0,2.9)	2.7 (2.3,3.1)	3.1 (2.7,3.6)
18:1/18:1 & 18:0/18:2	1.4 ± 0.3	1.1 ± 0.4	4.2 ± 1.4	3.6 (3.6,3.7)	4.0 (3.6,4.4)	5.0 (4.8,5.3)
18:0/20:4	1.0 ± 0.6	1.6 ± 0.3	4.9 ± 0.9	0.3 (0.2,0.3)	0.5 (0.4,0.6)	0.7 (1.1,0.3)

Values are expressed as mole% total PC and standard deviations are shown for the rat data where n=4, individual values are shown in parentheses for the rabbit data as n=2.

Lung surfactant is secreted into the airways from type II cells, and is packaged into lamellar bodies prior to secretion into the alveoli. The comparison of the phospholipid composition of purified lamellar bodies and purified lung surfactant from the same animal should facilitate the identification of the proportions of the starting material that are surface active and those that are from the limiting membrane of the lamellar bodies.

In the rat there is proportionally less 16:0/16:0PC and 14:0/16:0PC in the lamellar bodies when compared to the lung surfactant. Rat lamellar bodies also contain less16:0/16:1PC

than in surfactant. However, the lamellar bodies have relatively higher proportions of 16:0/18:2PC, 16:0/18:1PC, 18:1/18:2PC, 18:0/18:2PC, 18:1/18:1PC and 18:0/20:4PC than surfactant. There are a few possible reasons for the decrease in the disaturated phospholipids and the rise in the polyunsaturated phospholipids in the lamellar bodies when compared to surfactant. The most likely explanation for the lamellar body fraction containing substantial amounts of PC species characteristically found in cell membranes (e.g. 16:0/18:1PC and 18:0/20:4PC) is the presence of a limiting membrane encompassing the lamellar bodies. The membrane surrounding the lamellar bodies is likely to be composed of such PC species that are uncharacteristic of surfactant PC but typically found in membranes. Another possible reason may be that complete purification of the lamellar bodies may not have been achieved and some cellular derived PC from the lung tissue is also present. Finally, due to the nature of 16:0/16:0PC being solid at body temperature, having a high concentration in the lamellar bodies would result in a very viscous (turbid) cell and lamellar bodies that are unlikely to be able to secrete their contents into the alveoli. Therefore the presence of other PC species may assist in secretion to the alveolar space.

4.3.1.2 Phosphatidylglycerol molecular species

Table 4.2 presents the phosphatidylglycerol (PG) molecular species compositions of BALF, purified surfactant and lamellar bodies for both the rat and the rabbit. The same PG species were present in both the rat and the rabbit. However the PG compositions were different between the two animals, for instance the rat has higher proportions of 16:0/16:0PG and 20:4 containing PG species. The rabbit though has a greater proportion of 16:0/18:1PG than the rat. The differences between these animals in the fatty acid compositions of the PG species were also reflected in the PC compositions and will be discussed in greater depth in section 4.3.2.2.

When comparing the PG compositions of BALF and purified surfactant for both the rat and rabbit it becomes apparent that the composition of the BALF is very similar to that of purified surfactant. The rat has marginally more 20:4 containing PG species in the purified surfactant, following the same trend as the PC composition. However a slightly higher proportion of 16:0/18:2PG was observed in the purified surfactant of both animals. The exact physiological role of the higher proportion of 16:0/18:2PG in the

purified surfactant is hard to establish but may be due to it being an active component at the air:liquid interface.

Table 4.2 Rabbit and rat lamellar bodies, BALF and purified surfactant phosphatidylglycerol molecular species composition

mole %	Rat (n=4)			Rabbit (n=2)		
PG Species	**BALF**	**Surfactant**	**Lamellar bodies**	**BALF**	**Surfactant**	**Lamellar bodies**
16:0/16:1	10.1 ± 0.6	8.3 ± 0.9	8.1 ± 0.9	7.8 (7.5,8.1)	10.4 (10.6,10.2)	8.7 (8.9,8.5)
16:0/16:0	34.9 ± 1.0	30.3 ± 1.3	25.1 ± 2.2	20.0 (17.2,22.9)	20.4 (20.4,20.5)	17.4 (14.9,20.0)
16:0/18:2	17.0 ± 1.2	20.0 ± 2.7	17.9 ± 2.0	10.2 (8.3,12.1)	11.8 (9.8,13.9)	11.1 (9.4,12.9)
16:0/18:1	21.0 ± 1.4	17.8 ± 1.3	17.0 ± 1.4	37.4 (39.4,35.5)	36.8 (41.8,31.9)	34.9 (35.8,34.0)
16:0/18:0	1.9 ± 0.5	2.4 ± 0.4	5.4 ± 2.2	0.9 (1.6,0.2)	1.7 (1.5,1.9)	1.6 (1.6,1.7)
18:1/18:2	3.7 ± 0.7	4.9 ± 0.6	5.1 ± 0.4	6.8 (7.6,6.0)	5.2 (3.5,6.9)	8.7 (10.3,7.2)
18:1/18:1 & 18:0/18:2	4.7 ± 0.8	5.5 ± 0.4	6.8 ± 1.0	10.2 (9.0,11.3)	7.9 (6.9,8.9)	10.9 (10.8,11.1)
18:0/18:1	2.3 ± 0.5	2.5 ± 0.2	2.9 ± 0.9	4.9 (5.8,4.0)	4.0 (4.6,3.4)	3.9 (4.2,3.6)
16:0/22:6	1.4 ± 0.5	2.8 ± 0.4	5.2 ± 1.8	0.4 (0.9,0.0)	1.1 (0.5,1.6)	0.7 (1.5,0.0)
18:1/20:4	1.5 ± 1.1	2.8 ± 0.4	4.2 ± 2.1	1.0 (2.0,0.0)	0.3 (0.1,0.5)	1.5 (2.0,1.0)
18:0/20:4	1.5 ± 0.2	2.8 ± 0.5	2.3 ± 1.8	0.4 (0.8,0.0)	0.3 (0.3,0.4)	0.4 (0.7,0.1)

Values are expressed as mole% total PG and standard deviations are shown for the rat data where n=4, individual values are shown in parentheses for the rabbit data as n=2.

In both animals there are some differences in the PG composition of the lamellar bodies when compared to purified surfactant. For instance the lamellar bodies contain a greater percentage of the PG species with longer chain fatty acids (for example 18:1/20:4, 18:0/18:2 and 18:1/18:1). Nevertheless surfactant contains more 16:0 PG species, to be precise there is proportionally more 16:0/16:0PG in the purified surfactant than in the

lamellar bodies, which suggests that 16:0/16:0PG gathers in the surfactant complex and may be an integral component of surfactant.

4.3.1.3 Phosphatidylinositol molecular species

The rat phosphatidylinositol (PI) compositions of BALF, purified surfactant and lamellar bodies are very different to the rabbit and are shown in table 4.3. The predominant PI species in rabbit BALF, surfactant and lamellar bodies is 16:0/18:1PI. Whereas 18:0/20:4PI is the major PI species in rat BALF and surfactant, and the main species in rat lamellar bodies is 18:0/20:3PI. The differences in the PI composition between rabbit and rat surfactant will be discussed in more detail in section 4.3.2.3.

Table 4.3 Rabbit and rat lamellar bodies, BALF and purified surfactant phosphatidylinositol molecular species composition

mole %	Rat (n=4)			Rabbit (n=2)		
PI Species	**BALF**	**Surfactant**	**Lamellar bodies**	**BALF**	**Surfactant**	**Lamellar bodies**
16:0/16:1	5.2 ± 3.6	3.2 ± 1.8	1.2 ± 0.9	4.5 (4.1,4.9)	5.7 (5.8,5.5)	4.3 (4.1,4.5)
16:0/16:0	9.1 ± 6.3	4.6 ± 1.5	0.6 ± 0.4	1.4 (0.8,2.0)	2.0 (2.2,1.8)	1.4 (1.1,1.7)
16:0/18:2	12.1 ± 7.5	18.1 ± 1.5	3.3 ± 0.7	15.4 (13.1,17.7)	18.3 (17.5,19.0)	13.1 (10.4,15.8)
16:0/18:1	12.2 ± 1.4	13.6 ± 1.5	3.6 ± 1.5	42.8 (47.9,37.6)	43.9 (46.7,41.2)	29.8 (25.8,33.8)
18:1/18:1 & 18:0/18:2	15.2 ± 4.2	14.8 ± 2.3	3.3 ± 1.0	15.1 (14.2,15.9)	16.2 (14.0,18.4)	15.5 (11.5,19.5)
18:0/18:1	4.1 ± 5.8	6.2 ± 3.3	1.8 ± 0.8	9.7 (8.2,11.2)	7.8 (7.7,7.8)	6.5 (5.4,7.6)
18:0/20:4	31.9 ± 11.4	28.6 ± 3.1	26.6 ± 2.4	6.1 (6.0,6.2)	2.6 (2.6,2.6)	14.0 (18.4,9.7)
18:0/20:3	10.2 ± 8.9	10.8 ± 2.7	59.6 ± 6.2	5.2 (5.8,4.6)	3.6 (3.4,3.7)	15.4 (23.4,7.4)

Values are expressed as % total PI and standard deviations are shown for the rat data where n=4, individual values are shown in parentheses for the rabbit data as n=2.

The PI compositions of the BALF and purified surfactant are very similar and this is true for both the rabbit and the rat. Nevertheless the PI composition of the lamellar bodies is extremely different to that of the purified surfactant. The lamellar body PI composition

of the rat contains 60% 18:0/20:3PI and 18% 16:0/18:2PI compared with 11% and 3% respectively for purified surfactant. The exceptionally high % of the typically membrane derived 18:0/20:3PI in rat lamellar bodies fraction probably is present as a main constituent of the membrane encircling the lamellar bodies. The rabbit does not have such dramatic differences, however the membrane PI species 18:0/20:3PI and 18:0/20:4PI are in higher proportions in the lamellar bodies when compared to the surfactant. However, rabbit surfactant has higher proportions of 16:0/18:1PI and 16:0/18:2PI when compared to the lamellar bodies of the same animal.

The PC, PG and PI molecular species compositions of the BALF are very similar to that of the purified lung surfactant. Therefore BALF could be used as an indicator for any changes that have occurred in the lung surfactant. The lamellar bodies composition differs significantly from the purified surfactant suggesting that surfactant undergoes substantial sorting prior to or after adsorption to the air:liquid interface.

4.3.2 Comparison of different mammalian species BALF phospholipids

The phospholipid molecular species composition of purified surfactant from the rat, rabbit, and human along with guinea pig BALF were analysed using ESI-MS. Purified surfactant was used where possible in order to be able to directly compare the results with previous studies [22,245-247]. At present no study has compared the PC, PG and PI molecular species composition of surfactant from these three laboratory animals with human samples using the same technique. These results set to answer whether there is any variation in mammalian lung surfactant as reports about other animal species suggest differences in phospholipid composition. For instance the disaturated phospholipid content of surfactant from amphibians, birds and most reptiles is lower than that reported from mammals, however no study has directly compared inter-mammal variation of PC, PG and PI molecular species.

4.3.2.1 Phosphatidylcholine molecular species

The PC molecular species composition of the rabbit, rat and human purified lung surfactant is shown in figure 4.1. Comparison of the lung surfactant composition of these animals with guinea pig BALF was not possible for all the PC species analysed due both the molecular ion and the sodium adduct of the PC species being present in the

mass spectra from the guinea pig samples (see section 3.2.2) that unfortunately means it was not possible to analyse three of the ten PC species (namely 16:0/18:0PC, 16:0/20:4PC and 18:0/20:4PC). However the PC composition of guinea pig BALF was compared with the rabbit for 7 PC species as they have essentially identical PC compositions and the results are shown in figure 4.2.

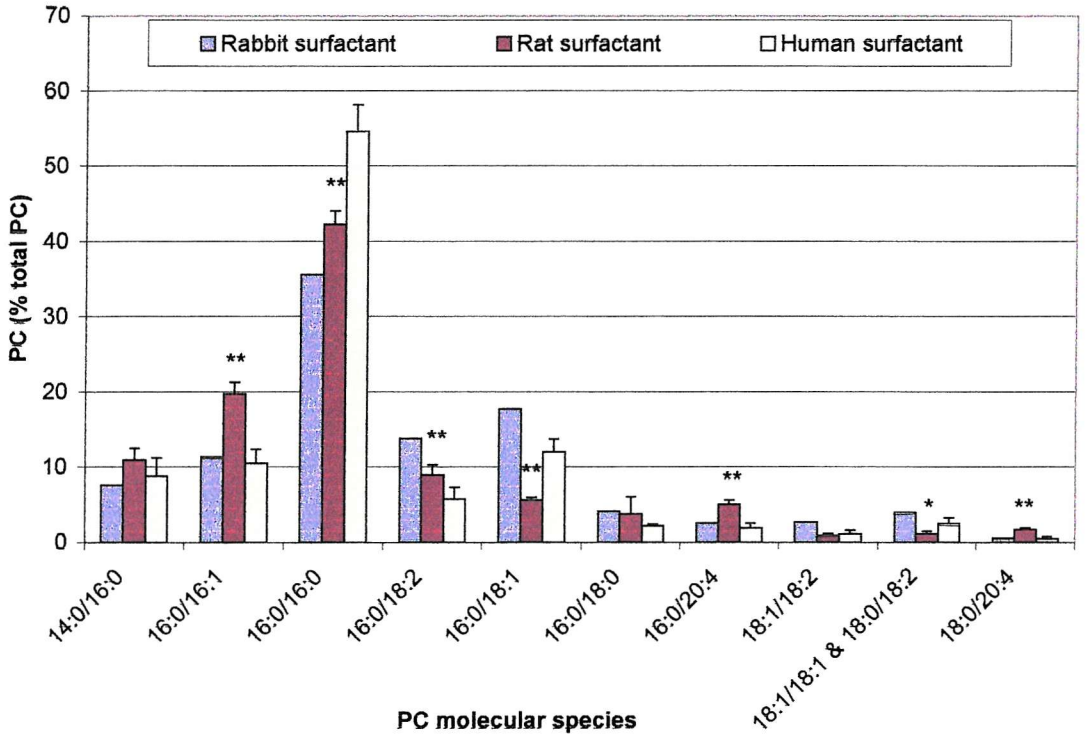


*Figure 4.1 The composition of the major PC molecular species from purified rabbit, rat and human lung surfactant. Error bars show standard deviations were applicable. Statistical significance is denoted by ** $P<0.005$ and * $P<0.05$ when compared by an independent t-test to human surfactant. n=2 rabbits, n=4 rats and n=10 human control subjects*

The most striking difference in the PC composition between the rabbit, rat and human is that the human has the highest proportion of 16:0/16:0PC. Although the value of 54% (± 3.4) 16:0/16:0PC for human purified surfactant in this section is higher than 44% (range 40.4-51.8) in BALF of human control subjects from a separate study (figure 6.2). The exact reason for this difference is unknown but the subjects are different and this may be the reason. It can be noted that 16:0/16:0PC is the proposed surface-active component of surfactant and these results show that it is the major PC species for all these animals. Therefore this implies that 16:0/16:0PC has been conserved through the evolution of the mammalian species and is essential for mammalian lung function.

Other significant differences between these animals include that the rat has the highest proportion of 16:0/16:1PC in addition to the highest proportion of 20:4 containing PC species. This high proportion of 20:4 containing species is typical of the rat and is reflected in the lung surfactant overall metabolism. The rabbit however has the highest proportion of 16:0/18:2PC and 16:0/18:1PC when compared to rat and human purified lung surfactant PC compositions. The proportion of 16:0/18:1PC varies considerably among the animals but rat surfactant contains the lowest percentage. The PC composition of guinea pig BALF is very similar to that of purified rabbit surfactant (figure 4.2). The only slight difference is that rabbit surfactant has a slightly higher percentage of 16:0/18:2PC than guinea pig BALF.

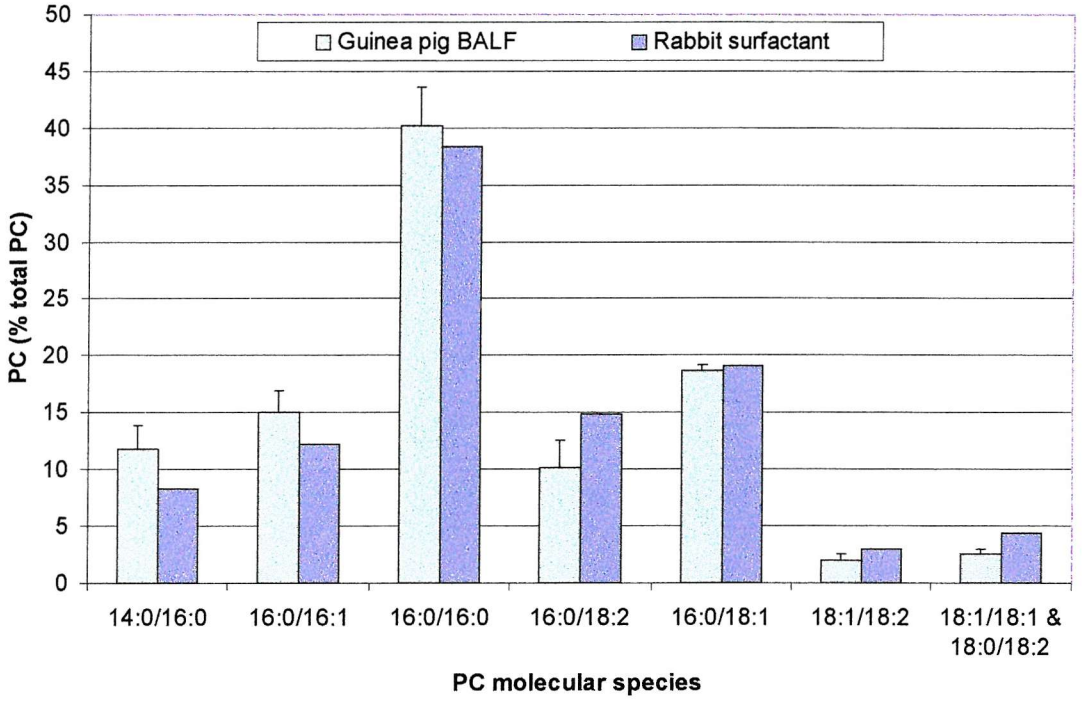


Figure 4.2 The PC molecular species composition of purified rabbit lung surfactant and guinea pig BALF. Error bars show standard deviations were applicable. n=10 guinea pigs and n=2 rabbits

To highlight that 16:0/16:0 is the major PC species in all the mammals studied; the five major PC species for each animal are shown in figure 4.3. The other 4 main PC species are the same for all the mammals considered, namely, 14:0/16:0, 16:0/16:1, 16:0/18:2 and 16:0/18:1, and are in varying proportions in each animal. It is clear from these results either that there are different requirements for surfactant PC species between

these animals or that the precise composition of non-16:0/16:0PC is not critical for effective surfactant function in the mammalian lung.

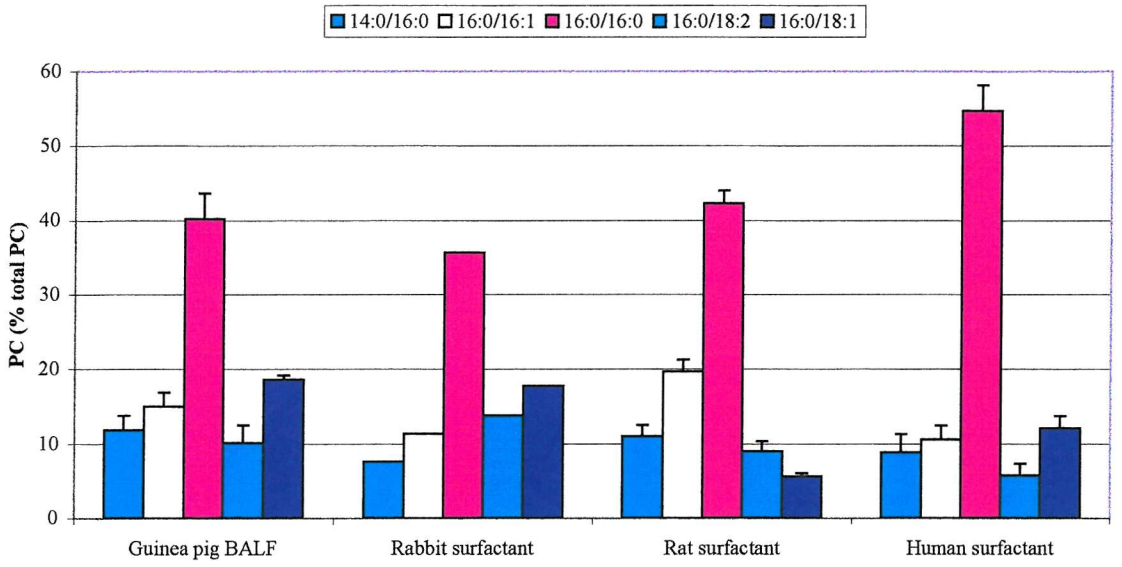


Figure 4.3 The composition of the five main PC molecular species from guinea pig BALF and purified rabbit, rat and human lung surfactant. Error bars show standard deviations were applicable. n=2 rabbits, n=4 rats and n=10 human control subjects

4.3.2.2 Phosphatidylglycerol molecular species

The phosphatidylglycerol (PG) molecular species composition of rat, rabbit and human purified lung surfactant as well as guinea pig BALF is shown in figure 4.4. There is great variability in the PG composition between the animals. One of the most significant differences is the very low proportion of 16:0/16:0PG in human lung surfactant. The virtual absence of 16:0/16:0PG in human lung surfactant has been reported previously [248,249] but has never been shown when comparing it to other animals lung surfactant. It is not clear why human lung surfactant has very little 16:0/16:0PG and why it is a major PG species in rabbit, rat and guinea pig surfactant.

There are many other differences in the PG composition between these animals. For instance 16:0/18:1PG is the major PG species in guinea pig BALF, and human and rabbit lung surfactant. However the main PG species in rat surfactant is 16:0/16:0PG with 16:0/18:2PG and 16:0/18:1PG being the next most prevalent respectively. Rat surfactant contains high proportions of 16:0 and 20:4 containing PG species. This high prevalence

of 20:4 containing species was also reflected in the PC composition of rat lung surfactant but is also evident in other rat tissues.

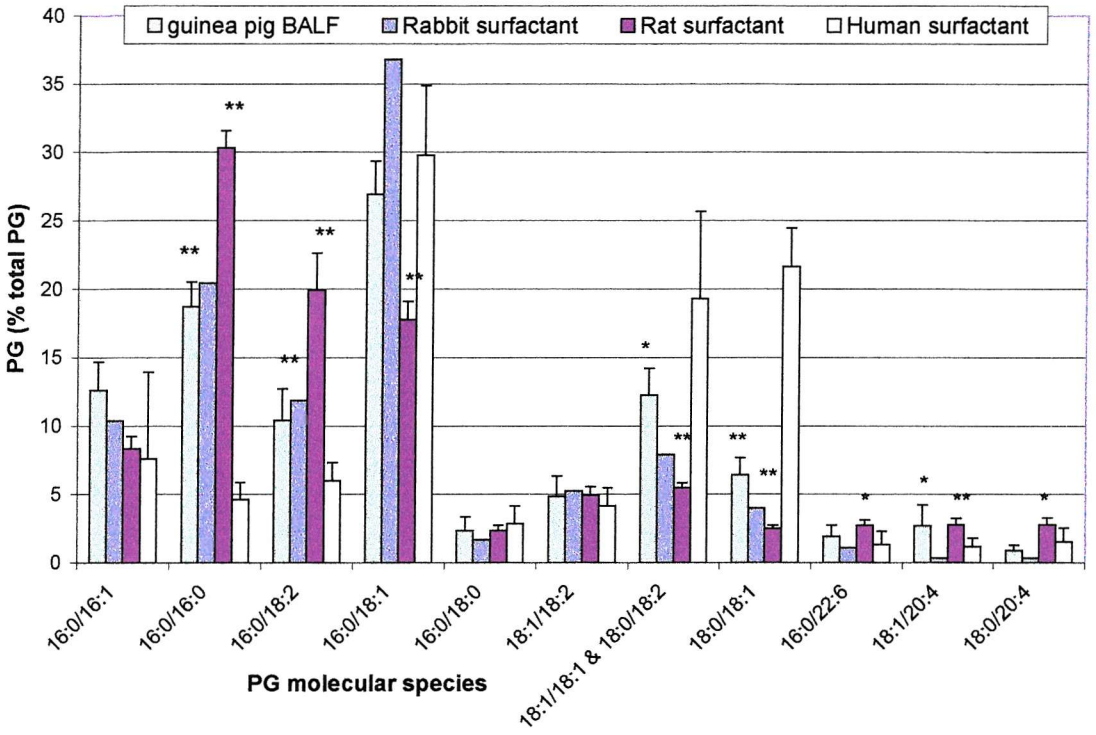


*Figure 4.4 PG molecular species composition of guinea pig BALF and rabbit, rat and human purified lung surfactant. Error bars denote standard deviations were applicable. Statistical significance is denoted by ** $P<0.005$ and * $P<0.05$ when compared by an independent t-test to human surfactant. n=10 guinea pigs, n=2 rabbits, n=4 rats and n=10 human control subjects.*

Human lung surfactant PG composition is very different to that of the rat. Human lung surfactant contains large amounts of the 18 carbon chain PG species, namely 18:1/18:1PG and 18:0/18:1PG. These two PG species were present in proportions significantly higher than in all the other animals studied. The physiological significance of human lung surfactant containing mainly 16:0/18:1PG, 18:1/18:1PG and 18:0/18:1PG is unknown but these PG species may prove to be involved in the immunological function of surfactant. A recent report studying group IIa $sPLA_2$ synthesis in alveolar macrophages has shown that 18:1/18:1PG was the most effective surfactant phospholipid tested at inhibiting group IIa $sPLA_2$ synthesis and that this effect can be explained, at least in part, by an impairment of TNF-α secretion from alveolar macrophages [250].

Guinea pig BALF and rabbit lung surfactant have very similar PG compositions, the major species being 16:0/18:1PG and 16:0/16:0PG respectively. The only slight differences are that guinea pig BALF contains somewhat higher proportions of 18:1/18:1PG, 18:0/18:1PG and 18:1/20:4PG than rabbit surfactant. The similarities between the rabbit and the guinea pig PG compositions were also apparent for the PC species.

The PG composition of lung surfactant varies between these 4 animals and is emphasized in figure 4.5, illustrating the five major PG species. Each animal species has a different PG composition, which suggests that the composition of PG is not tightly regulated. This substantial variation in the PG composition is a contrast to the PC composition where the PC species for all the animals follow a similar trend, with 16:0/16:0PC being the major species. There is also great variability in the human lung surfactant PG composition, which is in contrast to the other animals studied. This difference within the PG species of human lung surfactant is not surprising even though the subjects were control subjects with no history of lung disease. The absence of such variability in the laboratory-bred animals is likely to be due to the animals being in-bred in order to be the same. Therefore the laboratory-bred animals are unlikely to produce the variability seen in the human population, as they are all exposed to the same conditions and have similar genetic make-ups.

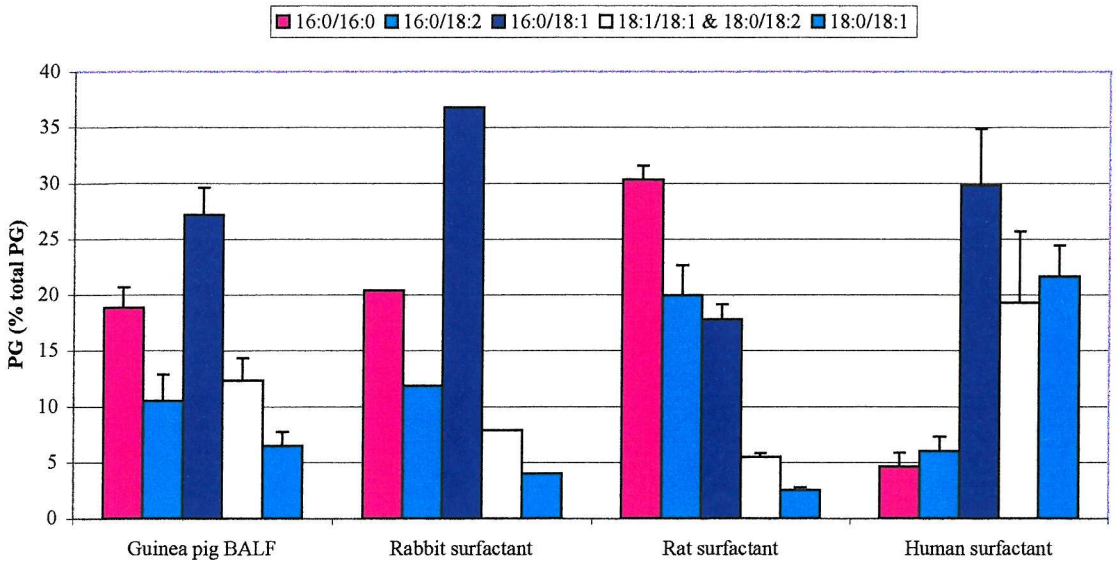


Figure 4.5 The composition of the five main PG molecular species from guinea pig BALF and purified rabbit, rat and human lung surfactant. Error bars show standard deviations were applicable. n=2 rabbits, n=4 rats and n=10 human control subjects.

4.3.2.3 Phosphatidylinositol molecular species

The phosphatidylinositol (PI) molecular species composition of guinea pig BALF and rabbit, rat and human purified lung surfactant are shown in figure 4.6. There is a large variation in the PI compositions between the mammals measured, although similarities were seen between the rabbit and guinea pig PI composition that was also evident in the PC and PG compositions. The major PI species varied dependent upon the animal, for instance 16:0/18:1 was the main PI species of rabbit surfactant and guinea pig BALF while 18:0/18:1 and 18:0/20:4 were the major PI species of human and rat surfactant respectively.

The PI composition of guinea pig BALF is most like rabbit surfactant and this is a trend that has been reflected in all of the phospholipid classes studied. However there are distinct differences between the PI composition of the guinea pig BALF and rabbit surfactant for instance guinea pig BALF contains higher proportion of 16:0/16:0PI, 16:0/16:0PI, 18:0/18:1PI, 18:0/20:4PI and 18:0/20:3PI. However, the three main PI species of guinea pig BALF namely, 16:0/18:1, 16:0/18:2, 18:0/18:2 and 18:1/18:1 are identical to the three major PI species of rabbit surfactant.

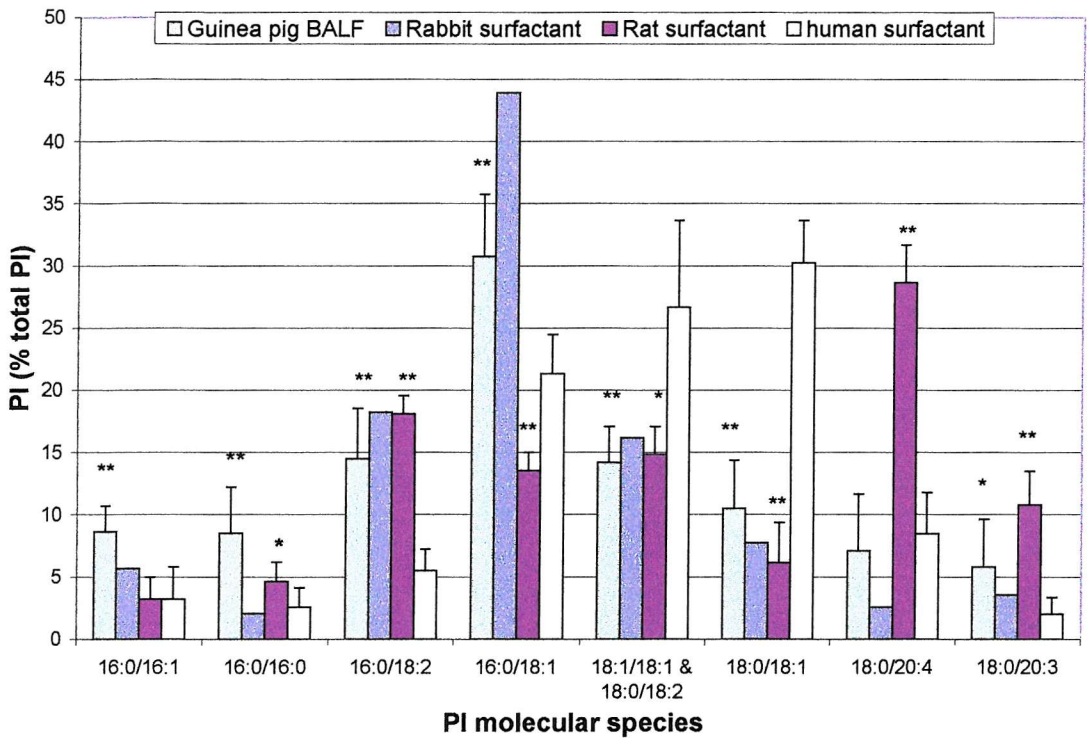


*Figure 4.6 PI molecular species composition of guinea pig BALF and rabbit, rat and human purified lung surfactant. Error bars denote standard deviations were applicable. Statistical significance is denoted by ** $P<0.005$ and * $P<0.05$ when compared by an independent t-test to human surfactant. n=10 guinea pigs, n=2 rabbits, n=4 rats and n=10 human control subjects.*

The PI molecular species composition of rabbit surfactant resembles the PG composition already reported for this animal (compare figure 4.4 and 4.6). There are many similarities between the PI and the PG compositions of rabbit surfactant, for instance the major species for both PG and PI is 16:0/18:1. There is however, a relative lack of the second most abundant PG species 16:0/16:0 in rabbit surfactant PI composition. Although all the animals studied also had a much lower proportion of 16:0/16:0PI when compared to the percentage of 16:0/16:0PG. Irrespective of this 16:0/18:2 is the third major PG species and is also the second main PI species.

Like the rabbit, human lung surfactant PI composition is very similar to the corresponding PG composition. The three main PI species in human lung surfactant namely 18:0/18:1PI, 18:1/18:1PI and 16:0/18:1PI are the same molecular species as the three major PG species but in slightly different proportions. These similarities in the PI and the PG composition suggest that they are synthesised from a common CDP-DAG pool (see section 4.3.2.4). The only differences from the PG composition are those that

are reflected in all the animals namely the lack of 16:0/16:1PI and 16:0/16:0PI and the presence of PI species associated with membranes (18:0/20:4PI and a very small amount of 18:0/20:3PI).

Rat surfactant PI composition appears to be very different to the PG composition with the major species being 18:0/20:4PI then 16:0/18:2PI, 18:1/18:1PI, 16:0/18:1PI and 18:0/20:3PI. The PI composition of rat surfactant is very different to that of all the other animals studied. Although like the other animals only a small proportion of 16:0/16:0PI is present but this amount is significantly higher than that present in human lung surfactant.

Figure 4.7 shows the five major PI species specifically 16:0/18:2, 16:0/18:1, 18:0/18:1, 18:0/20:4, 18:1/18:1 and 18:0/18:2 for guinea pig BALF and rabbit, rat and human purified lung surfactant. The figure illustrates the differences in the proportions of the five main PI species between these animals. For example rat surfactant contains 29% 18:0/20:4PI, whereas guinea pig, rabbit and human surfactant contains only 8%, 3% and 8% respectively. However rabbit surfactant and guinea pig BALF have very similar patterns of PI distribution both have 16:0/18:1PI as the main species and identical order of the next 4 main PI species.

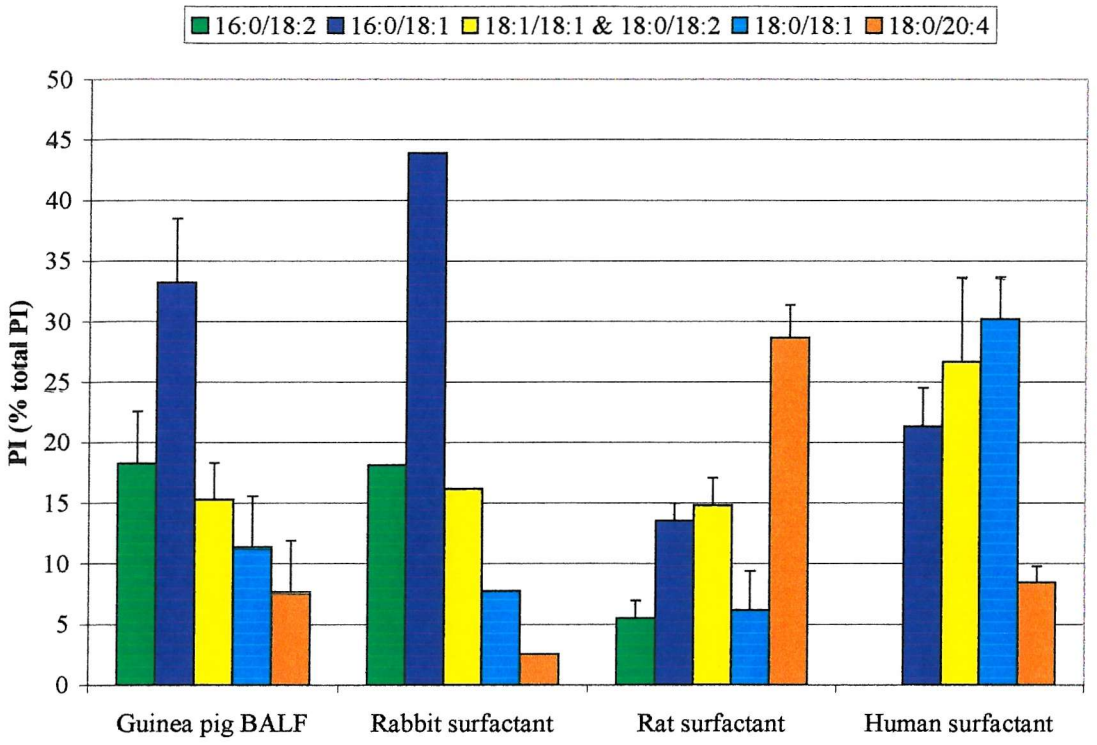


Figure 4.7 The composition of the five main PI molecular species from guinea pig BALF and purified rabbit, rat and human lung surfactant. Error bars show standard deviations were applicable. n=2 rabbits, n=4 rats and n=10 human control subjects.

4.3.2.4 Investigation as to whether PG and PI are synthesised from common CDP-DAG pool.

The similarities in the PG and PI compositions of rabbit and human lung surfactant tend to suggest that they are synthesised from a common CDP-DAG pool. It is important to establish whether laboratory animals have similar pathways for the metabolism and synthesis of surfactant phospholipids as humans, in order to ascertain which laboratory animals are good models to study alterations in lung surfactant. By identifying a good animal model to study alterations in lung surfactant during disease process, such as ARDS, it is then possible using the data gathered to extrapolate such changes to humans. However, if an animal model is used that has very different pathways for the metabolism and synthesis of lung surfactant to humans, the results obtained would be very misleading if it was extrapolated to humans. Although, *in-vivo* labelling studies using ^{13}C labelled glycerol or other similar isotopes are the only real way to understand the metabolism and synthesis of phospholipids, important information may be gathered from studying the similarities between the PI and PG compositions. Therefore using the data available 7 molecular species were identified that were present in both the PG and PI

analyses, namely 16:0/16:1, 16:0/16:0, 16:0/18:2, 16:0/18:1, 18:1/18:1 (and 18:0/18:2), 18:0/18:1 and 18:0/20:4 and then their relative percentages as a percent of total PI and PG were then correlated.

Figure 4.8 shows the correlation of human lung surfactant PG and PI species, the correlation is significant ($R=0.829$, $P<0.05$). Therefore because the PG and PI molecular species compositions are very similar it suggests that lung surfactant PG and PI are synthesised from the same CDP-DAG pool in the human lung. There is also a strong correlation ($R=0.769$, $P<0.05$) for rabbit surfactant PG and PI composition and this is shown in figure 4.9. Although there was not a significant correlation between the PG and PI compositions of guinea pig BALF ($R=0.751$, $P=0.052$), there was a trend that suggests may be other pathways of synthesis are taking place as well as being synthesised from a common CDP-DAG pool. Rat surfactant PG and PI compositions did not correlate implying that PG and PI species are synthesised from separate CDP-DAG pools in the rat. This has already been suggested and previous studies carried out on rats that have also suggested separate CDP-DAG pools [251,252].

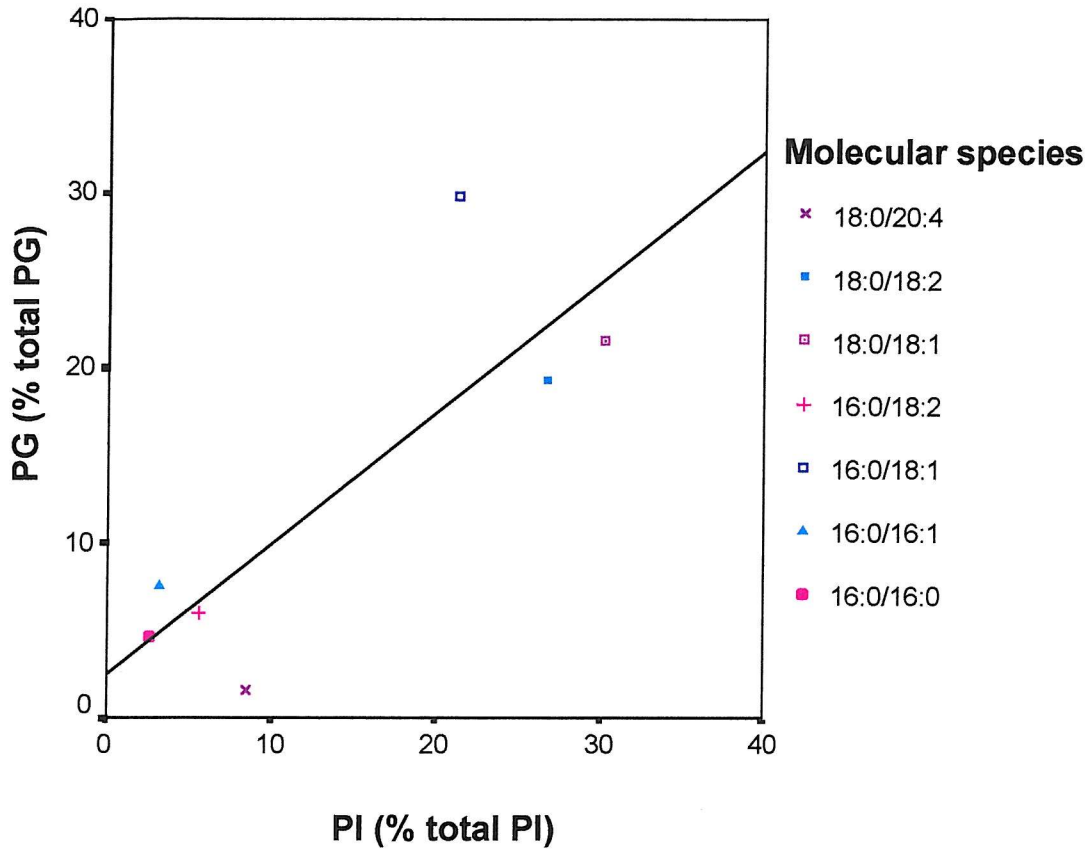


Figure 4.8 Correlation between PI and PG molecular species of human purified lung surfactant. Mean values of each molecular species are shown. Significance was determined by a Pearsons correlation, R=0.829 and P< 0.05

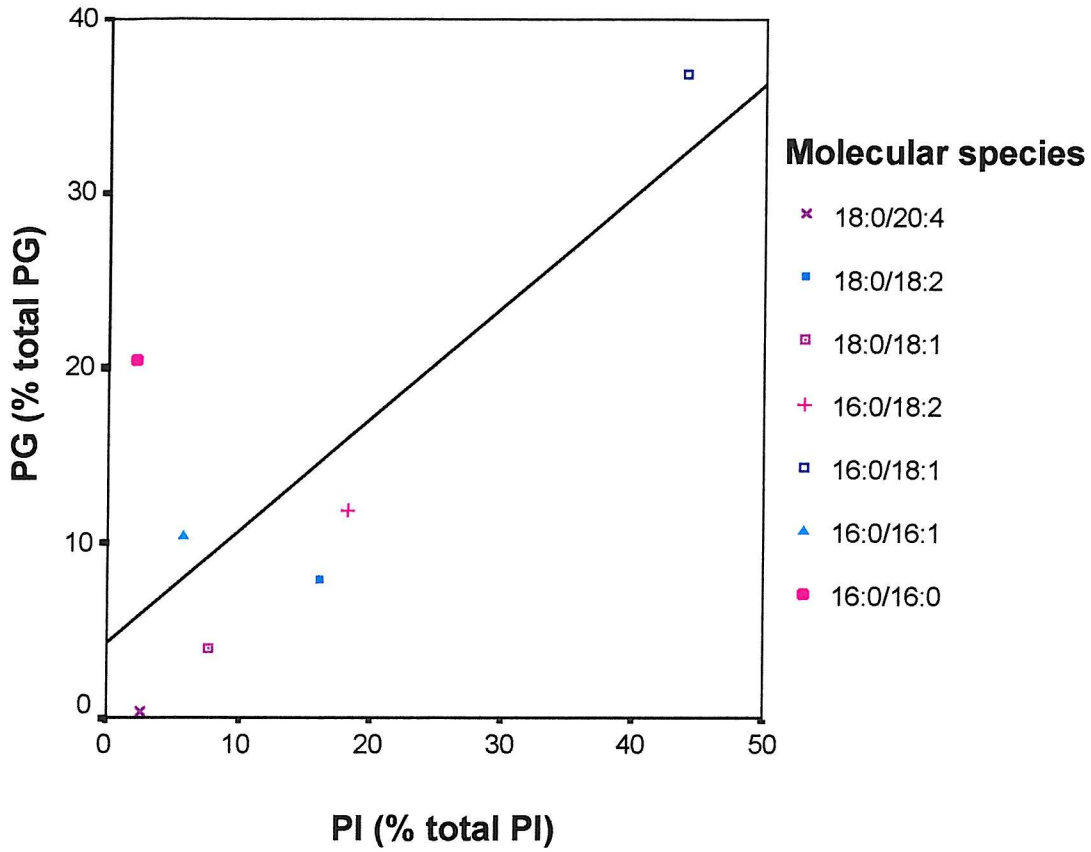


Figure 4.9 Correlation between PI and PG molecular species of rabbit purified lung surfactant. Mean values of each molecular species are shown. Significance was determined by a Pearsons correlation, R=0.769 and P< 0.05

4.3.2.5 Total Phospholipid composition

All the individual phospholipid molecular species were summed to give the total amount of each phospholipid class; these values were expressed as a percentage of the total phospholipid and are shown in figure 4.10. Rabbit surfactant contains the highest proportion of PC, then human and rat lung surfactant followed by guinea pig BALF respectively. Guinea pig BALF is significantly different to human surfactant for all the phospholipid classes (PC and PG $P<0.005$, PI $P<0.05$). The anionic phospholipids PG and PI appear to be interchangeable for instance rat surfactant has a high proportion of PG and low PI, whereas rabbit surfactant has a low proportion of PG and high PI. As Beppu suggested a low proportion of PG could be functionally compensated for by a higher proportion of PI [16]. This balance between the proportion of PI and PG appears to be evident when comparing the lung surfactant composition of these four animals.

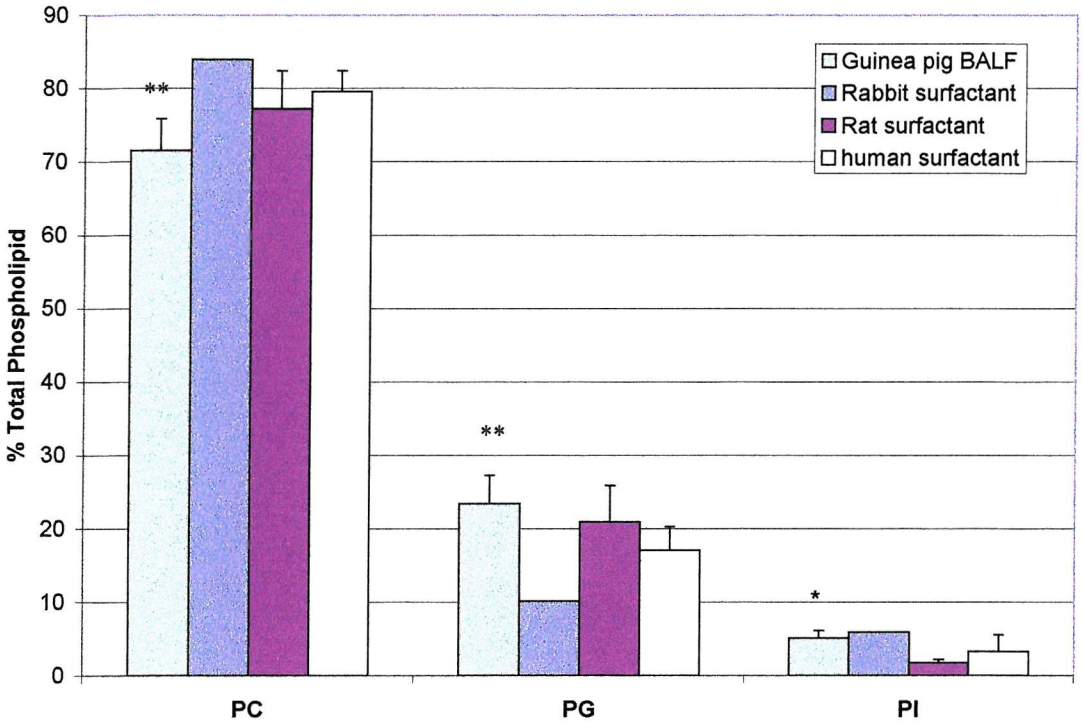


*Figure 4.10 The percent of PC, PG and PI of total phospholipid of guinea pig BALF (n=10) and rabbit (n=2), rat (n=4) and human purified lung surfactant (n=10). Error bars show standard deviations where applicable. Statistical significance is denoted by ** $P<0.005$ and * $P<0.05$ when compared by an independent t-test to human surfactant.*

The proportion of anionic phospholipids as a percentage of the total phospholipid for each of the mammals studied was calculated by the addition of the total PG and PI. The anionic phospholipids account for approximately 28% of guinea pig BALF total phospholipid and 16%, 22% and 20% of rabbit, rat and human surfactant respectively. This is very interesting as rabbit surfactant had the lowest proportion of PG and the highest proportion of PI also has the lowest percentage of total anionic phospholipid. The potential physiological significance is that PI may be more effective functionally in the lung and therefore less PI is required to fulfil the same function as PG. This observation is further supported by a study on the foetuses of myo-inositol treated rats that found the lamellar bodies to be smaller than those of control foetuses, suggesting the amount of surfactant stored is reduced as the PG content decreased [253]. Another study looked at the surface activity of PG deficient surfactant in adult rats, caused by myoinositol-treatment. They found no difference in the surface activity of PG deficient surfactant when compared to normal surfactant [251]. This study agreed with others by concluding that surfactant PG could be replaced by PI without altering normal lung function [16-18].

4.4 Discussion

These studies have shown that the analysis of BALF phospholipid composition does represent that of purified surfactant. The use of BALF as opposed to purified surfactant, as a diagnostic marker for surfactant abnormalities in the lung is a useful tool, enabling the faster generation of results from smaller aliquots of BALF. Although the results comparing BALF with purified surfactant in the rabbit and the rat were very similar they did reveal a few small differences, for instance that purification does not enhance the proportion of 16:0/16:0PC and 16:0/16:0PG. However 16:0/18:2PG did accumulate in the purified surfactant, which is deemed to be the surface-active fraction.

Unlike BALF, the phospholipid composition of purified lamellar bodies differed considerably from that of purified lung surfactant in the rat. This finding was interesting as surfactant is packaged into lamellar bodies in the alveolar type II cell prior to secretion into the alveolar subphase [254]. Lamellar bodies have been described as consisting of a dense proteinaceous core with lipid bilayers arranged in parallel, stacked lamellae surrounded by a limiting membrane [255]. The lamellar body material can be taken as the starting material and the purified surfactant as the resultant material after all the regulatory processes have occurred. There is some controversy as to what these regulatory processes are, for instance whether there is sorting of the surfactant after secretion from the type II cell and prior to adsorption to the surface-associated phase or selective catabolism of the phospholipids in the alveolar space. However what is certain is that some regulatory processes must occur, as the phospholipid composition of the lamellar bodies is very different to the purified surfactant.

Lamellar bodies have been shown to have less disaturated phospholipid species and more polyunsaturated species than purified surfactant. The presence of polyunsaturated phospholipid species in lamellar bodies is likely to be there as part of the membrane surrounding the lamellar bodies. For instance there are high proportions of PI and PC species that are typically found in membranes such as 18:0/20:3PI, 18:0/20:4PI, 16:0/18:1PC and 18:0/20:4PC. The proportion of 16:0/16:0PC the surface-active component and 16:0/16:0PG is higher in the purified surfactant which suggests an accumulation of these disaturated species at the air-liquid interface. The reason for the accumulation of 16:0/16:0PC at the air liquid interface is likely to be due to the

squeezing out of other less saturated phospholipid species. The exact role of 16:0/16:0PG is unknown but a recent report suggested that it may stabilise the surfactant structure. The presence of 16:0/16:0PG in a reconstituted surfactant of 16:0/16:0PC reduced the conversion rate to the less surface active light subtype of surfactant, whereas the addition of a mixture of PG to 16:0/16:0PC increased the conversion rate compared to solely 16:0/16:0PC [256]. Therefore the higher proportion observed in the purified surfactant compared to the lamellar bodies is likely to be beneficial and this might be achieved by selective sorting in the alveolar subphase.

The main aim of this chapter was to compare the PC, PG and PI molecular species composition of surfactant from rats, rabbits, guinea pigs and humans. This is the first time that such a detailed analysis of PC, PG and PI composition of surfactant from these animals has been performed. The surface-active component 16:0/16:0PC is the major phospholipid species in all of the mammals studied. The PC compositions (figures 4.1 and 4.2) illustrate that human lung surfactant has the greatest percentage of 16:0/16:0PC. The composition of the other molecular species of PC is not identical for all of the mammals, however the PC composition is very similar with any differences being characteristic of the phospholipid compositions of the animals concerned. For instance rat surfactant has a high proportion of 20:4 containing species and rat platelets have been shown to have more 20:4 containing species [257] than human platelets [258]. The differences in the PC molecular species composition between these mammals suggests that, while a normal level of 16:0/16:0PC is required for normal surfactant function there is no apparent need for an exact metabolic control of each molecular species of PC.

The PG compositions for these mammals vary extensively, which suggests that the acidic head group may be of more importance than the acyl chain attached. Possibly the most notable difference was that human lung surfactant contained only a very small proportion of 16:0/16:0PG, whereas it was the major PG species in rat lung surfactant. This dramatic variation in the PG compositions has been reported before in another study that looked at the fatty acid compositions of lung surfactant phospholipids in different animal species. They found that the fatty acid composition of PG varied significantly among the mammals studied. Rabbit, dog and rat surfactant contained 50-60% 16:0, while human and cat contained much lower levels of saturated fatty acids [245].

No reason was proposed for these differences. Nevertheless this wide variation does imply that the PG composition is not as crucial as PC for these animals and that the PG molecular species composition has not been conserved through evolution.

The PI molecular species composition also varied between the animals studied with major PI species being different for each animal studied. However similarities were noticed in the molecular species composition between the PI and the PG composition of rabbit and human lung surfactant. The resemblance between the PG and the PI compositions suggest that these acidic phospholipid molecular species were synthesised from a common CDP-DAG pool. The proposed hypothesis that both PG and PI in lung surfactant are synthesised from a common CDP-DAG pool [77] has been subject to some controversy. Studies have been performed on either rabbits or rats using different techniques to measure the fatty acid compositions. In this chapter the molecular species compositions of seven PG and PI species were correlated for all the animals studied. Human and rabbit lung surfactant showed a good correlation (R= 0.829 and 0.769 respectively) which indicates that the PG and PI species have a similar distribution of molecular species that is likely to have occurred through both PG and PI being synthesised from a common CDP-DAG pool. However the composition of rat surfactant PG was distinctly different from the corresponding PI composition and no correlation was shown indicating that probably the pathways for the synthesis of surfactant PG and PI in the rat lung are different from that of the human and rabbit. The PG and PI compositions of guinea pig BALF were similar but the correlation was not significant (R=0.751, P=0.052), which may indicate that a combination of pathways are involved.

The relationship between PG species and PI species in the lung has as yet not been clearly established. What is accepted is that a high concentration of myoinositol suppresses PG synthesis and correspondingly increases the PI content of lung surfactant in adult animals, *in vivo* as well as *in vitro* [259-261]. However the fatty acid compositions of lung surfactant both before and after administration of myoinositol to rabbits and rats have been disputed in different reports. For instance Rustow et al [22] reported that the PG and PI species pattern was very similar in the adult rat lung and concluded that PG and PI species were synthesized from a common CDP-DAG pool. However another study in rats treated with myoinositol [251], found that PG and PI had dissimilar fatty acid

compositions, and concluded that it agreed with other findings that suggest that these lipids are likely to be synthesised in different subcellular organelles and not from a common CDP-DAG pool [252]. These findings suggest that the regulation of the synthesis of PG and PI may be more complex than originally envisioned, and that substantial rearrangement of 1 or both of these lipids must occur. The results in this chapter agree with this hypothesis for the separate synthesis of surfactant PG and PI in the rat lung. However in contrast to the rat, human and rabbit lung surfactant have very similar PG and PI species patterns which suggests that they are synthesised from a common CDP-DAG pool and undergo very little rearrangement of the lipids prior to secretion into the alveolar subphase. Whereas PG and PI synthesised in the guinea pig lung could be synthesised from either a common CDP-DAG pool and then undergo modification prior to secretion or synthesised from different CDP-DAG pools or most likely a combination of both pathways.

When looking at the proportions of each phospholipid class of lung surfactant in the animals studied in this chapter it becomes apparent that PG and PI seem to be interchangeable. For instance rat lung surfactant has a high proportion of PG and low PI when compared to the other animals (figure 4.10) and rabbit surfactant has a low proportion of PG yet the highest percentage of PI. These differences indicate that there is a form of co-regulation of the acidic phospholipids and it might possibly be due to different levels of myoinositol in the animals. But other studies in turtles, monkeys and chickens have reported PG virtually absent in surfactant and suggested that PG can be replaced by PI without altering normal lung function [16-18](see section 1.2.3.2). No explanation was given for these varying amounts of PG and the corresponding change in the proportion in PI lung surfactant. It does appear that anionic phospholipids are required for normally functioning lung surfactant whether it is PG or PI seems not to matter.

There are a few fundamental differences in the phospholipid compositions between the animals studied, such as rat lung surfactant containing a high proportion of 20:4 species. Whereas human lung surfactant has very little 16:0/16:0PG and more 18 carbon chain fatty acid species. Rabbit lung surfactant is dominated by 16:0/18:1 and 16:0/18:2 PG and PI species. The PC, PG and PI compositions of guinea pig BALF were very similar

to that of the rabbit and suggest that these two animals have very similar surfactant systems.

The lack of any good comparative data on the lung structure of these animals has made it difficult to assess whether there is any association with the differences in phospholipid composition and the lung structures of these animals. However a recent study into alveoli size of mouse, hamster, rat, rabbit, monkey, baboon and human reported that the number of alveoli per lung increased with body weight to the 0.59 power according to a regression analysis [262]. The general trend was for larger lungs to be composed of both larger and more numerous alveoli. Although the alveoli of the rabbit were of comparable size to those of the rat (diameter of rabbit alveoli 97± 5 versa rat alveoli 94±4μm), which has a six- to seven fold smaller lung, either the rabbit has more proportionally smaller alveoli or the rat has less proportionally larger alveoli. The differences in alveolar size would suggest that larger alveoli would require a surfactant that is more effective at reducing the increased surface tension when compared to smaller alveoli. However accompanying this increase in alveolar size was an increase in elastin and collagen fibres that increases lung elasticity and lung compliance, therefore the recoil forces due to surface tension will be lower in species with larger alveoli [262]. This finding accompanied by studies that demonstrated that the surface tension lowering properties of surfactant are also similar across theses animals, for instance in rats[263], rabbits [264,265], and cats [266], suggests that surfactant from all the mammals studied in this chapter will have the same surface tension lowering abilities. Therefore the variation in surfactant phospholipid composition observed in this chapter has very little to do with the structure of the mammals lungs as they are all fairly similar. The differences in phospholipid composition observed must be for another reason.

Animal models have been used to study the pathophysiology and treatment of surfactant deficiency on the assumption that this material is essentially identical in various species. Rabbits, rats and guinea pigs are typical laboratory animals and the comparative analyses presented above demonstrate that their phospholipid compositions differ to humans and in the case of the rat significantly. Other studies in rats investigating the changes in the lung function with age demonstrated that over an equivalent portion of the adult life span, the lung function and structure of rats lungs does not deteriorate in the same

manner as does that of man [267,268]. This together with the observation that rat surfactant phospholipid composition is significantly different to human suggests that the rat would not be a good animal model to study surfactant alterations that are thought to occur in man. However rabbit surfactant phospholipid composition is the most like human surfactant compared to the rat and guinea pig. Although the guinea pig phospholipid molecular species composition was very similar to that of the rabbit, the guinea pig did have significantly less PC and more PG than both the rabbit and human. The lung structure of the rabbit also appears to undergo similar age related changes as the human lung, although only one study has been performed [269]. Therefore I propose that the rabbit is a good animal model for studying surfactant related disease. The use of such animal models provides invaluable data and is very important tool for studying human diseases. However animal studies tend to use animals that have originated and were maintained in controlled environments largely free of many of the environmental insults to which man is exposed to. These additional factors may also play a role in lung diseases.

Chapter Five

Phospholipase A_2

5.1 Introduction

Phospholipase A_2 enzymes catalyse the hydrolysis of the fatty acid ester at the *sn*-2 position of glycerophospholipids releasing lysophospholipids and free fatty acids. When the fatty acid is arachidonic acid, several pro-inflammatory lipid mediators (e.g. prostaglandins and leukotrienes) can be formed. The lysophospholipids may also serve as the precursor for the pro-inflammatory agent platelet activating factor (PAF). The understanding of the PLA_2 catalysed reaction is important to many physiological processes such as lipid metabolism, the inflammatory response, and digestive reactions [116]. In this chapter the role of $sPLA_2$s in inflammation will be considered along with the possible effect that increased levels of $sPLA_2$ would exert on lung surfactant phospholipid composition. Human group IIa $sPLA_2$s are released from inflammatory cells (e.g. macrophages) in response to cytokines (e.g. TNF_α) and are thought to be involved in the inflammatory response.

Lung surfactant is a mixture of phospholipids and apoproteins that lines the air:liquid interface of the lungs. The main function of lung surfactant is to reduce the surface tension in alveoli thereby preventing fluid entering the alveoli and enabling efficient gas exchange to occur. Lung surfactant has an unusual phospholipid composition as it contains 10-15% PG and approximately 80% PC. Alterations in the phospholipid composition of lung surfactant have been linked with Acute Respiratory Distress Syndrome (ARDS), a very severe lung inflammatory disease[64,77] in which the most notable change is a dramatic increase in the PC:PG ratio suggesting a decrease in the proportion of PG.

The relatively high proportion of PG in lung surfactant is unique in mammalian tissues and may make it a potential target for human group IIa $sPLA_2$ hydrolysis as this enzyme has a preference for anionic phospholipids such as PG. In contrast human group IIa $sPLA_2$ has been reported to be essentially inactive against pure PC vesicles as well as cell plasma membranes [200,270], which do not normally contain anionic phospholipids in the outer monolayer. However the presence of PG in the PC interface should greatly enhance the activity of the group IIa $sPLA_2$. This presence of PG would therefore suggest that lung surfactant might be susceptible to hydrolysis by the group IIa enzyme.

The human group IIa $sPLA_2$ has been reported to be released in the alveolar space in lung inflammatory processes such as acute asthma [4,208] and ARDS [3].

Taken together this information suggests the possibility of human group IIa $sPLA_2$ playing a significant role in lung surfactant degradation during acute inflammation. However there have been conflicting reports as to whether lung surfactant phospholipid is a substrate for mammalian group IIa $sPLA_2$ hydrolysis. Recombinant human group II $sPLA_2$ was reportedly essentially inactive against native porcine surfactant [271], while the equivalent recombinant enzyme from guinea pig readily hydrolyzed the phosphatidylcholine (PC) in sonicated guinea pig surfactant [272].

It has been previously reported that the human group IIa $sPLA_2$ exhibits little acyl chain selectivity [118-120]. However the enzyme does have a well-documented need for anionic phospholipids as part of the substrate aggregate such as vesicles and membranes. It has been proposed that the cationically charged enzyme (pI>10.5) [273] requires the negatively charged interface in order to bind to the substrate prior to any hydrolysis occurring. The requirement for anionic lipids is because PLA_2 acts at a lipid-water interface, and the action of PLA_2 necessitates the binding of protein to the phospholipid interface. This interfacial binding is a unique and important step in the interfacial catalysis of PLA_2 and occurs prior to the binding of a substrate to the active site [197]. Human group IIa $sPLA_2$ has a reported active site preference that is proposed to explain why PE is hydrolysed more than PC and this is likely to be due to the relative inability of the active site to accommodate the bulkier choline headgroup [274]. In addition this paper demonstrated a preference of this enzyme for the anionic phospholipids, PG and PA. Therefore the presence of PG could enhance human group IIa $sPLA_2$ activity both by enhancing interfacial binding and also by being a preferred substrate for the enzyme.

The aim of this chapter is not only to investigate whether lung surfactant can act as a substrate for PLA_2 mediated hydrolysis but also to identify if the human group IIa $sPLA_2$ and the *Naja naja* enzyme show selectivity in terms of hydrolysis of the substrate. The comparison of the human group IIa enzyme with the *Naja naja* enzyme provides an important positive control. The venom enzyme is well known as a highly penetrating

enzyme able to hydrolyse PC rich vesicles and cell membranes and demonstrates activity 100-1000 fold higher than the group IIa enzyme on such substrates [144]. It has now been possible by utilizing ESI-MS to analyse the individual phospholipid molecular species in complex phospholipid mixtures both before and after incubation with an enzyme. This experimental method has not been available previously as ESI-MS is a relatively new technique for phospholipid analysis. As stated previously, the group IIa enzyme shows a marked preference for phospholipid interfaces containing a significant proportion of anionic lipid. Partly the specificity may be explained by interfacial binding and partly because of an active site preference for phospholipids with smaller and anionic headgroups. The use of ESI-MS to analyse surfactant phospholipid composition before and after incubation with $sPLA_2$ provides a unique opportunity to investigate enzyme specificity. In addition previous work on the enzyme suggests that the enzyme demonstrates little acyl chain specificity.

5.2 Methods

The methods used in this chapter have already been described in chapter 2. The PC and PG vesicles were analysed as described in section 2.7.1, using a mobile phase of methanol: chloroform: water (7:2:1 v/v) and an injection solvent of methanol: chloroform: water (7:2:1 v/v) containing 1% (w/v) NH_4OH. PC, PG and PI compositions of rabbit surfactant were analysed after the classes had been separated as in section 2.6.1. The PC compositions were analysed as in section 2.7.2, using an injection solvent of methanol: chloroform: water (7:2:1 v/v) containing 20mM sodium acetate, while PG and PI compositions were analysed as described in section 2.7.1.

5.3 Results

The overall aim of this chapter is to determine the ability of human group IIa $sPLA_2$ to hydrolyse lung surfactant as a possible crucial event in inflammatory lung diseases. The following chapters will then assess whether the hydrolysis of lung surfactant by PLA_2 enzymes is part of the disease process in asthma (chapter 6) and ARDS (chapter 7). There are two methods available that will allow the detailed assay of this hydrolysis using lung surfactant in its native form. The first method (see section 2.9.1) involves a fluorescence displacement assay in which released fatty acids are detected in real-time.

This method provides a measure of the overall rate of phospholipid hydrolysis and therefore is a measure of the overall ability of a PLA_2 to hydrolyse lung surfactant. The method will give no indication of phospholipid substrate specificity. The second method (see sections 2.10 and 2.11) utilises ESI-MS to detail both the loss of phospholipid molecular species and the appearance of lysophospholipid products. Therefore the method provides a unique measure of what phospholipids are being hydrolysed. Initially the results obtained from using the fluorescent displacement assay will be discussed. The second part of the chapter will detail results obtained using ESI-MS.

5.3.1 Effect of increasing PG in PC vesicles on $sPLA_2$ rates of hydrolysis as measured by the fluorescent displacement assay

Human group IIa $sPLA_2$ has a well-documented inability to hydrolyse pure PC vesicles, but the ability of the enzyme to hydrolyse the zwitterionic phospholipid is greatly enhanced with the presence of anionic phospholipids [200]. A very wide range of biological activities at the membrane interface require the presence of anionic phospholipids and such phenomena are characterised by a dramatic enhancement of activity with between 10-20 mol% anionic phospholipid. This phenomenon is due to multiple non-specific electrostatic interaction between protein and membrane required a minimum anionic charge density [275]. Lung surfactant consists of mainly PC but contains between 10-15% of the anionic phospholipid PG so it is important to investigate the proportion of PG that is required to initiate this enzyme and identify whether lung surfactant is a potential substrate for human group IIa $sPLA_2$ hydrolysis.

The consequence of increasing proportions of 18:1/18:1PG in 18:1/18:1PC SUVs on human group IIa $sPLA_2$ activity was investigated using the fluorescent displacement assay. The measurements were performed in triplicate and the results are shown in figure 5.1. The advantage of a simple vesicle system of PC and PG with the same molecular species (18:1, oleic acid) is it is possible to identify the proportion of PG that is required for any initial hydrolysis to occur independent of the fatty acids attached.

The results demonstrate that human group IIa $sPLA_2$ has almost zero activity in 100% PC vesicles. A small amount (about 10%) of PG is required in the vesicles to enable

significant hydrolysis of the phospholipid vesicles to occur. The rate of hydrolysis of the vesicles thereafter increases proportionally with increasing molar % of PG present. These results indicate that the enzyme requires an anionic interface with a minimal anionic density (10 mole%) in order to bind to the phospholipid substrate via non-specific electrostatic interactions. The highly cationic nature of the enzyme in terms of interfacial surface potential has been highlighted that will promote such non-specific electrostatic interactions [276]. Once the enzyme is bound to the surface of the vesicle it then permits the hydrolysis of the phospholipids at the active site.

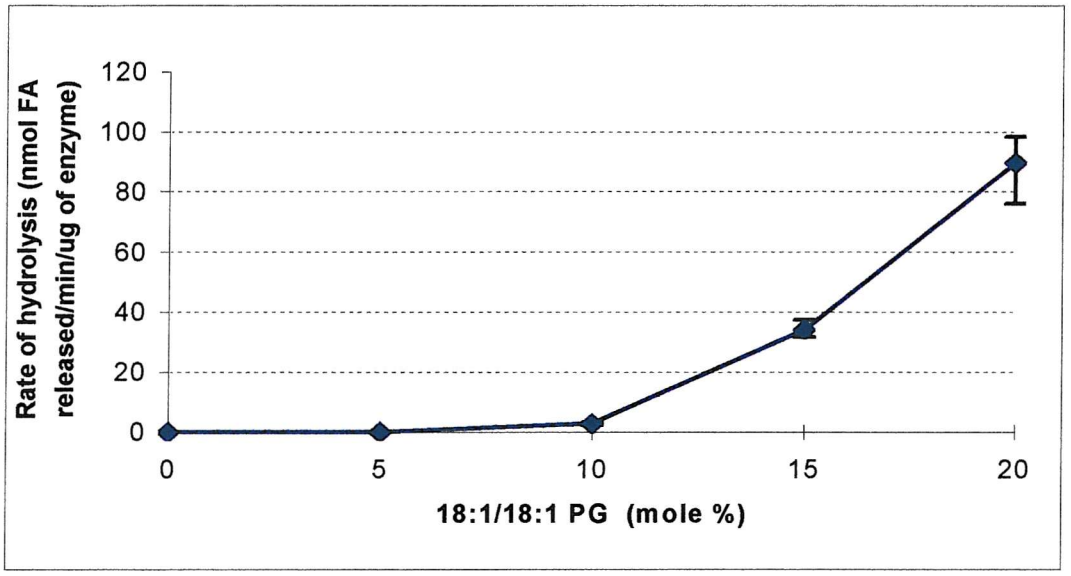


Figure 5.1 Human group IIa $sPLA_2$ activity on 18:1/18:1PC vesicles containing an increasing the proportion of 18:1/18:1PG. The initial rate of hydrolysis was determined by the rate of fall in fluorescence. This fall in fluorescence was calibrated in terms of released fatty acid (oleic acid). All values were the mean of triplicate values and the error bars are ± standard deviations.

The almost zero rate of hydrolysis of a pure PC vesicle by the human group IIa $sPLA_2$ is well known [200]. This lack of activity is of particular importance as PC is the major phospholipid in the outer monolayer of most eukaryotic cell membranes and lipoproteins and under normal conditions would render them not susceptible to hydrolysis by group IIa $sPLA_2$. Lung surfactant however contains a particularly high proportion of the anionic phospholipid PG that is present at about 15% of the total phospholipid in human lung surfactant and should facilitate hydrolysis (see figure 5.1). PG is not a normal constituent of mammalian biological membranes and its presence in surfactant is

unusual. The very low activity of the human group IIa $sPLA_2$ enzyme against PC interfaces is not a unique characteristic of $sPLA_2$s because the structurally very similar *Naja naja* enzyme is very active against such interfaces and can provide an important positive control in these studies.

5.3.2 The use of the fluorescent displacement assay to study the action of PLA_2s on lung surfactant

Due to the inherent difficulties in obtaining large amounts of human lung surfactant, purified rabbit lung surfactant (see section 2.3 for preparation) was used to ascertain whether lung surfactant is a substrate for PLA_2 hydrolysis. The extent of hydrolysis of the lung surfactant was measured using the fluorescent displacement assay. Three different types of PLA_2 enzymes were used with different interfacial characteristics, namely human group IIa secreted PLA_2 ($sPLA_2$), snake venom *Naja naja* PLA_2 and Group I porcine pancreatic PLA_2 (PP PLA_2). The rates of hydrolysis of the rabbit lung surfactant in its native form, sonicated and as a lipid extract was measured using the fluorescent displacement assay. As mentioned above, *Naja naja* $sPLA_2$ provides a positive control while the porcine pancreatic enzyme demonstrates intermediate activity against membranes and vesicles [144].

5.3.2.1 Incubation of purified rabbit surfactant with PLA_2 enzymes

The purified surfactant was not hydrolysed by either the PP PLA_2 or the human group IIa $sPLA_2$ and this lack of hydrolysis was unaffected by increasing amounts of substrate (up to 100nmoles of phospholipid phosphorous) and enzyme (up to 10μg). However the snake venom enzyme *Naja naja* PLA_2 was capable of hydrolysing the native rabbit surfactant at a rate of 1.2 nmol of fatty acid released/minute/μg of enzyme after the addition of 0.1μg of the enzyme to 7nmoles of rabbit surfactant per ml in individual assay mixtures.

5.3.2.2 The effect of sonication on the hydrolysis of purified rabbit surfactant by PLA_2 enzymes

The sonication of rabbit surfactant increased the initial rate of hydrolysis by the *Naja naja* PLA_2 nearly five fold from 1.2 to 5.8 nmol of fatty acid released/minute/μg of

enzyme. The porcine pancreatic PLA_2 enzyme was able to hydrolyse the sonicated surfactant phospholipids at a detectable initial rate of 0.04 nmol of fatty acid released /minute/μg of enzyme whereas no rate was detectable using native surfactant. These observed increases in initial rates of hydrolysis after sonication are probably due to a change in the physical structure of the substrate converting the large aggregates of surfactant into smaller vesicles; this would increase the surface area available for phospholipid hydrolysis and also would decrease the packing density. Human group IIa $sPLA_2$ demonstrated an inability to hydrolyse the sonicated rabbit surfactant, even after increasing the amount of enzyme present in the assay from 2 to 24 μg and repeated sonications of the rabbit surfactant. A level of 24μg/ml of the human enzyme is at least 10 fold higher than serum levels seen in the most acute inflammatory conditions.

5.3.2.3 Differential rates of PLA_2 hydrolysis of a lipid extract of rabbit surfactant

Lung surfactant comprises of not only phospholipids but also specific surfactant proteins (SP) and SP-A is reported to inhibit the action of human group IIa $sPLA_2$ [272]. The removal of the hydrophilic proteins such as SP-A and D by organic solvent extraction of the phospholipid allows the comparison of initial rates of hydrolysis of lung surfactant without these inhibitory proteins. The lipid extract of purified rabbit surfactant was dissolved in methanol (see section 2.9.3) and injected into the assay cocktail mixture to form SUVs. The SUVs were then used as substrates to see the hydrolysis rates of different PLA_2s. The results are shown in figure 5.2 and illustrate that the SUVs were susceptible to hydrolysis by all the PLA_2 enzymes tested but gave varying initial rates of hydrolysis. The rates of hydrolysis of the SUVs are faster than those achieved when the substrate was presented in its native and sonicated form for all the enzymes. This increase in rates may be due to the removal of any inhibitory proteins such as SP-A during the lipid extraction process. However the physical structure of the SUVs will be different to that of surfactant, presenting the phospholipids as a curved interface (vesicle) and thus creating a readily accessible substrate. It is well known that the sonication of phospholipid vesicles creates highly curved SUVs that produce dramatic rate enhancement when used to assay the activity of PLA_2s.

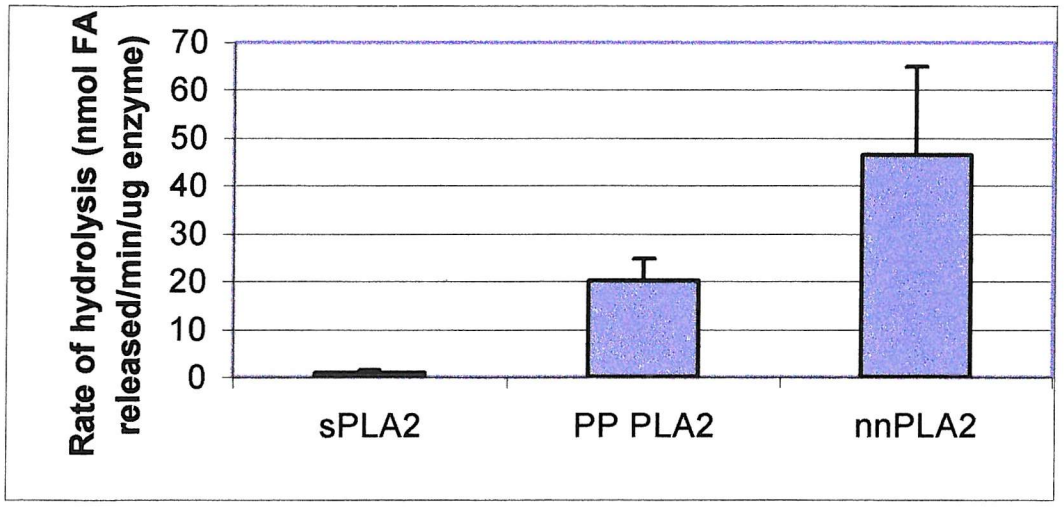


Figure 5.2 The effect of different PLA_2 enzymes on the rates of hydrolysis of a lipid extract of rabbit surfactant. Human group IIa $sPLA_2$ ($sPLA_2$), Porcine pancreatic PLA_2 (PP PLA_2) and the snake venom Naja naja PLA_2 ($nnPLA_2$) were all assayed to determine their rates of hydrolysis on a lipid extract of rabbit surfactant (7 nmoles/ml) using the fluorescent displacement assay. The initial rates of hydrolysis were determined by the rate of fall in fluorescence. This fall in fluorescence was calibrated in terms of released fatty acid (18:1, oleic acid). All values are the mean of triplicate measurements ± standard deviations.

The snake venom *Naja naja* PLA_2 again demonstrated the fastest rate of hydrolysis, followed by the porcine pancreatic PLA_2. The human group IIa $sPLA_2$ was now able to hydrolyse the substrate but at a very low rate. The differential rates of hydrolysis are not surprising as the snake venom enzyme *Naja naja* PLA_2 has been reported to exhibit a much higher ability than mammalian group IIa $sPLA_2$s to hydrolyse phospholipids on packed monolayer structures [277,278], while the same order of preference was seen on biological membranes [144] as now observed with lung surfactant phospholipids.

5.3.3 The effect of increasing 18:1/18:1PG in 18:1/18:1PC vesicles on the extent of human group IIA $sPLA_2$ hydrolysis as monitored by electrospray ionisation mass spectrometry.

The presence of the anionic phospholipid PG in a PC vesicle increases the rate of hydrolysis by human group IIa $sPLA_2$ (see figure 5.1). The proposed reason for the increase in rate is that the anionic phospholipids provide a negative charge at the interface allowing the enzyme to bind and then hydrolyse the phospholipids. A study of a simple system of 18:1/18:1PC vesicles containing an increasing proportion of

18:1/18:1PG using the mass spectrometer, will allow the determination of phospholipid composition before and after hydrolysis by the PLA_2. It is possible, therefore, to investigate the phospholipids hydrolysed and study whether the enzyme selectively hydrolyses PG or just requires the presence of anionic phospholipids to bind to the surface.

In order to investigate enzyme specificity, approximately 53nmoles of phospholipid containing increasing proportions of PG were incubated with 100ng of human group IIa $sPLA_2$ for 0, 30, 60, 300, 600 and 1800 seconds. The results are expressed as a percentage of phospholipid when compared to the amount present after incubation in the absence of enzyme. The effects of increasing the molar proportion of PG are shown in figures 5.3 to 5.8.

The inability of human group IIa $sPLA_2$ to hydrolyse pure PC vesicles is illustrated in figure 5.3. However after a long incubation with the enzyme (i.e. 30 minutes) a small amount of the PC was hydrolysed and the LPC has risen correspondingly. The small relative increase in PC after incubation with enzyme over a short period of time can be put down to experimental errors and due to the inherent variability. However the visible lag period before any hydrolysis occurred has been previously reported [279,280]and is believed to be due to the build up of a critical proportion of products, fatty acid and lysophospholipid, in the membranes. The accumulation of the anionic fatty acid is believed to play a major role in the process and has a dramatic effect as these products reach a certain molar concentration (see figure 5.1).

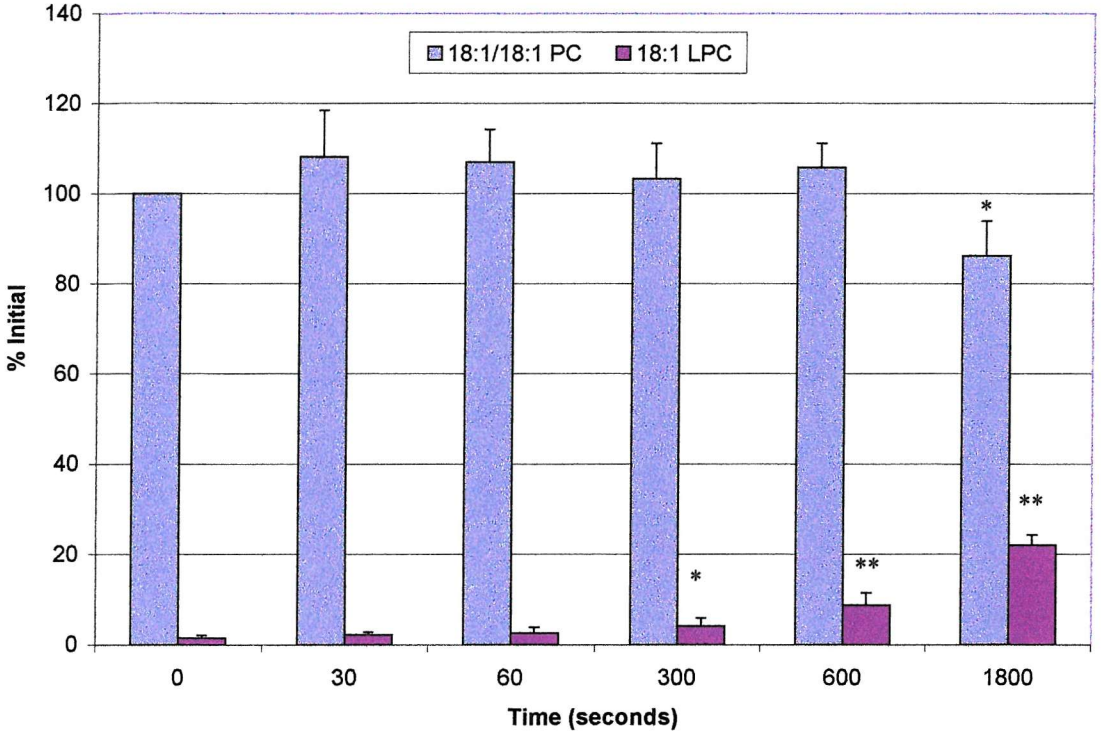


*Figure 5.3 The PC species present after pure 18:1/18:1PC vesicles were incubated with human group IIa $sPLA_2$ over an increasing time period. 53nmoles of phospholipid vesicles were incubated with 100ng of human group IIa $sPLA_2$ at 37℃ for the set time period prior to extraction and analysis by ESI-MS. The bars indicate the mean of three experiments that were performed in duplicate and the error bars are standard deviations. Statistical significance was determined by the independent t-test comparing all values to time zero where no enzyme was added, * denotes $p<0.05$ and ** $p<0.005$.*

The presence of 5% 18:1/18:1PG in the PC vesicles makes them more susceptible to hydrolysis by human group IIA $sPLA_2$ (figure 5.4). A greater fraction of the PC was hydrolysed when 5% PG was present when compared to solely PC vesicles. Although approximately the same amount of PG and PC were hydrolysed, because only 5% of the phospholipid was PG this indicates the preferential hydrolysis of the anionic phospholipid. The errors in these experiments were much larger than with higher proportions of PG and this may be due to the low level (5%) of anionic phospholipids being at a critical point for the enzyme (figure 5.1), which leads to such large variability in the results. This problem will be most apparent at early time points where the error is large compared with the amount of hydrolysis that has occurred.

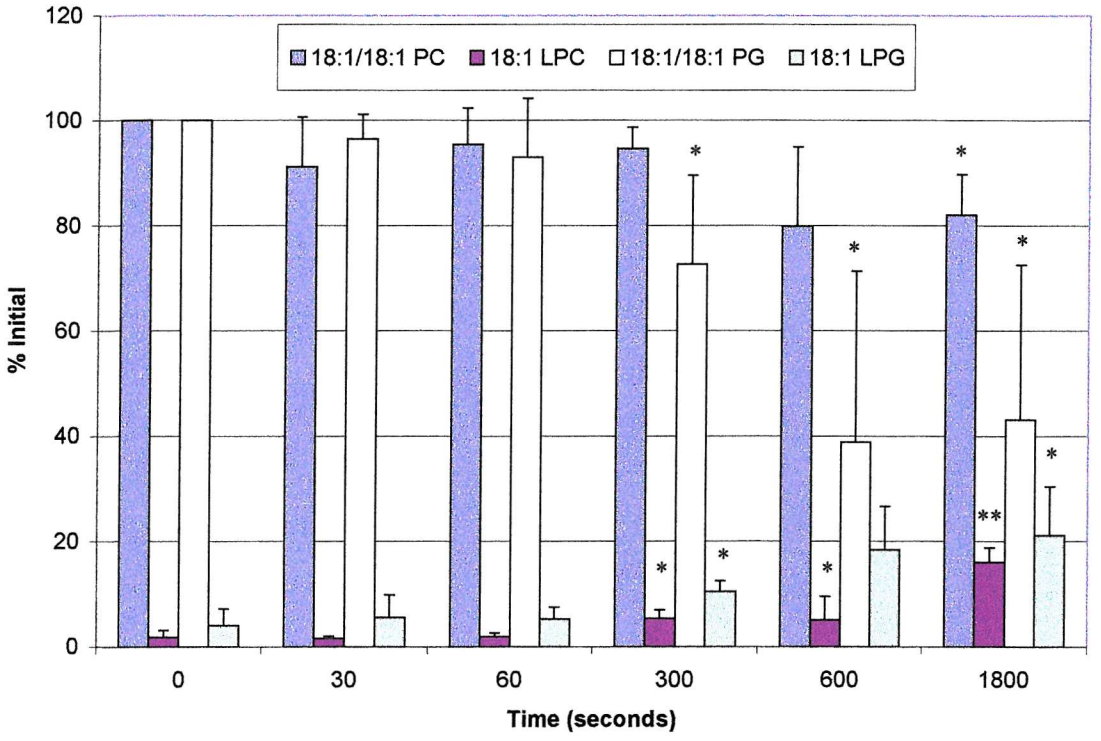


*Figure 5.4 The relative proportions of the PC and PG species of 18:1/18:1PC vesicles containing 5% 18:1/18:1PG after incubation with human group IIa $sPLA_2$. Conditions were the same as for figure 5.3. The bars indicate the mean of three experiments that were performed in duplicate and the error bars are standard deviations. Statistical significance was determined by the independent t-test comparing all values to time zero where no enzyme was added, * denotes $p<0.05$ and ** $p<0.005$*

As the proportion of PG in a PC vesicle was increased from 5% to 10% there was a decreased variability in the proportion of phospholipids present after hydrolysis by the enzyme (figure 5.5). The results showed more clearly that there was increased hydrolysis of both PC and PG over time and a corresponding increase in the percentage of lysophospholipids produced. PG again was hydrolysed to a greater extent than the PC, demonstrating a preferential hydrolysis of the PG as opposed to PC. For instance, if the enzyme had no substrate preference then the proportion of PC and PG hydrolysed would be the same as there should be equal distribution of these phospholipids throughout the vesicles. This trend continues with the use of vesicles containing 20% PG as shown in figure 5.6.

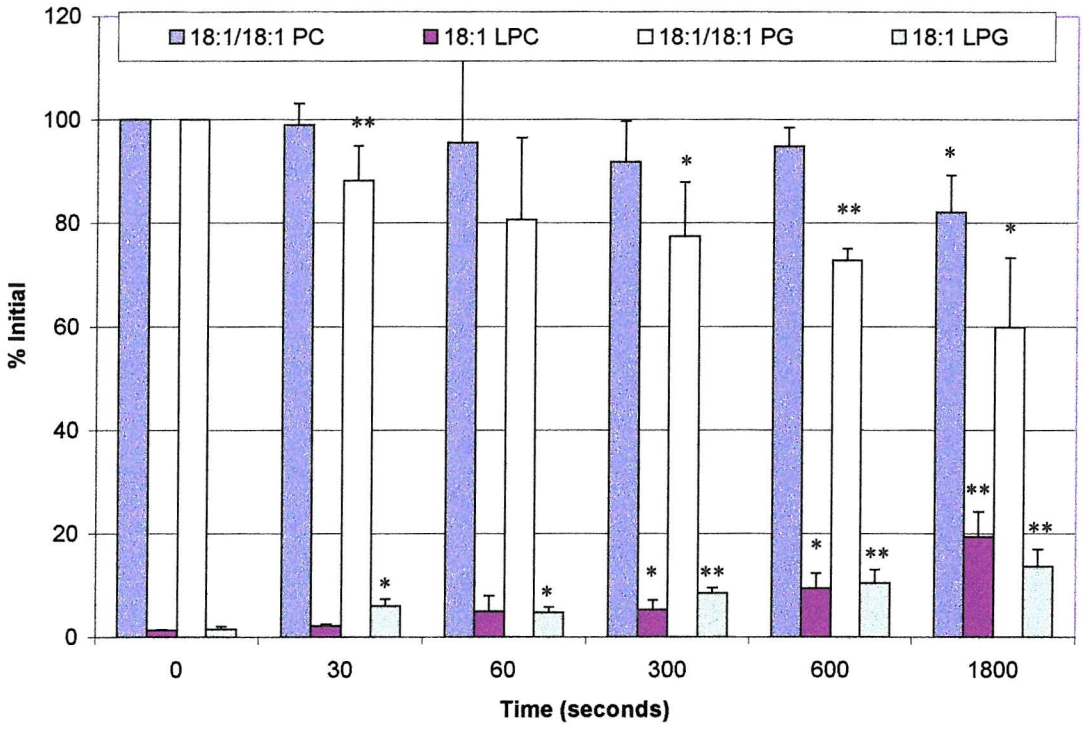


*Figure 5.5 The relative proportions of the PC and PG species of 18:1/18:1PC vesicles containing 10% 18:1/18:1PG after incubation with human group IIa $sPLA_2$. Conditions were the same as for figure 5.3. The bars indicate the mean of three experiments that were performed in duplicate and the error bars are standard deviations. Statistical significance was determined by the independent t-test comparing all values to time zero where no enzyme was added, * denotes $p<0.05$ and ** $p<0.005$*

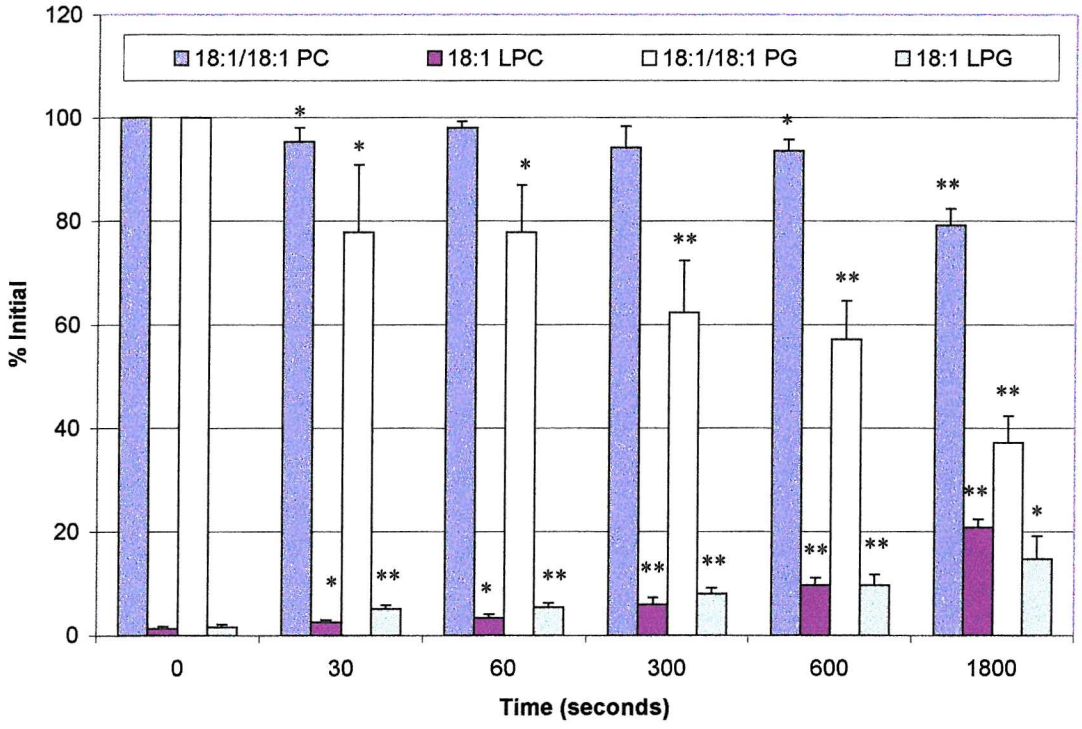


*Figure 5.6 The relative proportions of the PC and PG species of 18:1/18:1PC vesicles containing 20% 18:1/18:1PG after incubation with human group IIa $sPLA_2$. Conditions were the same as for figure 5.3. The bars indicate the mean of three experiments that were performed in duplicate and the error bars are standard deviations. Statistical significance was determined by the independent t-test comparing all values to time zero where no enzyme was added, * denotes $p<0.05$ and ** $p<0.005$*

The results so far have indicated that human group IIa $sPLA_2$ preferentially hydrolyses PG instead of PC. However previous assays have involved vesicles that contain a higher molar proportion of PC that complicates the quantification of this specificity. By studying an equimolar mix of PC and PG it is possible to establish the active-site preference for human group IIa $sPLA_2$. Therefore an equimolar mix of PC and PG was incubated with human group IIa $sPLA_2$ over increasing time and is shown in figure 5.7. The PG was hydrolysed to a greater extent than the PC; forming conclusive evidence that the human group IIa $sPLA_2$ not only requires the presence of anionic phospholipids to interfacially bind to the substrate but also preferentially hydrolyses PG at the active site. This active site preference for PG is examined in more detail in figure 5.10 and Table 5.1.

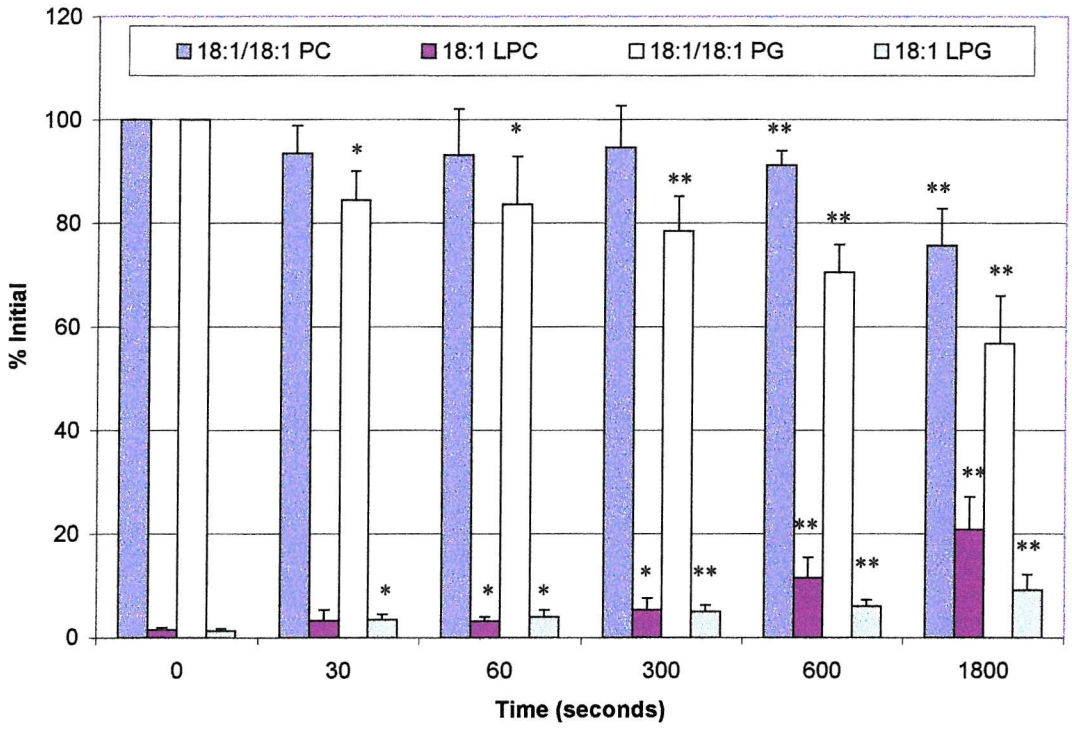


*Figure 5.7 The relative proportions of the PC and PG species of an equimolar 18:1/18:1PC and 18:1/18:1PG vesicles after incubation with human group IIa $sPLA_2$. Conditions were the same as for figure 5.3. The bars indicate the mean of three experiments that were performed in duplicate and the error bars are standard deviations. Statistical significance was determined by the independent t-test comparing all values to time zero where no enzyme was added, * denotes $p<0.05$ and ** $p<0.005$.*

The extent of hydrolysis of a solely PG vesicle by human group IIa $sPLA_2$ is shown in figure 5.8 in which after 1800 seconds just over 40% of the PG was hydrolysed. There was a corresponding increase in the LPG but this had a large discrepancy that can be attributed to the lack of an available internal standard for LPG. Therefore, it was not possible to quantitatively analyse the LPG as it could only be related to 14:0/14:0PG. Lysophospholipids are much more soluble in the aqueous phase than the corresponding phospholipid and hence extraction into solvent is difficult and variable.

The rate of hydrolysis of PG is not linear with time, the initial rate over the first 60 seconds is very fast and then the rate slows down and starts to plateau out at 1800 seconds (figure 5.9). The rate profile is not unexpected and reflects the diminishing availability of substrate. Moreover the 40% hydrolysis seen at the last time point may reflect almost complete hydrolysis of the outer monolayer of the vesicle.

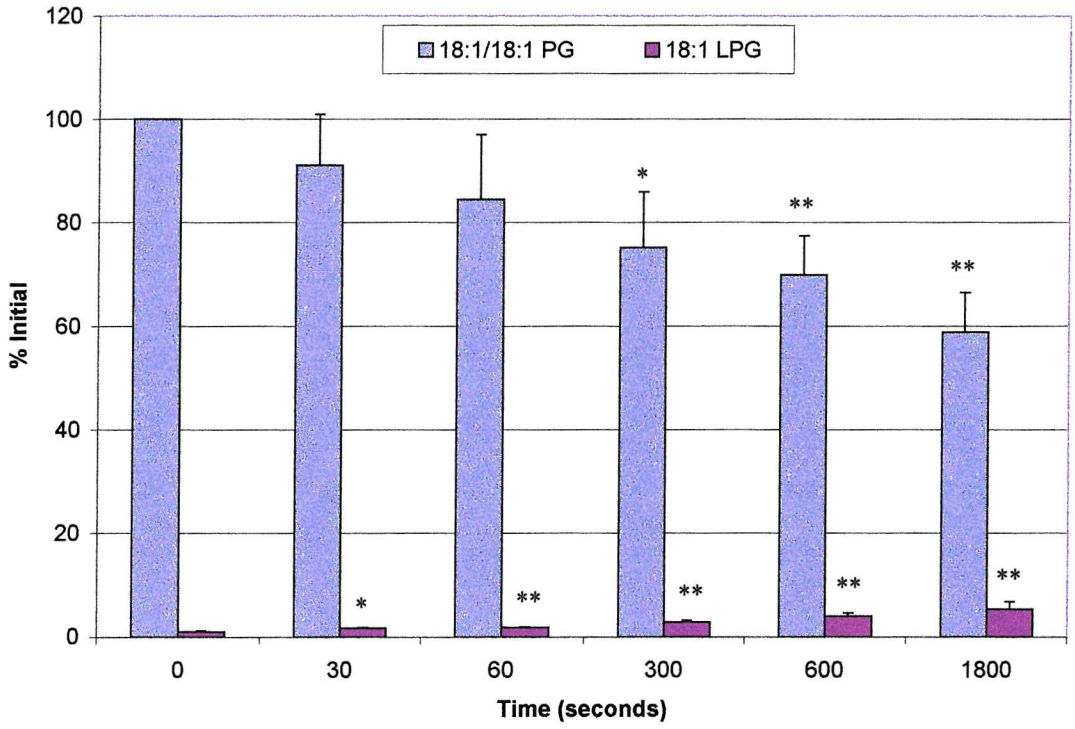


*Figure 5.8 The PG species present after pure 18:1/18:1PG vesicles were incubated with human group IIa $sPLA_2$ over an increasing time period. Conditions were the same as for figure 5.3. The bars indicate the mean of three experiments that were performed in duplicate and the error bars are standard deviations. Statistical significance was determined by the independent t-test comparing all values to time zero where no enzyme was added, * denotes $p<0.05$ and ** $p<0.005$.*

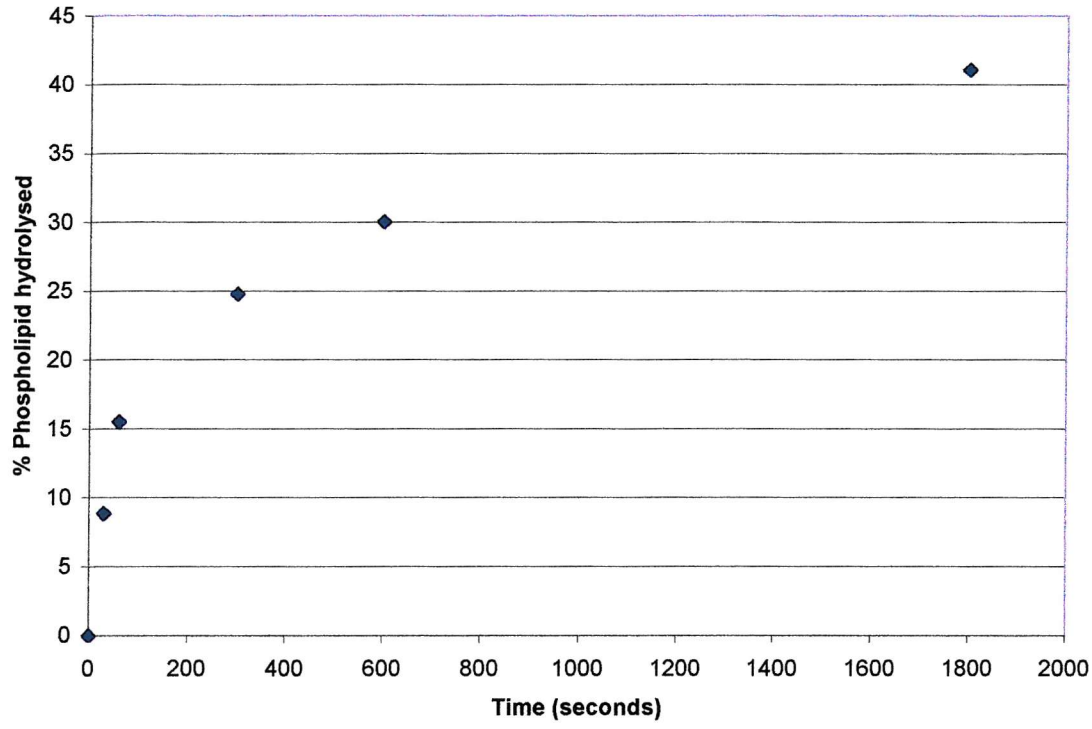


Figure 5.9 A real time plot of % phospholipid hydrolysed after incubation of human group IIa $sPLA_2$ with a pure 18:1/18:1PG vesicle. The %phospholipid hydrolysis was measured using ESI-MS under the same conditions as figure 5.3. The values are the mean of three experiments performed in duplicate.

5.3.3.1 The outcome of increasing human group IIa $sPLA_2$ concentration on the subsequent hydrolysis of equimolar 18:1/18:1PC and 18:1/18:1PG vesicles

The effect of increasing the concentration of human group IIa $sPLA_2$ on phospholipid hydrolysis measured over 60 seconds are shown in figure 5.10, where it is seen that the extent of hydrolysis increases as the enzyme concentration rises. The largest percentage of hydrolysis occurs with an enzyme concentration of 6250ng/ml when 75 ± 6.2 % of the PG was hydrolysed. While in comparison only 22 ± 7.3 % of the PC was hydrolysed. The lysophospholipids increased as the phospholipids were hydrolysed but due to the lack of an internal standard for LPG the proportion of LPG generated and PG hydrolysed do not equate unlike LPC and PC. It is possible to conclude that human group IIa $sPLA_2$ preferentially hydrolyses PG as opposed to PC.

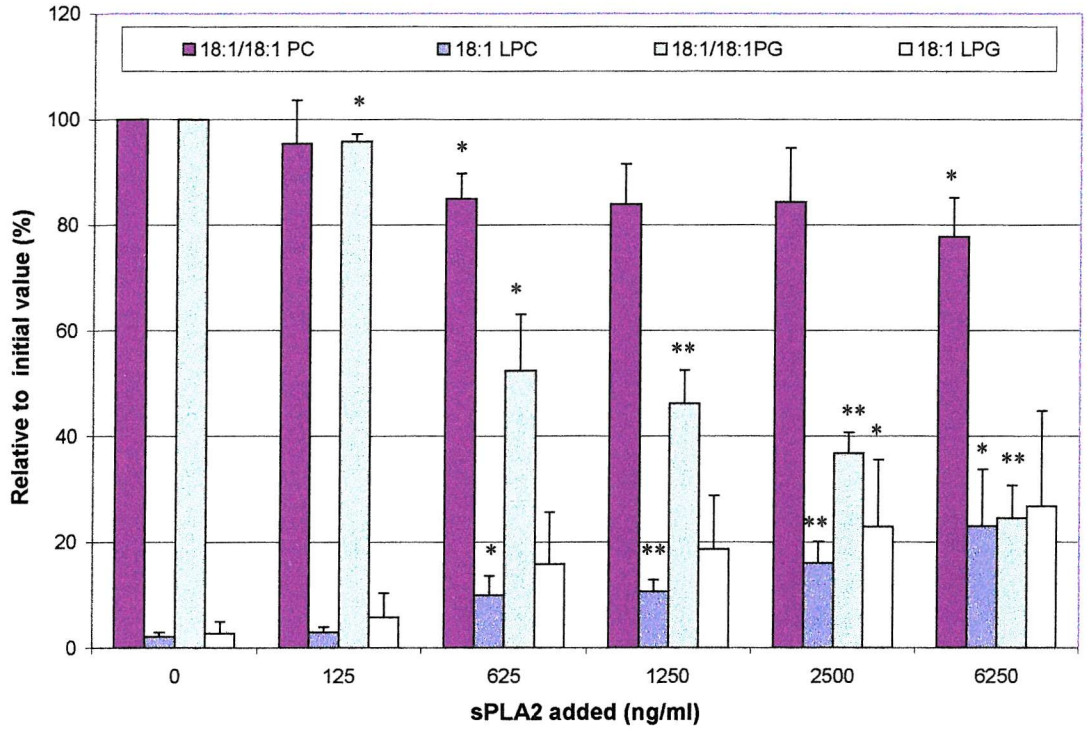


*Figure 5.10 Equimolar 18:1/18:1PC and 18:1/18:1PG vesicles incubated with increasing amounts of human group IIa $sPLA_2$ for 60 seconds. 53nmoles of equilmolar PC and PG vesicles were incubated with increasing amounts of enzyme for 60 seconds. The phospholipids were analysed by ESI-MS and the % of phospholipid present was calculated relative to the initial value when no enzyme was present. The bars indicate the mean of three experiments that were performed in duplicate and the error bars are standard deviations. Statistical significance was determined by the independent t-test comparing all values to when no enzyme was added, * denotes $p<0.05$ and ** $p<0.005$.*

Although substrate specificity can reflect interfacial binding and/or active site preference, under conditions where the vesicle is composed of 50% anionic phospholipid (PG) it may be assumed that the enzyme is completely bound to the vesicle. Hence the difference seen between the rates of hydrolysis of PG and PC is a measure of active site preference. Comparison of PG and PC hydrolysis at all concentration of enzyme, where significant hydrolysis was observed indicates an approximate 3-fold (3.5 ± 0.4) preference for PG in terms of hydrolysis rates (see table 5.1)

Table 5.1 Comparison of the rates of hydrolysis of PG and PC from equimolar 18:1/18:1PC and 18:1/18:1PG vesicles at differing concentrations of $sPLA_2$

$sPLA_2$ concentration (ng/ml)	**% PC hydrolysed per minute**	**% PG hydrolysed per minute**	**% PG hydrolysed / % PC hydrolysed**
625	15.0 ± 4.8	47.5 ± 10.6	3.17
1250	16.1 ± 7.6	53.7 ± 6.2	3.35
2500	15.6 ± 10.2	63.3 ± 4.0	4.04
6250	22.2 ± 7.3	75.4 ± 6.2	3.39

The mean values of the percentage of phospholipid that has been hydrolysed after 30 seconds from triplicate experiments are shown ± standard deviations. The ratio of % PG hydrolysed compared to % PC hydrolysed is also shown and illustrates an average of 3.5 ± 0.4 preference for PG as opposed to PC in equimolar vesicles.

The demonstration of an apparent 3-fold preference of the human group IIa $sPLA_2$ for PG over PC is the first time that a true active site preference for PG has been reported using otherwise similar substrates. In the only equivalent previous study on the group IIa $sPLA_2$ enzyme, Bayburt et al reported a 2.7 ± 1.2 fold preference of 14:0/14:0PG over 18:0/20:4PC [120]. In this case trace amounts of the two phospholipids were incorporated into vesicles composed of 18:1/16:0PC and 18:1/18:1PA to which the enzyme will bind with high affinity. The relative rates of hydrolysis of the 14:0/14:0PG and 18:0/20:4PC were determined by measuring the released 14:0 and 20:4 fatty acids by gas chromatography.

Of importance is the publication of Snitko et al where a very high preference of the human group IIa $sPLA_2$ for the PG and PA headgroups of phospholipids was reported compared with PC [274]. This study involved the use of substrates in which the *sn*-2 fatty acyl chain was labelled with pyrene and suggest a 100-fold preference for the PG substrate over PC. The major discrepancy between our results and those of Snitko would suggest that the presence of the non-physiological pyrene flurofore might be generating anomalous results. All this substrate specificity data obtained *in vitro* is as a result of doing experiments under "scooting conditions" whereby the enzyme is already bound to anionic vesicles. Under physiological conditions a lack of significant interfacial binding

as a result of the substrate aggregate having a low anionic charge density will tend to discriminate further against neutral phospholipids such as PC.

5.3.4 Use of the ESI-MS to assess the hydrolysis of surfactant lipids PLA_2s

The previous section described the use of ESI-MS to measure phospholipid hydrolysis in artificial vesicles by $sPLA_2$. In this section the technology was used to assess the hydrolysis when lung surfactant was provided as the substrate for these enzymes. The effect that human group IIa $sPLA_2$ and snake venom *Naja naja* PLA_2 has on the phospholipid composition of purified rabbit surfactant after a three-hour incubation with increasing concentrations of these enzymes was studied using ESI-MS. The results are discussed both in terms of activity and specificity of each enzyme.

5.3.4.1 The effect of increasing human group IIa $sPLA_2$ on rabbit surfactant PC

PC in rabbit surfactant proved to be a substrate for group IIa $sPLA_2$hydrolysis but only when the enzyme was present in high concentrations and over a 3-hour time period. The amount of rabbit surfactant PC hydrolysed increased with increasing amounts of enzyme present for both the native and the sonicated purified surfactant (figure 5.11 and 5.12). Both the native and the sonicated surfactant show similar amounts of PC hydrolysis at the highest enzyme concentration (10μg/ml) with approximately 15% of total PC being hydrolysed.

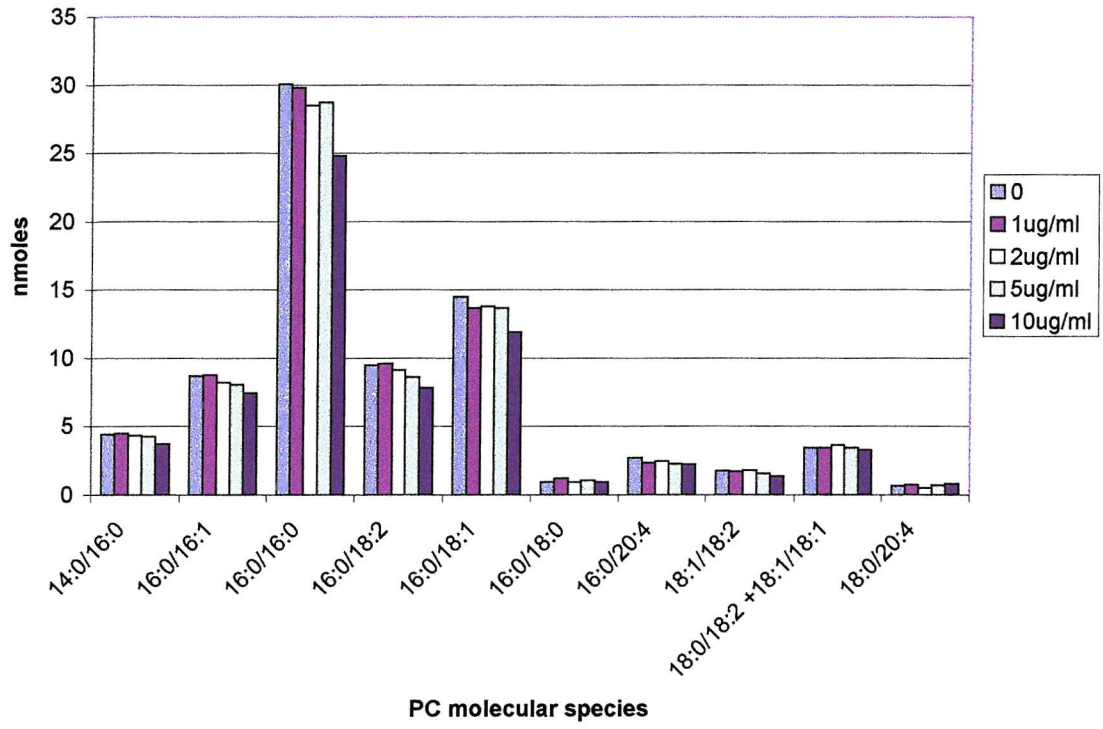


Figure 5.11 Effect of increasing human group IIa $sPLA_2$ on the amount of PC molecular species in native rabbit surfactant. Native rabbit surfactant was incubated with increasing concentrations of human group IIa $sPLA_2$ for 3 hours at 37℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PC species were calculated relative to the internal standard 14:0/14:0PC. The bars shown are the mean of duplicate values.

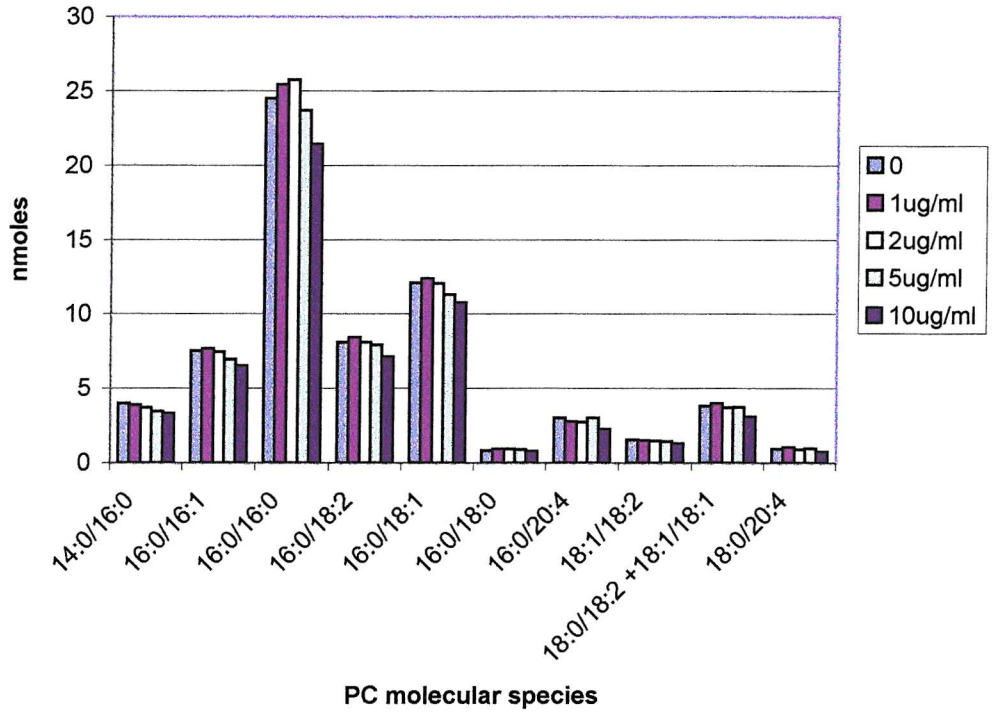


Figure 5.12 Effect of increasing human group IIa $sPLA_2$ concentration on the amount of PC molecular species present in sonicated rabbit surfactant. Rabbit surfactant was sonicated prior to being incubated with increasing concentrations of human group IIa $sPLA_2$ for 3 hours at 37 ℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PC species were calculated relative to the internal standard 14:0/14:0PC. The bars shown are the mean of duplicate values

There was no change in the PC composition for either native or sonicated surfactant (data not shown) and this suggests that group IIa $sPLA_2$ shows very little specificity for the fatty acids attached at the *sn-1* and *sn-2* position of the PC species

5.3.4.2 The effect of increasing human group IIa $sPLA_2$ concentration on rabbit surfactant PG

In contrast to the PC in rabbit surfactant the amount of PG hydrolysed was considerably greater in the surfactant that had been sonicated prior to incubation with the enzyme compared with the native surfactant (figure 5.13 and 5.14). An explanation for this dramatic difference is that the sonication causes the formation of vesicles and made the PG more accessible to the enzyme. This is consistent with a report that has shown that

when PG was co-sonicated with equal molar quantities of PC inverted vesicles are formed which contain on the outer surface twice as many PG than PC molecules [281].

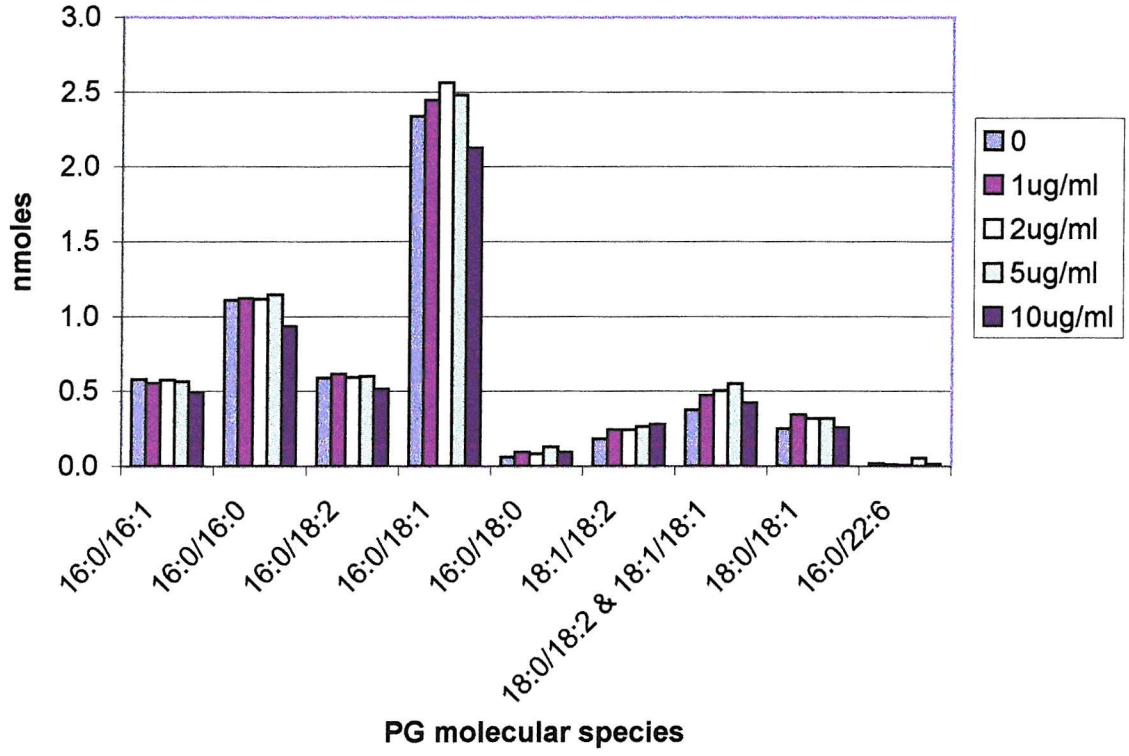


Figure 5.13 Effect of increasing human group IIa sPLA2 on the PG molecular species present in native purified rabbit surfactant. Native rabbit surfactant was incubated with increasing concentrations of human group IIa $sPLA_2$ for 3 hours at 37 ℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PG species were calculated relative to the internal standard 14:0/14:0PG. The bars shown are the mean of duplicate values.

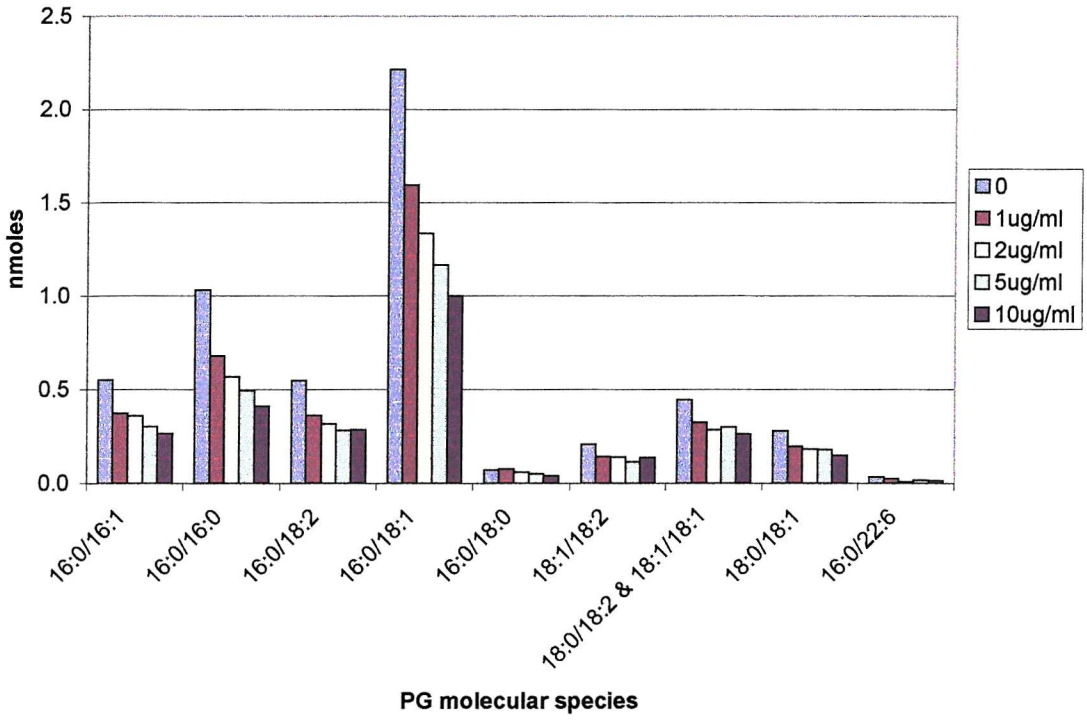


Figure 5.14 Effect of increasing human group IIa $sPLA_2$ concentration on the PG molecular species present in sonicated rabbit surfactant. Rabbit surfactant was sonicated prior to being incubated with increasing concentrations of human group IIa $sPLA_2$ for 3 hours at 37℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PG species were calculated relative to the internal standard 14:0/14:0PG. The bars shown are the mean of duplicate values.

When studying the PG composition of both native and sonicated surfactant (figure 5.15 and 5.16) after incubation with the human group IIa $sPLA_2$ it appears that the enzyme preferentially hydrolyses 16:0/18:1PG and 16:0/16:0PG. This is a surprising result as it is accepted that this family of enzyme does not show obvious acyl chain specificity when hydrolysing native phospholipids. The substrate specificity is more apparent in the sonicated surfactant because more of the total PG was hydrolysed. Because the enzyme exhibits substrate specificity in both the native and sonicated surfactant this suggests this is a genuine preference and not due to poor distribution of PG in the native surfactant. However this preference is only seen with the two most abundant species 16:0/18:1PG and 16:0/16:0PG.

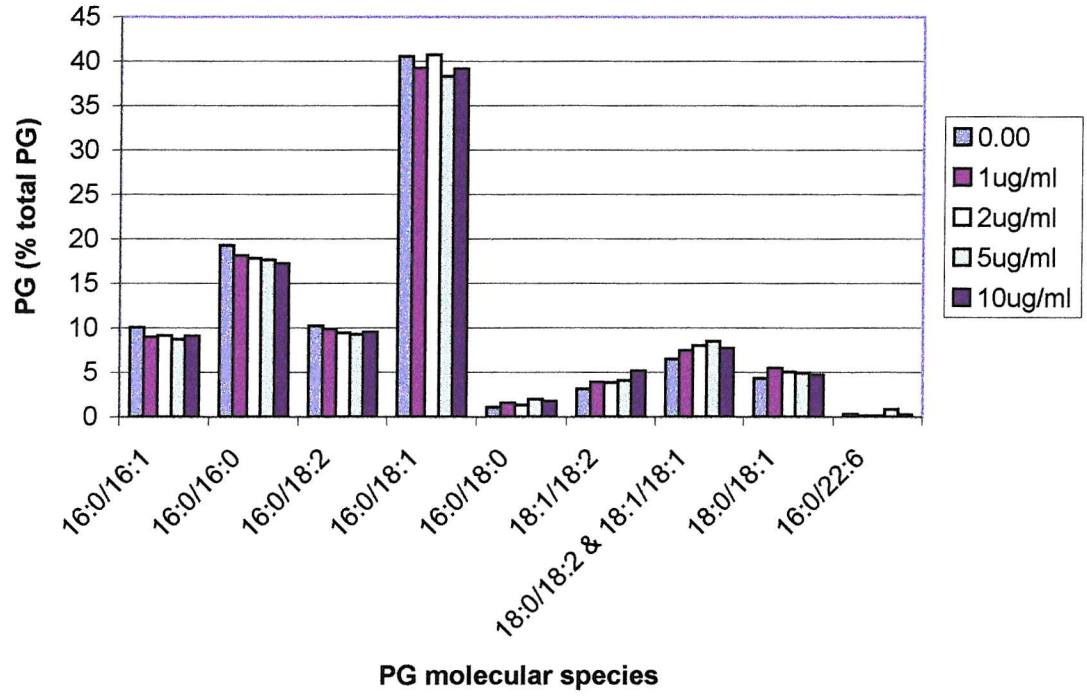


Figure 5.15 The % composition of rabbit surfactant PG after incubation with increasing concentrations of human group IIa $sPLA_2$. The conditions were the same as figure 5.13.

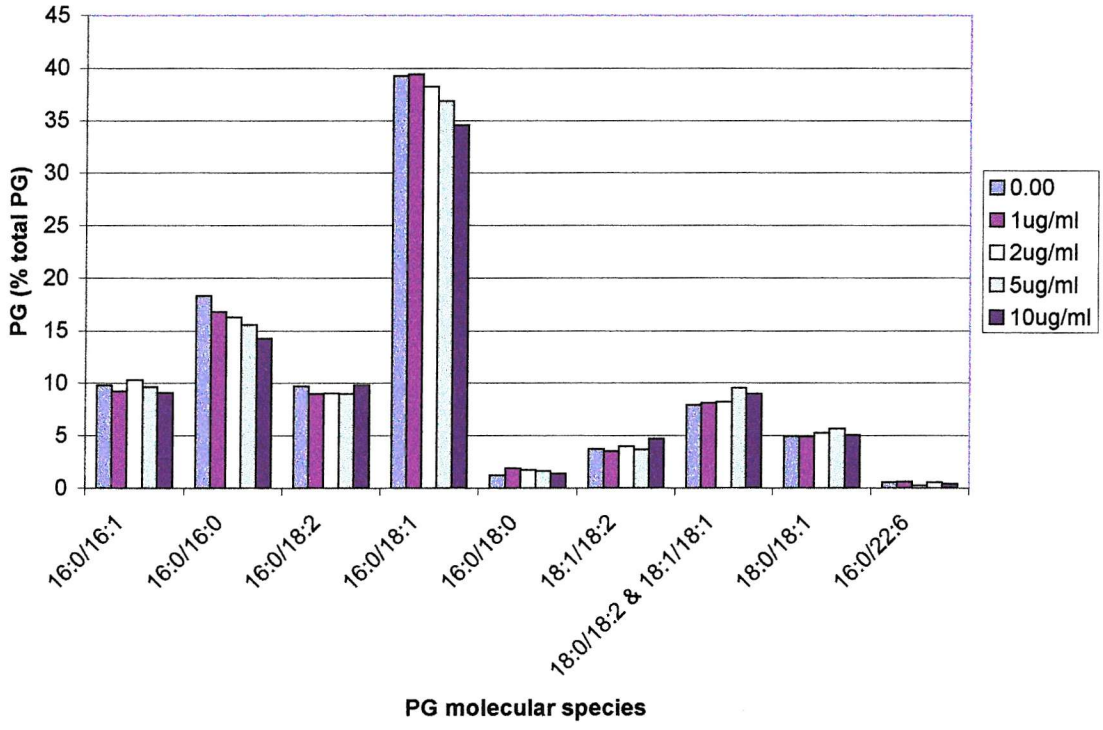


Figure 5.16 The % composition of sonicated rabbit surfactant PG after incubation with increasing concentrations of human group IIa $sPLA_2$ for 3 hours. The conditions were the same as figure 5.14.

5.3.4.3 The effect of increasing human group IIa $sPLA_2$ concentration on rabbit surfactant PI

There is very little hydrolysis of surfactant PI by human group IIa $sPLA_2$ in either the native or the sonicated surfactant (figures 5.17 & 5.18). The results are somewhat inconsistent and can be attributed to the lack of an internal standard for PI. A lack of internal standard means it is not possible to allow for any variation in the extraction of PI relative to PG or account for any differential response on the mass spectrometer. The PI percentage composition results are not shown, as there were no changes in the PI composition, this is not surprising as very little hydrolysis occurred. Although the PI content of rabbit surfactant is low (<6%) and the results are limited by the lack of internal standard, a lack of preference for PI by the human $sPLA_2$ is still indicated. The data of Bayburt et al when comparing the hydrolysis 18:0/20:4PI with 16:0/16:0PC indicated a low preference for PI phospholipids with preferential hydrolysis of PC phospholipids [120]. This is an opposite effect to that seen when comparing PG and PC.

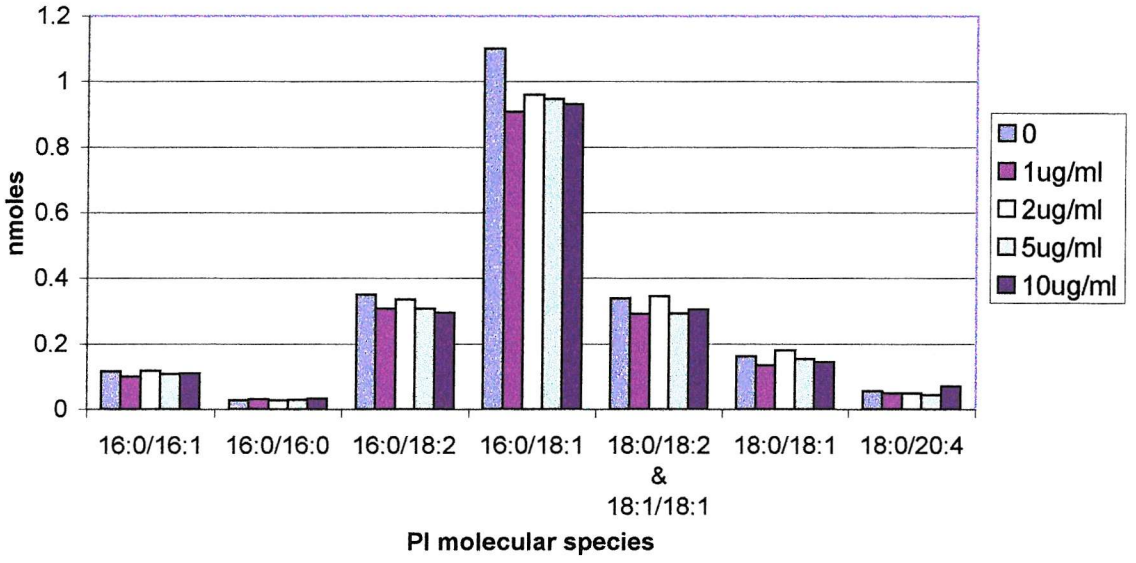


Figure 5.17 Effect of increasing human group IIA $sPLA_2$ concentration on the PI molecular species present in purified rabbit surfactant. Native rabbit surfactant was incubated with increasing concentrations of human group IIa $sPLA_2$ for 3 hours at 37 ℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PI species were calculated relative to the internal standard 14:0/14:0PG. The bars shown are the mean of duplicate values.

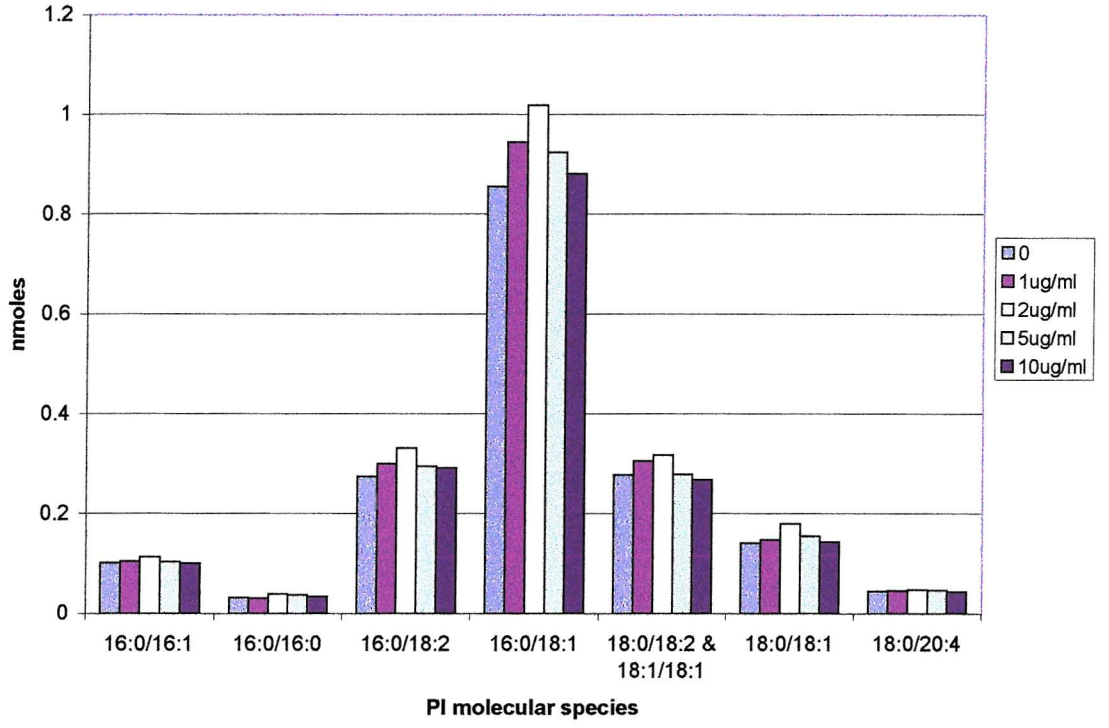


Figure 5.18 Effect of increasing human group IIa $sPLA_2$ concentration on the PI molecular species present in sonicated rabbit surfactant. Rabbit surfactant was sonicated prior to being incubated with increasing concentrations of human group IIa $sPLA_2$ for 3 hours at 37 ℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PI species were calculated relative to the internal standard 14:0/14:0PG. The bars shown are the mean of duplicate values.

5.3.4.4 Total PC and total PG hydrolysed by human group IIa $sPLA_2$

The ratio of total PC: total PG for both native and sonicated rabbit surfactant are shown in figure 5.19. The PC: PG ratio for native surfactant decreases with increasing amounts of enzyme present, this suggests that slightly more PC is hydrolysed than PG. However the PC: PG ratio of the sonicated surfactant increases dramatically with increasing enzyme concentration, indicating a large preferential hydrolysis of PG rather than PC. The most likely explanation for the differences seen in figure 5.19 is that in the native surfactant there is minimum exposure of PG and hence an apparent preference for PC hydrolysis. However, after sonication and the rearrangement of the phospholipid distribution the enzyme is able to demonstrate its preference for PG as seen by an increase in the PC: PG ratio. The effect sonication has on the phospholipid distribution

of the surfactant complex is unknown, however sonication is thought to produce small uniform vesicles. Interestingly there has been one study that reported sonicated equimolar PC and PG vesicles contained twice as much PG than PC in the outer monolayer [281]. It is difficult to ascertain the affect of sonication on the surfactant complex as it only contains 10%PG and 85%PC along with surfactant specific proteins. The increase in PC: PG ratio that occurs after sonicated but not native surfactant is incubation with the human group IIa enzyme is unlikely to be solely due to the rearrangement of phospholipids but also due to a change in the structure of the substrate.

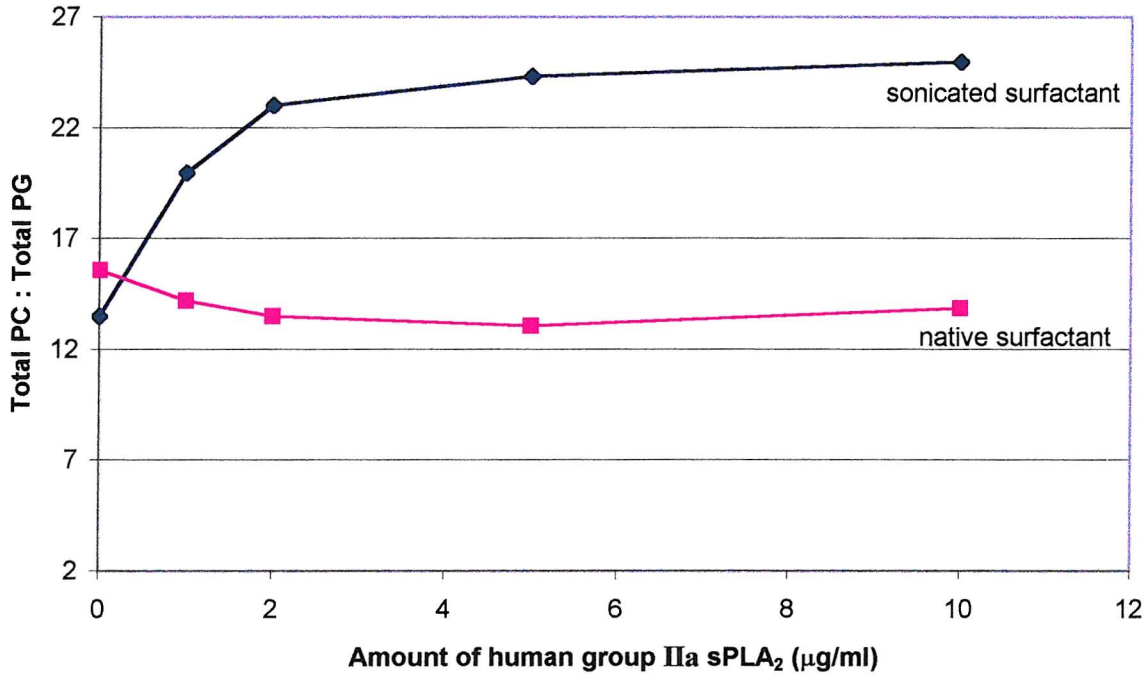


Figure 5.19 Total PC: total PG for native and sonicated purified rabbit surfactant after incubation with increasing amounts of human group IIa $sPLA_2$. Total PC and PG were calculated by summing all the individual molecular species measured. The values shown are the mean of duplicate values. The increase in total PC: total PG ratio seen after the addition of the enzyme to sonicated surfactant indicates that $sPLA_2$ preferentially hydrolyses PG over PC.

5.3.5 Hydrolysis of rabbit surfactant by *Naja naja* PLA_2 measured by ESI-MS

5.3.5.1 The effect on rabbit surfactant PC of increasing *Naja naja* PLA_2 concentration

After 3 hours incubation with 1μg/ml of *Naja naja* PLA_2 a large amount of the native and sonicated surfactant PC was hydrolysed (figure 5.20 and 5.21), this increased only slightly when the enzyme concentration was increased 10 fold suggesting that the enzyme is working at maximum rate at 1μg/ml. Approximately 80% of the total PC was hydrolysed in both the native and sonicated surfactant and would imply almost complete destruction of the phospholipid aggregate. Unlike human group IIa $sPLA_2$, *Naja naja* PLA_2 appears to be able to hydrolyse the surfactant phospholipids irrespective of their physical structure.

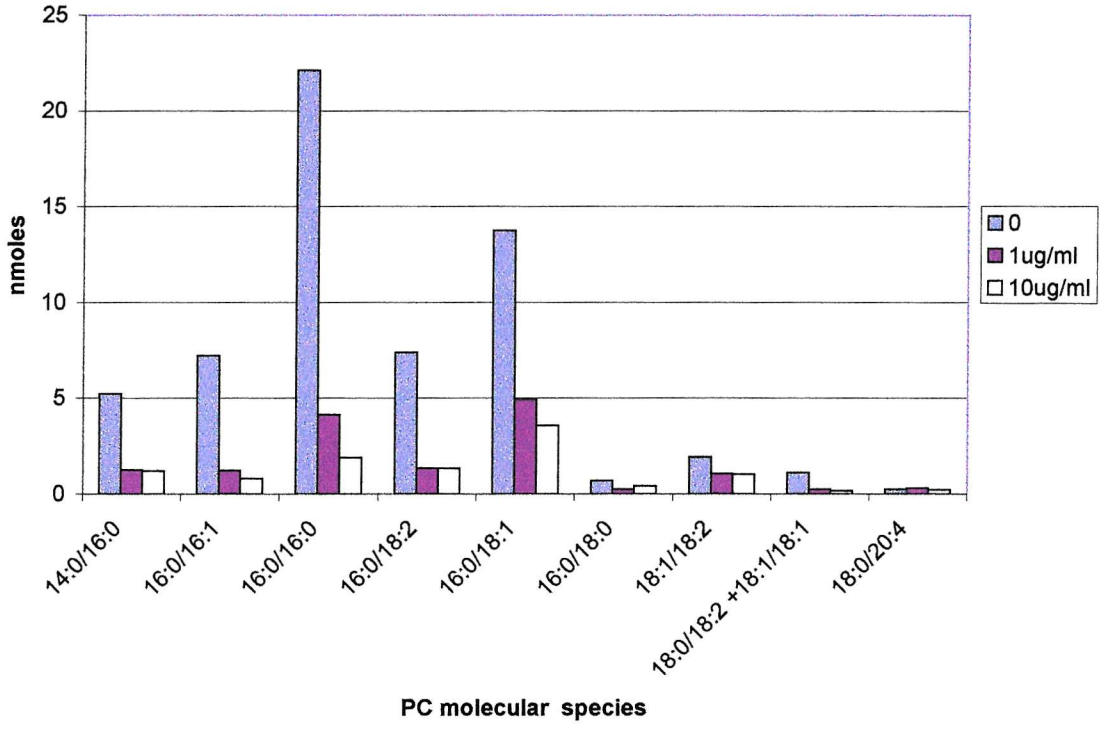


Figure 5.20 The PC species present in native purified rabbit surfactant after a three-hour incubation with 0, 1, and 10μg/ml Naja naja PLA_2 at 37℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PC species were calculated relative to the internal standard 14:0/14:0PC. The bars shown are the mean of duplicate values.

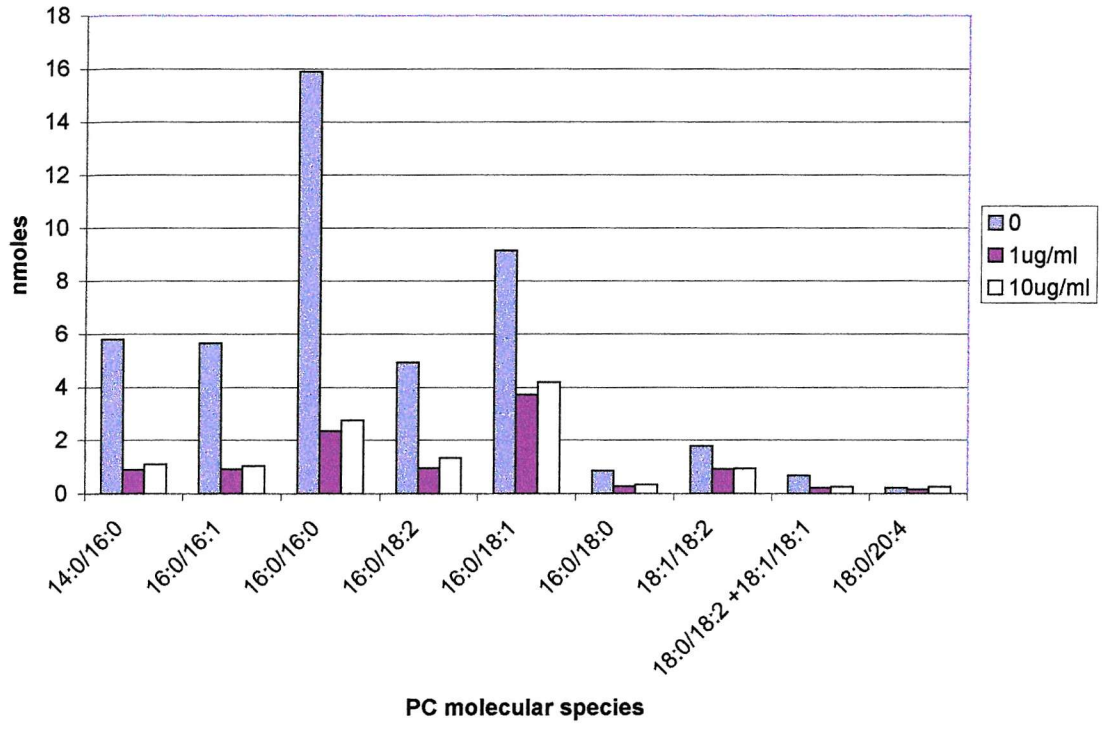


Figure 5.21 The PC species present in sonicated rabbit surfactant after a three-hour incubation with 0, 1, and 10μg/ml Naja naja PLA_2 at 37℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PC species were calculated relative to the internal standard 14:0/14:0PC. The bars shown are the mean of duplicate values.

Naja naja PLA_2 demonstrates a preference for PC species with 16:0 and 16:1 fatty acyl chains; this became apparent when looking at the PC compositions of the native and sonicated rabbit surfactant before and after the three-hour incubation with *Naja naja* PLA_2 (figures 5.22 and 5.23). The proportion of 16:0/16:0PC and 16:0/16:1PC to total PC decreased with increasing enzyme concentration while the relative amount of 16:0/18:1PC, 16:0/18:0PC and 18:1/18:2PC increased. The reason for the preferential hydrolysis of PC species with 16:0 and 16:1 acyl chains is unknown, but it is interesting as *Naja naja* PLA_2 preferentially hydrolyses 16:0/16:0PC the surface active component of lung surfactant. The apparent substrate specificity of the *Naja naja* PLA_2 has not been previously reported due to most of the studies in the literature on substrate specificity have been performed using mammalian $sPLA_2$s.

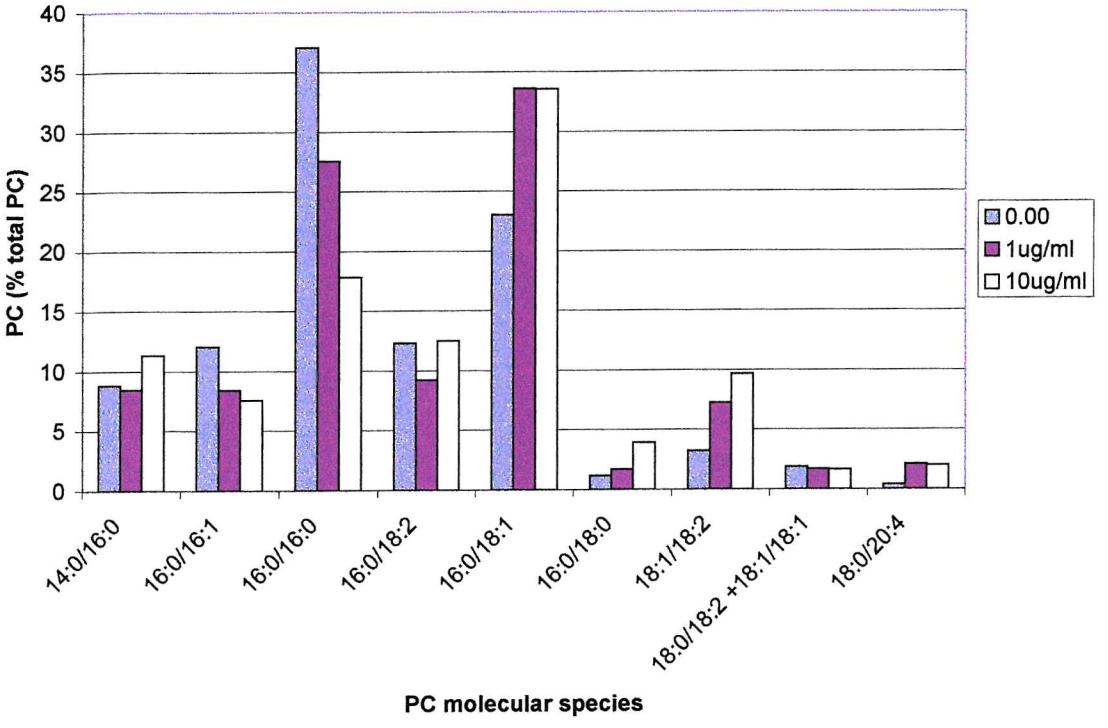


Figure 5.22 The PC composition of native purified rabbit surfactant after incubation with 0, 1 and 10μg/ml Naja naja PLA_2 for three hours at 37℃. The conditions were the same as figure 5.20.

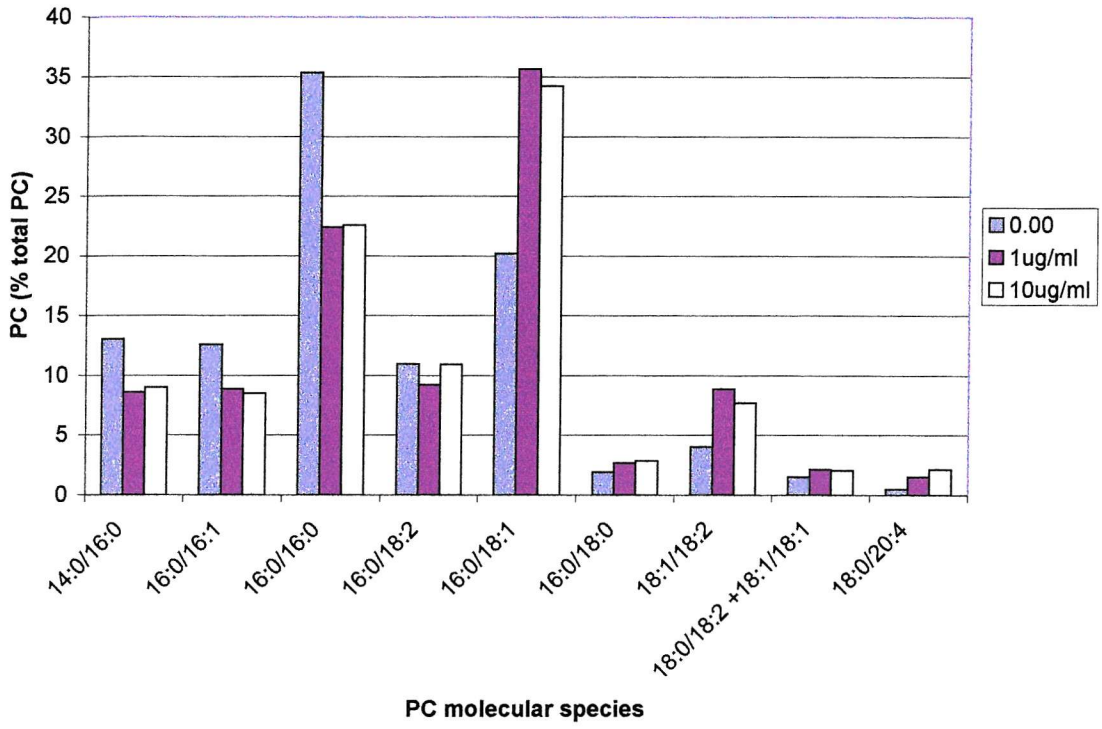


Figure 5.23 The PC composition of sonicated purified rabbit surfactant after a three-hour incubation with 0, 1 and 10μg/ml Naja naja PLA_2 at 37℃. The conditions were the same as figure 5.21.

5.3.5.2 The effect on rabbit surfactant PG of increasing *Naja naja* PLA_2 concentration

The increase in enzyme concentration did not increase the amount of PG hydrolysis that occurred, this is similar to the outcome for PC. Both the native and sonicated surfactant underwent similar amounts of total PG hydrolysis (approximately 60% of total PG was hydrolysed) (figures 5.24 and 5.25), which is considerably less hydrolysis than the 80% of PC hydrolysed. These results suggest that the *Naja naja* enzyme has a preference for PC over PG and this is an active site preference.

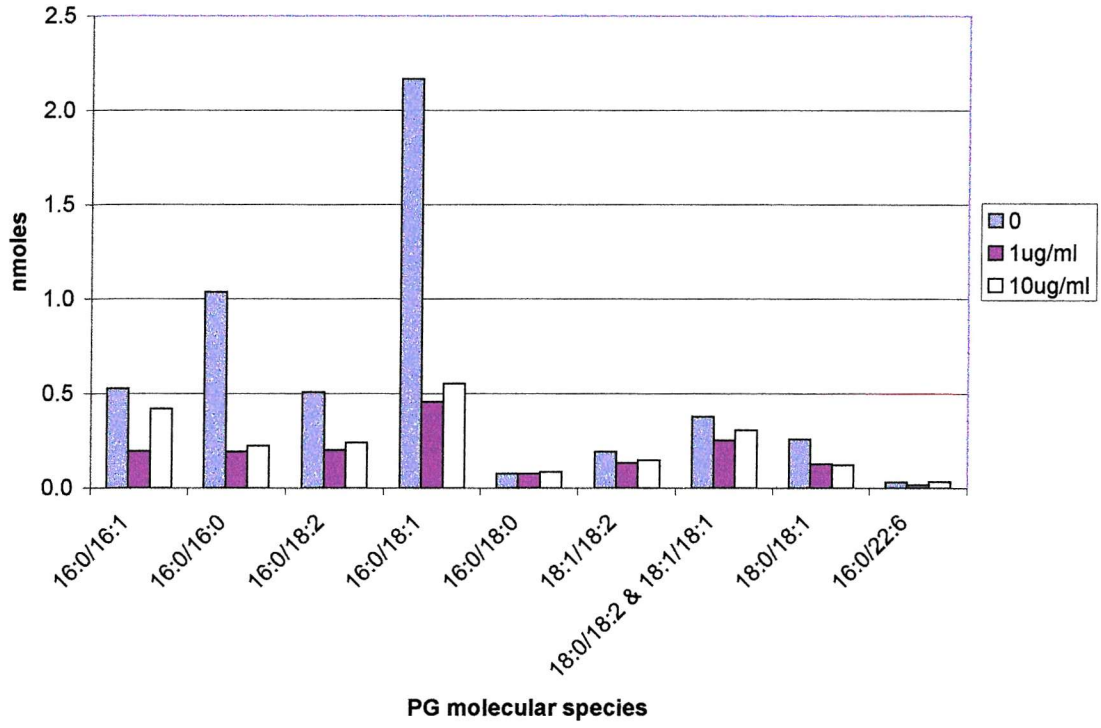


Figure 5.24 The PG species present in native purified rabbit surfactant after a three-hour incubation with 0, 1, and 10μg/ml Naja naja PLA_2 at 37℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PG species were calculated relative to the internal standard 14:0/14:0PG. The bars shown are the mean of duplicate values.

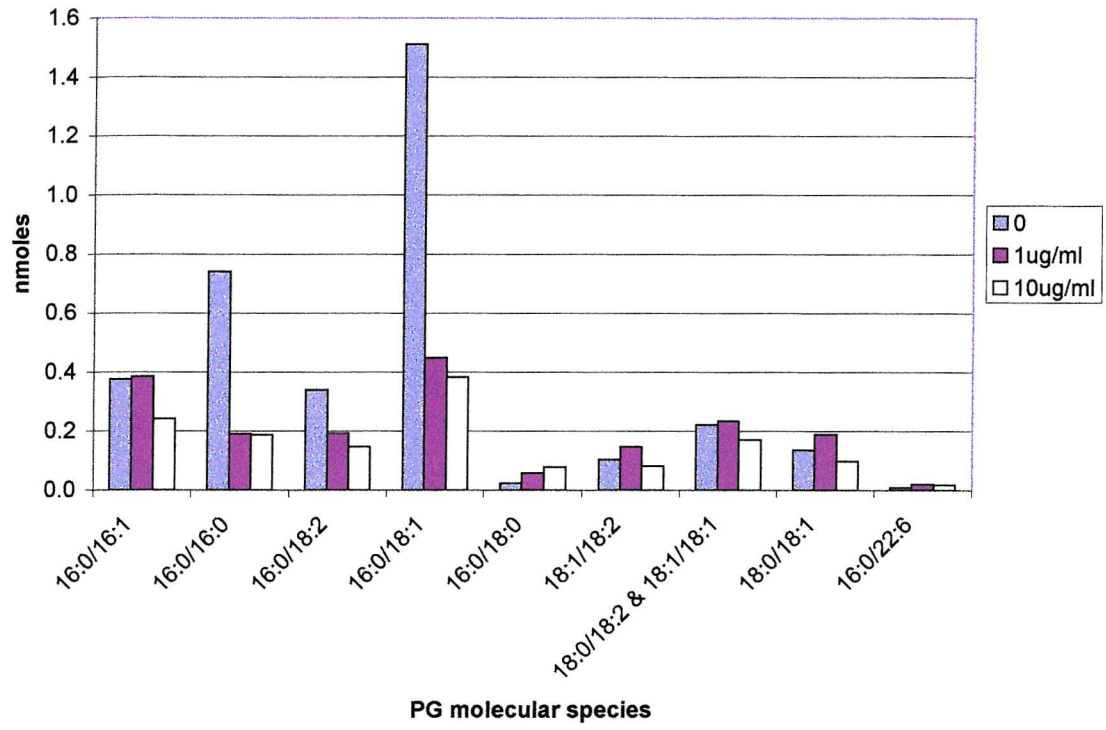


Figure 5.25 The PG species present in sonicated purified rabbit surfactant after a three-hour incubation with 0, 1, and 10μg/ml Naja naja PLA_2 at 37℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PG species were calculated relative to the internal standard 14:0/14:0PG. The bars shown are the mean of duplicate values.

Naja naja PLA_2 displayed the same preferential hydrolysis of the PG molecular species in both the native and sonicated surfactant (figures 5.26 and 5.27) specifically hydrolysing 16:0/18:1PG and 16:0/16:0PG. This preferential hydrolysis of the two major PG species with shorter fatty acyl chains was also evident after hydrolysis with the human group IIa $sPLA_2$ (section 5.12).

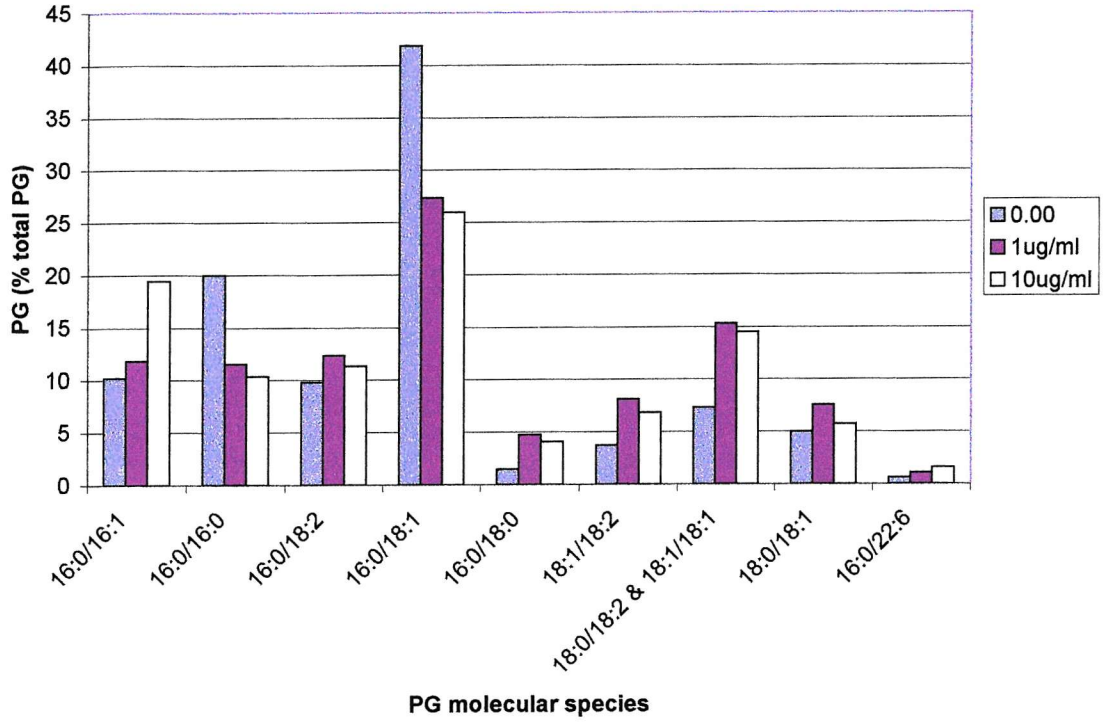


Figure 5.26 The PG composition of native purified rabbit surfactant after incubation with 0, 1 and 10μg/ml Naja naja PLA_2 for three hours at 37℃. The conditions were the same as figure 5.24 and the bars shown are the mean of duplicate values.

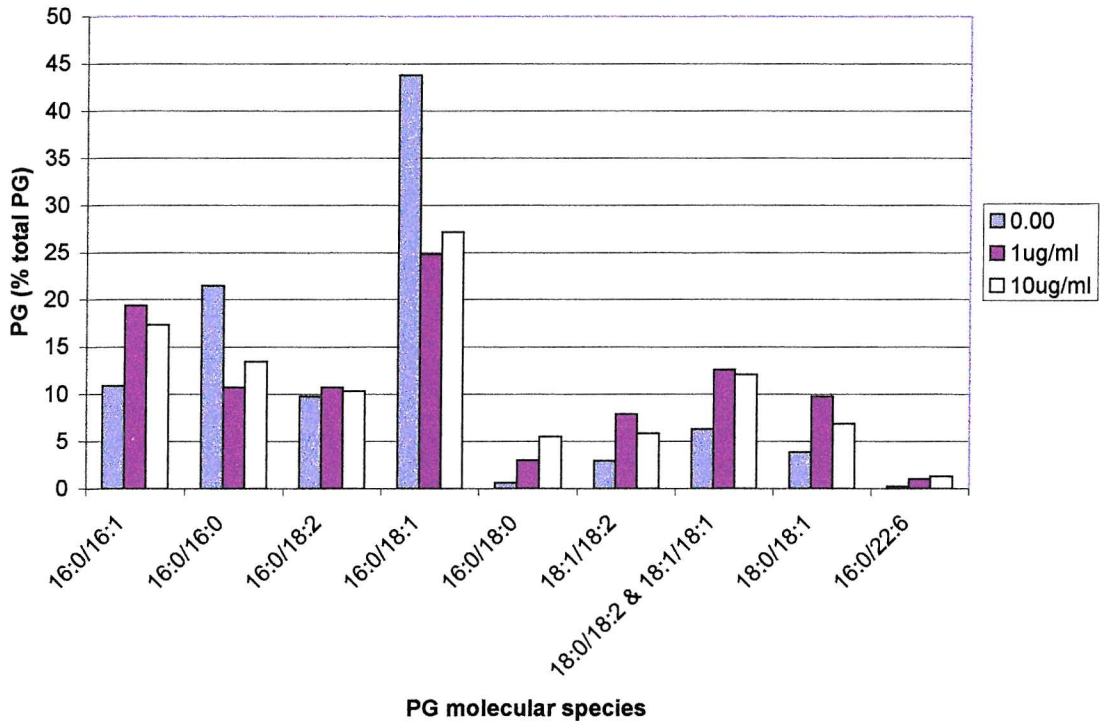


Figure 5.27 The PG composition of sonicated purified rabbit surfactant after incubation with 0, 1 and 10μg/ml Naja naja PLA_2 for three hours at 37℃. The conditions were the same as figure 5.25 and the bars shown are the mean of duplicate values.

5.3.5.3 The effect on rabbit surfactant PI of increasing *Naja naja* PLA_2 concentration

Naja naja PLA_2 readily hydrolysed rabbit surfactant PI in both the purified and sonicated surfactant (figures 5.28 and 5.29). The amount of hydrolysis hardly increased with the 10-fold increase in enzyme concentration for both the neat and sonicated surfactant. Fractionally more of the total PI was hydrolysed in the native surfactant as opposed to the sonicated (85% vs. 75% respectively), overall more of the total PI was hydrolysed than total PG (60%) suggesting a head group preference for PI with the *Naja naja* enzyme.

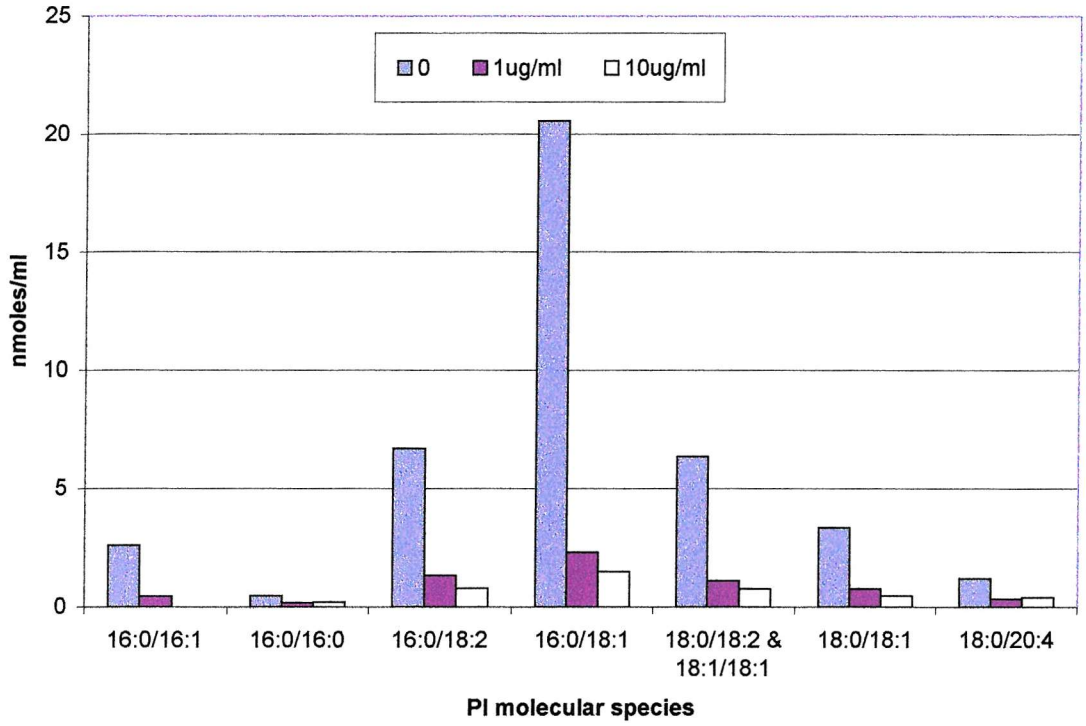


Figure 5.28 The PI species present in purified rabbit surfactant after a three-hour incubation with 0, 1, and 10μg/ml Naja naja PLA_2 at 37℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PI species were calculated relative to the internal standard 14:0/14:0PG. The bars shown are the mean of duplicate values.

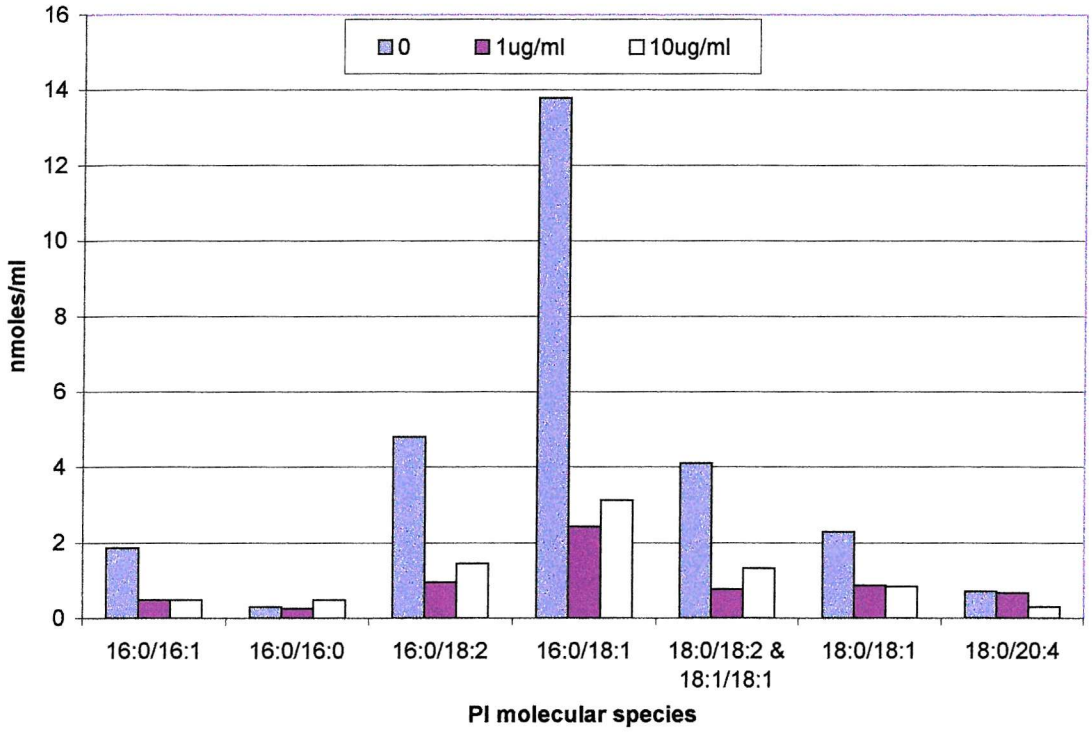


Figure 5.29 The PI species present in sonicated purified rabbit surfactant after a three-hour incubation with 0, 1, and 10μg/ml Naja naja PLA_2 at 37℃. After the incubation the phospholipids were extracted and the phospholipid classes were separated prior to analysis of the individual phospholipid molecular species by ESI-MS. The absolute amounts of the PI species were calculated relative to the internal standard 14:0/14:0PG. The bars shown are the mean of duplicate values.

The rabbit surfactant PI compositions after incubation with *Naja naja* PLA_2 were very similar for both native and sonicated purified surfactant (figures 5.30 and 5.31). The most evident difference in the PI composition after incubation with *Naja naja* PLA_2 was the decrease in 16:0/18:1PI accompanied by the relative increase in the other PI species. The apparent selective hydrolysis of 16:0/18:1PI by *Naja naja* PLA_2 also occurred in the PG species as 16:0/18:1PG was also selectively hydrolysed. No other studies have reported on the hydrolysis of phospholipids by the *Naja naja* enzyme and the reason for this selective hydrolysis of 16:0/18:1 containing anionic phospholipids is unknown.

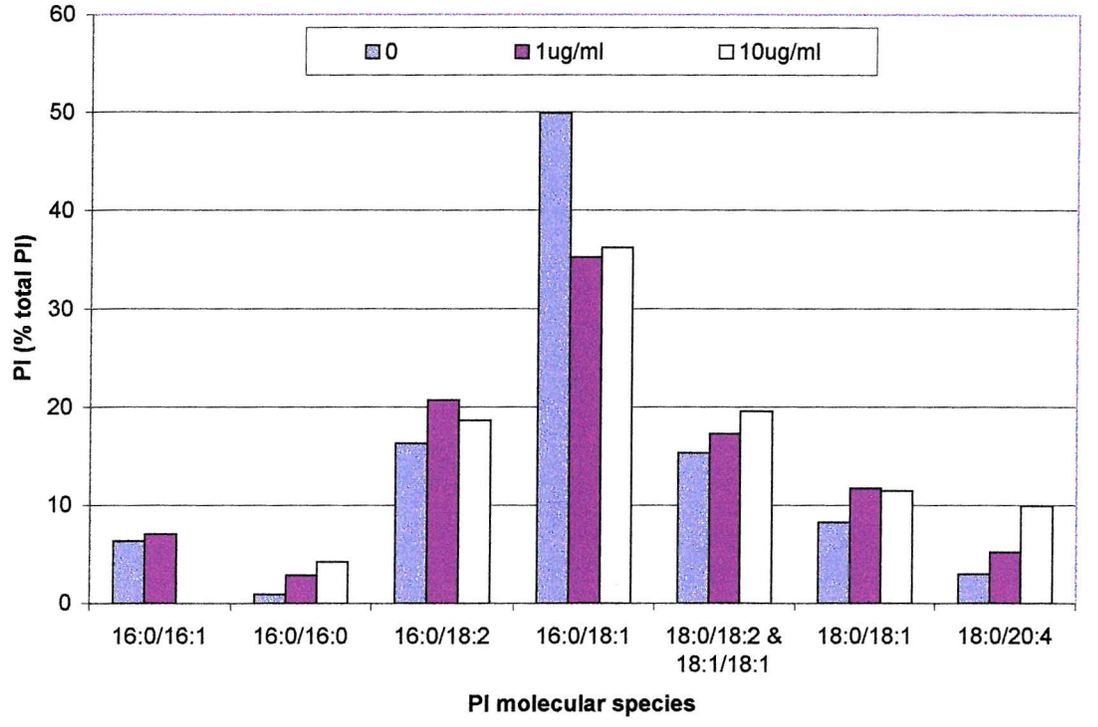


Figure 5.30 The PI composition of purified rabbit surfactant after incubation with 0, 1 and 10μg/ml Naja naja PLA_2 for three hours at 37℃. The conditions were the same as figure 5.28 and the bars shown are the mean of duplicate values.

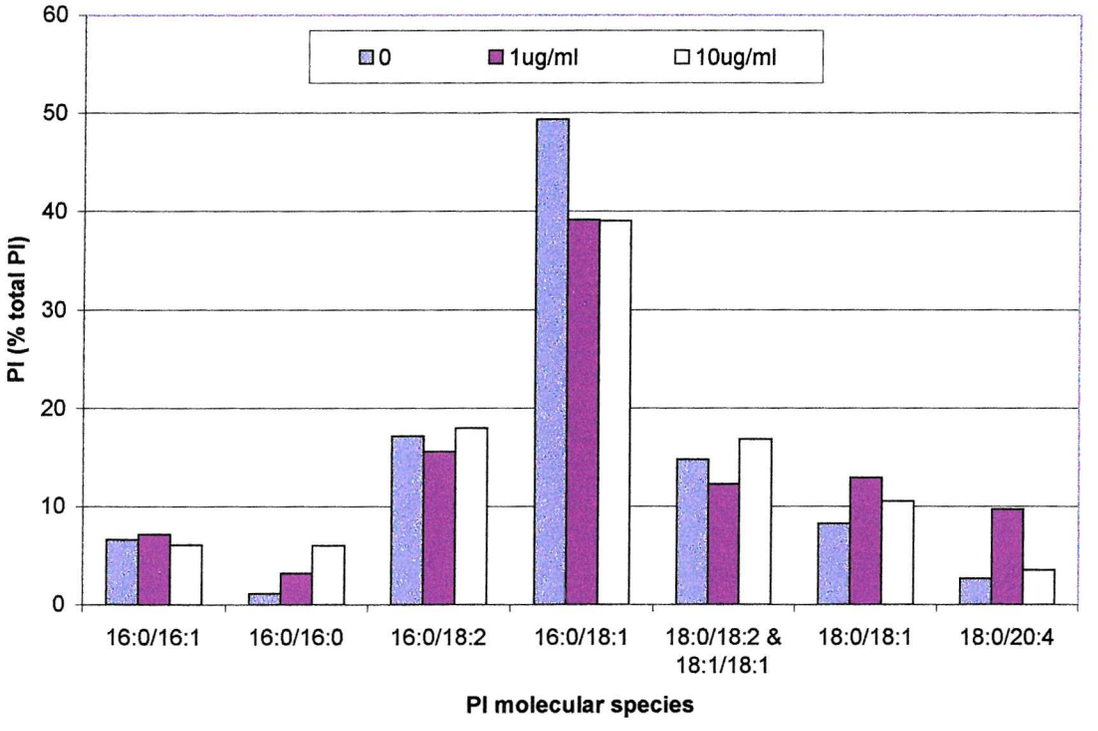


Figure 5 .31 The PI composition of sonicated purified rabbit surfactant after incubation with 0, 1 and 10μg/ml Naja naja PLA_2 for three hours at 37℃. The conditions were the same as figure 5.29 and the bars shown are the mean of duplicate values

5.3.5.4 Total PC and total PG hydrolysed by *Naja naja* PLA_2

After incubation with increasing amounts of *Naja naja* PLA_2, the ratio of total PC: total PG decreased for both the native and sonicated surfactant (figure 5.32). The most likely explanation for the decrease in the PC: PG ratio is due to preferential hydrolysis of PC at the active site of the enzyme. Asymmetric distribution of the phospholipids is unlikely to account for the preference for PC demonstrated by the enzyme, as it was apparent both before and after sonication. Sonication is thought to randomise the phospholipid distribution or as one report suggests cause more PG to be contained in the outer monolayer [281]. Consequently it is unlikely that unequal distribution of the phospholipids accounts for the selective hydrolysis of PC over PG by the *Naja naja* PLA_2 enzyme.

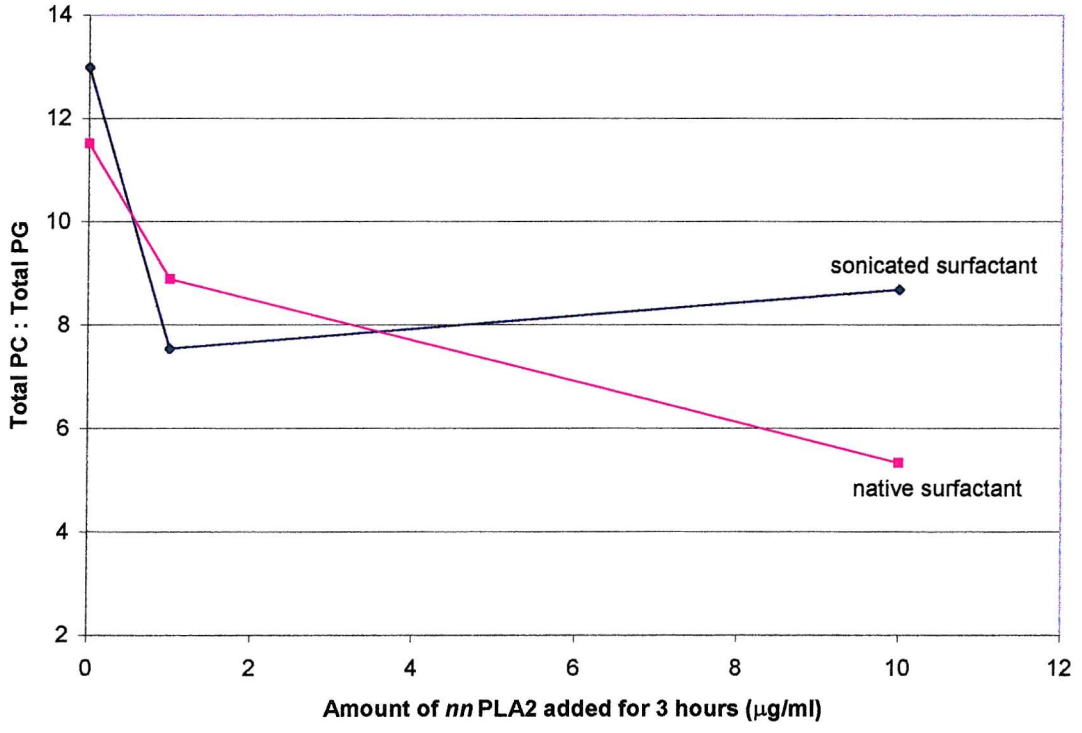


Figure 5.32 The ratio of total PC: total PG in both native and sonicated purified rabbit surfactant after incubation with Naja naja PLA_2. Total PC and PG were calculated by summing all the individual molecular species measured. The values shown are the mean of duplicate values. The decrease in total PC: total PG ratio seen after the addition of the enzyme to both sonicated and native purified surfactant indicates that Naja naja PLA_2 preferentially hydrolyses PC over PG.

The enzyme does have a net anionic charge due to its pI of about 5, however it still has cationic residues around the interface and therefore still has a high affinity for anionic

phospholipids, although not as great as human group IIa $sPLA_2$. Overall the studies with the *Naja naja* venom enzyme provide an important control for work with the human group IIa enzyme. The venom enzyme is able to hydrolyse the PC rich surfactant much more readily than the human enzyme (compare figure 5.11 & 5.20) and, as stated before, demonstrates a head group preference of PC over PG in sonicated surfactant (compare figure 5.19 & 5.32) whereas the reverse is true for the human group IIa enzyme. The fact that the human enzyme fails to demonstrate substrate preference with native surfactant indicates that the PG may reside either primarily in the inner monolayer of the surfactant or in complexes with the surfactant proteins and is only exposed with sonication.

5.4 Discussion

It is well known that human group IIa $sPLA_2$ requires the presence of anionic phospholipids to express its full activity and in particular a preference for PG has been demonstrated. The fact that in ARDS patients there is a dramatic loss of surfactant PG suggested that human group IIa $sPLA_2$ plays an important role in surfactant integrity under certain conditions. Therefore it is important to establish the ability of this enzyme to hydrolyse lung surfactant phospholipids and how the presence of the anionic phospholipids may facilitate the hydrolysis. In order to address this problem the hydrolysis of both phospholipid vesicles containing varying proportions of PG and also lung surfactant has been monitored using both a real-time fluorescent displacement assay and phospholipid analysis by ESI-MS.

The results from work on vesicles using the fluorescent displacement assay demonstrated that human group IIa $sPLA_2$ is very sensitive to the mol % of PG in the phospholipid interface (figure 5.1). A rapid increase in the initial rate of hydrolysis is seen as the mol % PG exceeds 10% in a PC vesicle and this dramatic enhancement of the rate above a critical mol % is characteristic of multiple non-specific electrostatic interactions between the enzyme and the anionic interface (reviewed in Buckland & Wilton, 2000)[275]. Because the fluorescence displacement assay only measures total fatty release, it was necessary to confirm and extend these findings using ESI-MS. This technique

allows the measurement of individual phospholipid molecular species of PC and PG that are being hydrolysed together with lysophospholipid hydrolysis products.

The results obtained by ESI-MS confirmed that total phospholipid hydrolysis was enhanced as the proportion of PG increased. Moreover, if the enzyme was incubated for longer periods of time with substrate hydrolysis occurred even with 100% PC vesicles, a result that could not be demonstrated with the fluorescence displacement assay because of the relatively unstable nature of the fluorescences signal over this time (hours). A lag period was observed before significant hydrolysis was detected by ESI-MS which can be explained by an accumulation of hydrolysis products, particularly anionic fatty acids, that facilitate interfacial binding and activation and is discussed below.

The preferential hydrolysis of PG over PC was observed in all cases where significant phospholipid occurred. For instance, in a phospholipid vesicle containing an equimolar ratio of PG and PC, the PG was hydrolysed on average 3.5 times faster than PC allowing the conclusion that human group IIa $sPLA_2$ shows an active site preference for PG. This preference for PG has already been discussed in detail in Section 5.3.3.10.

The fact that the human group IIa $sPLA_2$ will hydrolyse pure PC vesicles given a large enough concentration of enzyme over a long enough period of time has important physiological implications and therefore the basis for this hydrolysis must be considered. The enzyme is inactive against the neutral PC interface in normal initial rate assays and, as discussed previously, this lack of activity reflects two properties of this enzyme. The highly cationic enzyme has a requirement for an anionic interface to promote interfacial binding while the presence of anionic lipid will perturb the bilayer and facilitate interfacial activation and hydrolysis. The binding of the enzyme to PC vesicles will be greatly enhanced by the generation of hydrolysis products and it is the accumulation of these products to a critical level that leads to the characteristic lag period before a burst of hydrolysis is observed. The precise molecular basis of this lag period is complex and has still to be completely defined but the accumulation of a critical mol % of fatty acid appears to play an important role in vitro as does the presence of lysophospholipid [282-286].

More recently, this so called lag-burst behaviour of the enzyme activity was studied using atomic force microscopy where a pure 16:0/16:0PC bilayer was observed during the addition of the snake venom PLA_2 from *Agkistrodon piscivorous piscivorous* [279]. After the enzyme was added a lag phase was observed with only very few visible changes in the bilayer. Prior to the burst of enzyme activity small depressions in the bilayer were observed, the authors interpret these as product rich domains. The increased bilayer heterogeneity caused by the low levels of hydrolysis during the lag phase is assumed to play an important role in the triggering of the burst. The reported lag time was estimated by atomic force microscopy to be 8-10 minutes and is comparable to the lag time observed for vesicular substrates [287]. The results shown in figure 5.3 also demonstrate a similar lag time between 10-30 minutes. Because of these lag burst characteristics due to product accumulation it will be difficult to extrapolate *in vitro* measurements to the *in vivo* situation. Moreover, it must be remembered *in vivo* that product accumulation may not occur due to the high affinity of fatty acid and lysophospholipid for albumin in the circulation, while the concentrations of enzyme used *in vitro* to demonstrate hydrolysis are much higher than would be expected *in vivo*.

According to the results from the fluorescent displacement assay human group IIa $sPLA_2$ was unable to hydrolyse native and sonicated purified rabbit surfactant over a short period of time despite increasing enzyme concentration. However measurable initial rates were obtained using the *Naja naja* PLA_2 enzyme on both native and sonicated surfactant and the porcine pancreatic PLA_2 on the sonicated substrate thus confirming the validity of the assay. The lipid extract of the rabbit surfactant proved to be susceptible to hydrolysis by all three enzymes but with hugely varying rates. The *Naja naja* PLA_2 exhibited the fastest rate of hydrolysis followed by the porcine pancreatic PLA_2 and then the human group IIa $sPLA_2$ finally demonstrating an ability to hydrolyse the substrate (46, 20, 1 nmol of fatty acid released/minute/μg of enzyme respectively). These results agree with previous reports that the *Naja naja* enzyme is a highly potent enzyme against PC membranes [270].

The *in vitro* study of human group IIa $sPLA_2$ on rabbit surfactant involving the ESI-MS analysis of the surfactant phospholipid composition gave considerable insight into the

potential role of this enzyme in disease states. The enzyme hydrolysed the PC, PG and to a small extent PI of the native and sonicated substrate, however this was with relatively high enzyme concentrations of 10μg/ml. Sonication of the substrate led to a much larger proportion of the PG being hydrolysed but with the percentage of total PC remaining the same. It is proposed that in native surfactant the PG may be confined to the inner monolayer of the phospholipid aggregate. The effect of sonication on the surfactant complex is unknown but if it behaves in a similar way to equimolar PC and PG vesicles then it may be likely that the outer monolayer would contain more PG than PC [281]. Alternatively sonication may disrupt surfactant protein- phospholipid interactions and increase the availability of PG for hydrolysis.

The *Naja naja* enzyme was studied as a positive control to ensure it confirmed the results from the fluorescent displacement assay as an extremely efficient PLA_2 enzyme. The enzyme readily hydrolysed over 80% of the total PC in rabbit surfactant at the low concentration of 1μg/ml. However unlike the human group IIa $sPLA_2$ enzyme proportionally more of the PC and PI were hydrolysed than the PG. The reason for the *Naja naja* enzyme exhibiting a substrate specificity of PC>PI>PG is most likely due to an active site preference because all substrates are in the same vesicle. Therefore the probable effect of interfacial binding will affect the availability of all phospholipids equally.

In summary, it has been demonstrated that human group IIa $sPLA_2$ can hydrolysis native purified rabbit surfactant *in vitro.* However this hydrolysis was only achieved at very high enzyme concentrations over prolonged time periods that may be only of physiological relevance to critically ill patients who already have poor prognosis. The earlier vesicle work illustrated that over long periods of time the enzyme could eventually start slowly hydrolysing a pure PC vesicle and therefore potentially giving $sPLA_2$ a secondary role in prolonging the duration of inflammatory diseases. Although it has been reported that the PC/PG ratio of lung surfactant phospholipids dramatically rises in ARDS patients, the detailed study of the group IIa enzyme described in this chapter does not suggest an obvious role for the enzyme in producing the change in ratio

seen even though the enzyme has a preference for PG. An alternative explanation for the increase in the PC/PG ratio in ARDS patients is described in chapter 7.

Chapter Six

Asthma

6.1 Introduction

Asthma is a chronic lung disease with acute episodes that result in narrowing of the bronchial airways. While there is no clear-cut definition of asthma, it can be considered as a combination of three features that occur in the airways: inflammation, hyperresponsiveness to exogenous and endogenous stimuli (e.g. antigens and leukotrienes) and obstruction with spontaneous and pharmacological reversibility [288]. The role of surfactant in the pathogenesis of airway inflammation and the impact on asthma has not been clearly defined.

It has been postulated that inactivation of surfactant may contribute to the acute asthmatic response. Animal studies using aerosolized ovalbumin to challenge sensitized guinea pigs as a model for the acute asthmatic response demonstrated a surfactant dysfunction under these conditions [99]. This was validated by the surface activity of bronchoalveolar lavage fluid (BALF) being significantly reduced in the challenged animals when compared to the controls, accompanied by an increase in protein concentration in the BALF. The protein was deemed to be entering the airways as oedema fluid and thought to inhibit the surfactant function [99]. Both bronchoconstriction and oedema could be prevented by pre-treatment of animals with exogenous surfactant [289,290].

There are a number of proposed mechanisms responsible for surfactant inhibition in the acute asthmatic response. Firstly, inhibition of surface properties of surfactant by plasma proteins such as fibrin monomer has been proposed, but other components of oedema fluid may make significant contributions (e.g. albumin) [291]. Secondly, surfactant phospholipid may itself be susceptible to phospholipase-mediated hydrolysis. For instance, group IIa secretory phospholipase A_2 ($sPLA_2$) is secreted from many stimulated inflammatory cells, including mast cells and alveolar macrophages, and its activity is increased in BALF after allergen challenge. PLA_2 action produces fatty acids and lysophospholipids, and concentrations of arachidonic acid and lysophosphatidylcholine were elevated respectively in BALF 6 hours [208] and 20 hours [4] after allergen challenge in subjects with mild asthma.

It has been demonstrated in chapter 5 that phospholipids in purified rabbit surfactant were susceptible to $sPLA_2$ hydrolysis when incubated with high concentrations of the enzyme (see section 5.3.4). The hydrolysis of lung surfactant only occurred at very high concentrations of PLA_2, this finding could explain why two reports came to contradictory conclusions as to whether lung surfactant was a substrate for group IIa $sPLA_2$ hydrolysis. The recombinant enzyme from guinea pig readily hydrolyzed PC in native guinea pig surfactant [272]. However the human group IIa $sPLA_2$ were reportedly essentially inactive against native porcine surfactant [271], though this was at low enzyme concentrations and in comparison to Porcine Pancreatic group I $sPLA_2$ and snake venom *Naja naja* PLA_2 that have reported differences in substrate specificity [292]. It is still not clear whether the phospholipid in the intact surfactant complex *in vivo* is a substrate for $sPLA_2$ activity, and also if during the acute asthmatic response the concentration of $sPLA_2$ in the alveolar space reaches significant levels as to exert an effect on the lung surfactant. However $sPLA_2$ activity has been reported to be inhibited by SP-A [272]and also plasma derived proteins [293]. Decreased levels of SP-A in BALF from patients with idiopathic pulmonary fibrosis [294], cystic fibrosis [248], ARDS [295], severe respiratory syncytial virus (RSV) [296] and asthma [297] have been reported and may increase the potency of $sPLA_2$ in the lung during the asthmatic response.

Very few studies have been performed to study the acute asthmatic response in human subjects. One study compared the surface activity of sputum collected consecutively from patients during asthma attacks with that of normal subjects and patients with stable asthma[298]. The phospholipid content and total protein levels were measured and there were no significant differences between normal and stable asthmatic subjects, however during an acute asthmatic attack these levels increased. Surfactant function was measured on a pulsing bubble surfactometer; the increase in the minimal surface tension of sputum in acute asthma demonstrated the inability of surfactant to function normally during the acute asthmatic response. These parameters improved during the recovery phase from the asthma attack, suggesting that inactivation of surfactant may contribute to the early phase of an asthma attack.

A group in Germany have just reported that total protein increased and surface activity decreased in BALF 24 hours after local allergen challenge, and that these responses were not apparent in either sham-challenged asthmatic or in allergen-challenged control subjects [299]. I have had the opportunity to study the phospholipid composition of the BALF from these subjects and will present the results in this chapter.

6.2 Methods for allergen challenge study

Using the electrospray ionisation mass spectrometer (ESI-MS) the phospholipid composition of BALF before and after sham and local allergen challenge in control and mild asthmatic subjects were analysed to study the acute asthmatic response. A group from the Hannover Medical School in Germany kindly donated the BALF samples.

6.2.1 Study protocol

BALF samples were obtained by fiberoptic bronchoscopy from control and asthmatic subjects before and after local administration of sham or allergen challenge [for details see [299]]. At the first bronchoscopy, the initial BALF (100ml instilled volume) was collected for baseline values from one lobe, then another lobe was challenged with warmed saline solution (10ml) only, followed by a lobe in the other lung with warmed antigen solution (10ml). BALF was then obtained from both challenged lobes by a second bronchoscopy twenty-four hours later. BALF aliquots were pooled, filtered through sterile gauze and then centrifuged at 250xg for 10 minutes. An aliquot of the cell free supernatant was stored at –28°C and then sent to England on dry ice. Venous blood samples (10 ml) were taken on both days, allowed to clot, and serum stored at –28°C until analysis.

Nine healthy volunteers and 15 patients with mild asthma were enrolled on to the study. All patients had allergic asthma according to the criteria from the Heart, Lung and Blood Institute of the National Institutes of Health [300]. On the day of bronchoscopy, a skin wheal dose series was obtained with either *D. pteronyssinus* (house dust mite) or grass pollen, depending on which produced the largest response. The concentration used for endobronchial challenge was one-tenth of that which elicited a skin wheal with a diameter of 3mm. Current medication consisted of β2 agonists only, and no patient had

used glucocorticosteroids, sodium chromoglycate or theophyline for at least the previous six weeks.

The healthy volunteers had no history of allergic diseases or any other disorders; they had negative skin prick tests, normal IgE levels (<100 IU/ml), normal lung function tests, and no bronchial hyperresponsiveness. All study subjects were non-smokers, and none had suffered an episode of acute bronchitis during the four-week period preceding the challenge. The Ethic Committee of Hannover Medical School approved the study and informed consent was obtained from each person in the study.

6.2.2 Electrospray ionisation mass spectrometry (ESI-MS)

The BALF samples were analysed as described in section 2.7.1, using a mobile phase and injection solvent of methanol:chloroform:water (7:2:1 v/v) containing 1% (w/v) NH_4OH. PC compositions of the serum samples were analysed as in section 2.7.3 using an injection solvent of methanol:chloroform:water (7:2:1 v/v) containing 20mM sodium acetate, and a mobile phase of methanol:chloroform:water (7:2:1 v/v).

6.2.3 Surface tension analysis

Surface activity of BALF was measured in Hannover Medical School using a pulsating bubble surfactometer as described previously [299]. The large aggregate (48,000xg pellet) fraction of BALF was resuspended to 1 mg/ml of phospholipid phosphorous, and 40µl of this suspension used for surface tension analysis. The surface tension function reported here was the value at minimal bubble size, γ_{min}, registered after 5 minutes pulsation at 20 cycles/min at a temperature of 37°C.

6.2.4 Statistical analysis

Results are expressed in tables as median values, with ranges indicated. They are displayed in figures as median values, with inter-quartile and 95% confidence intervals indicated. Statistical differences between initial, sham and allergen-exposed groups were compared using a Friedman test for both control and asthmatic groups. Wilcoxon tests were then performed on paired data when p values were less than 0.05. Selected

parameters of BALF phospholipid composition were compared with protein concentration and surface tension measurements using Pearson's correlation.

6.3 Results from local allergen challenge in mild asthmatic subjects

6.3.1 Comparison of total phosphatidylcholine / phosphatidylglycerol

Total concentrations of PC and PG were calculated by addition of all the individual molecular species measured, and the ratios of total PC/PG in BALF are shown in figure 6.1. This ratio did not change in the control group after either sham or allergen challenge. Similarly, in the asthmatic group there was no significant difference in the PC/PG ratio following sham challenge. However, the PC/PG ratio increased significantly following allergen challenge to asthmatic subjects ($p<0.05$). Comparison of absolute concentrations suggested that this increase in PC/PG ratio of BALF following allergen challenge in asthmatic subjects was due to an increase in total PC. Total PC after allergen challenge (25.1nmoles/ml, (range 8.5- 115.2)) was significantly greater than after sham challenge (19.1nmoles/ml, (8.1-45.1)), with no difference between values for PG (5.3nmoles/ml (2.4-13.3) and 5.5nmoles/ml (2.4-10.5) respectively).

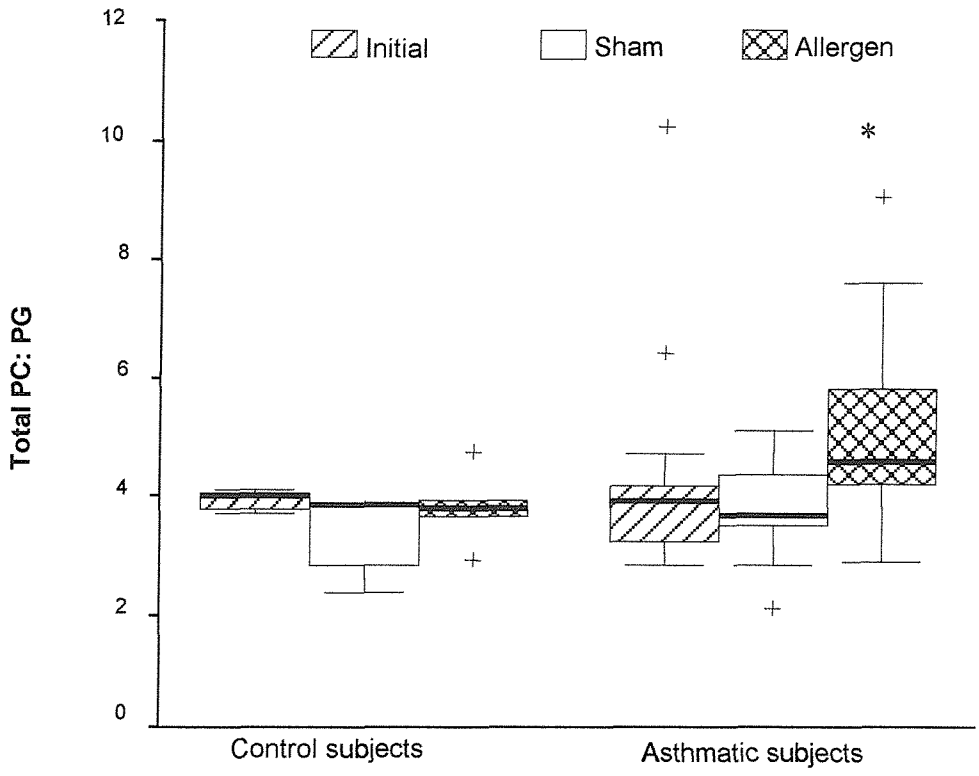


*Figure 6.1 The effect of local allergen challenge on the ratio of total PC: PG in BALF from control and asthmatic subjects. Total concentrations of PC and PG were calculated by addition of all the individual molecular species. Results are displayed, as median values together with interquartile ranges and 95% confidence limits, n=13 asthmatic subjects and n=5 control subjects. Extreme values are indicated by +. *p <0.05 for allergen vs. initial samples from asthmatic subjects*

6.3.2 Phosphatidylglycerol molecular species

The compositions of the 6 PG species identified in the BALF are summarized in table 6.1. While this composition varied considerably between individuals, the same major molecular species were predominant, namely 16:0/18:1PG, 18:1/18:1PG with smaller proportions of the isobaric components 18:0/18:2PG and 18:0/18:1PG. Median values for the PG species composition were identical for control and asthmatic subjects, and remained unchanged in both groups after either challenge procedure.

Table 6.1 BALF phosphatidylglycerol molecular species composition

mole %	Control Subjects			Asthmatic Subjects		
PG species	**Initial**	**Sham**	**Allergen**	**Initial**	**Sham**	**Allergen**
16:0/16:0PG	6.9 (4.2-9.5)	7.7 (0.6-14.7)	6.1 (5.4-13.8)	7.0 (0.6-21.5)	6.8 (1.0-8.9)	5.1 (1.3-9.3)
16:0/18:1PG	35.7 (32.1-42.5)	35.7 (29.3-40.5)	31.3 (29.0-39.3)	34.5 (4.0-42.7)	34.2 (30.1-44.4)	32.8 (26.0-41.2)
16:0/18:2PG	8.4 (2.9-11.4)	6.9 (2.0-15.0)	11.1 (6.1-11.7)	9.0 (1.7-15.8)	8.3 (1.1-15.7)	8.8 (3.5-14.6)
18:0/18:1PG	20.1 (13.1-21.6)	19.5 (16.2-22.0)	20.7 (20.3-22.1)	21.3 (17.8-51.9)	19.6 (16.1-27.6)	18.5 (16.8-26.3)
18:1/18:1PG & 18:0/18:2PG	23.2 (17.0-32.6)	20.5 (17.4-32.3)	24.5 (14.8-25.4)	21.3 (0.31-30.0)	24.1 (15.6-29.3)	22.4 (17.2-31.3)
18:1/18:2PG	7.0 (0.7-7.3)	6.6 (1.4-8.5)	7.5 (5.8-8.4)	7.1 (4.1-11.6)	5.4 (0.3-13.0)	7.6 (1.3-15.9)

PG molecular species composition of BALF from control and asthmatic subjects before challenge (initial) and 24 hours after either sham or allergen challenge. Values are expressed as mole % of total PG with median values and ranges indicated.

6.3.3 Phosphatidylcholine molecular species

Compared with results for PG, there was considerably less variation between subjects for the individual molecular species compositions of PC in BALF under baseline conditions, and this was identical for both control (Figure 6.2) and asthmatic groups (Figure 6.3).

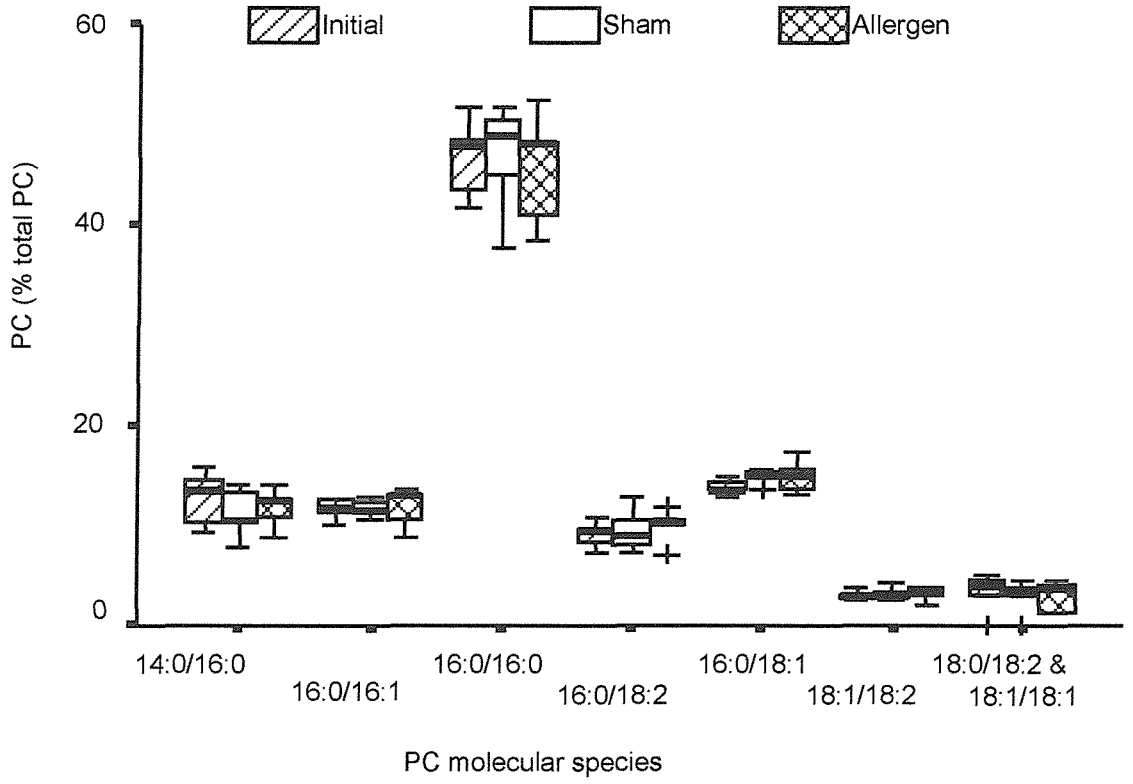


Figure 6.2 Compositions of individual PC molecular species in BALF for control subjects before challenge (initial), after sham challenge (sham) and after allergen challenge (allergen).

The molecular species composition of PC in BALF did not alter in control subjects after either sham or allergen challenge or in asthmatic subjects after sham challenge. In contrast there were significant changes in BALF PC composition in the asthmatic subjects following allergen challenge (Figure 6.3). The fractional concentration of 16:0/16:0PC decreased significantly following allergen challenge (36.6%, 13.8 – 50.7) compared to the before challenge lavage (47.1%, 40.1 – 54.7), and also displayed a much larger range of individual values. There was a corresponding increase in species containing 18:2, namely 16:0/18:2PC, 18:1/18:2PC and the isobaric components 18:0/18:2PC and 18:1/18:1PC. This increase in 18:2 containing species in the asthmatic subjects after allergen challenge is illustrated when comparing the initial and sham BALF positive ionisation mass spectra with the after allergen challenge from one of the extreme patients (figure 6.4).

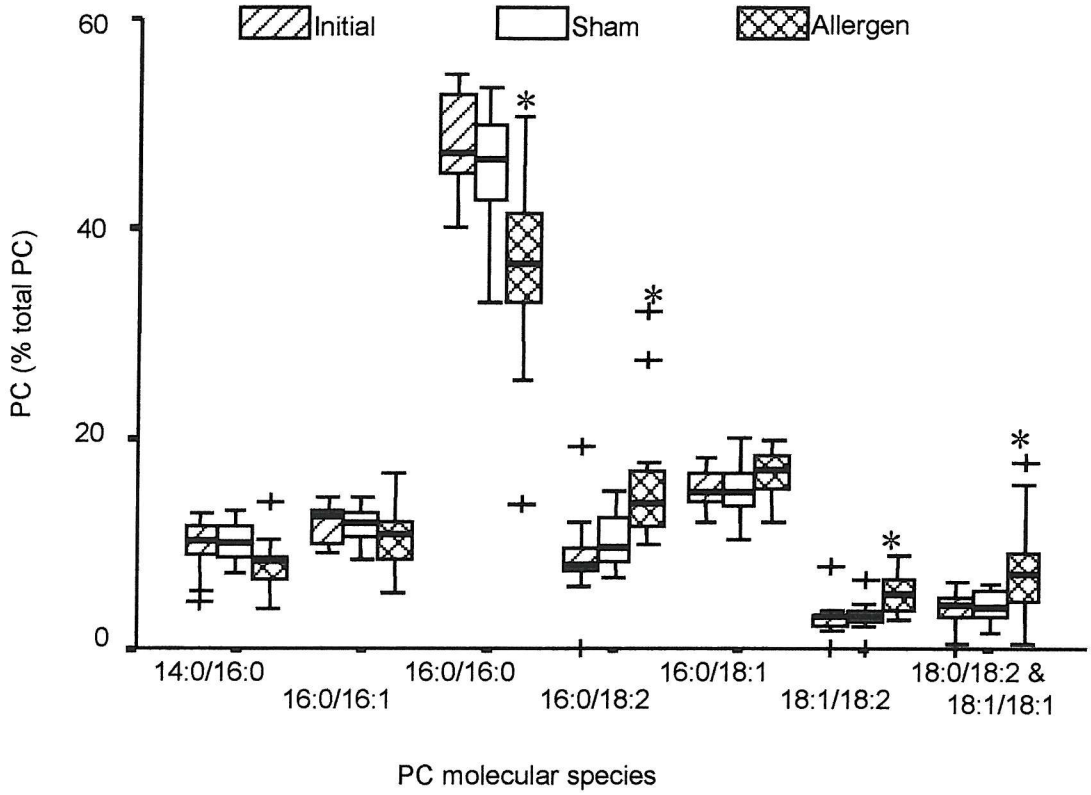


Figure 6.3 Compositions of individual PC molecular species in BALF for asthmatic subjects before challenge (initial), after sham challenge (sham) and after allergen challenge (allergen).

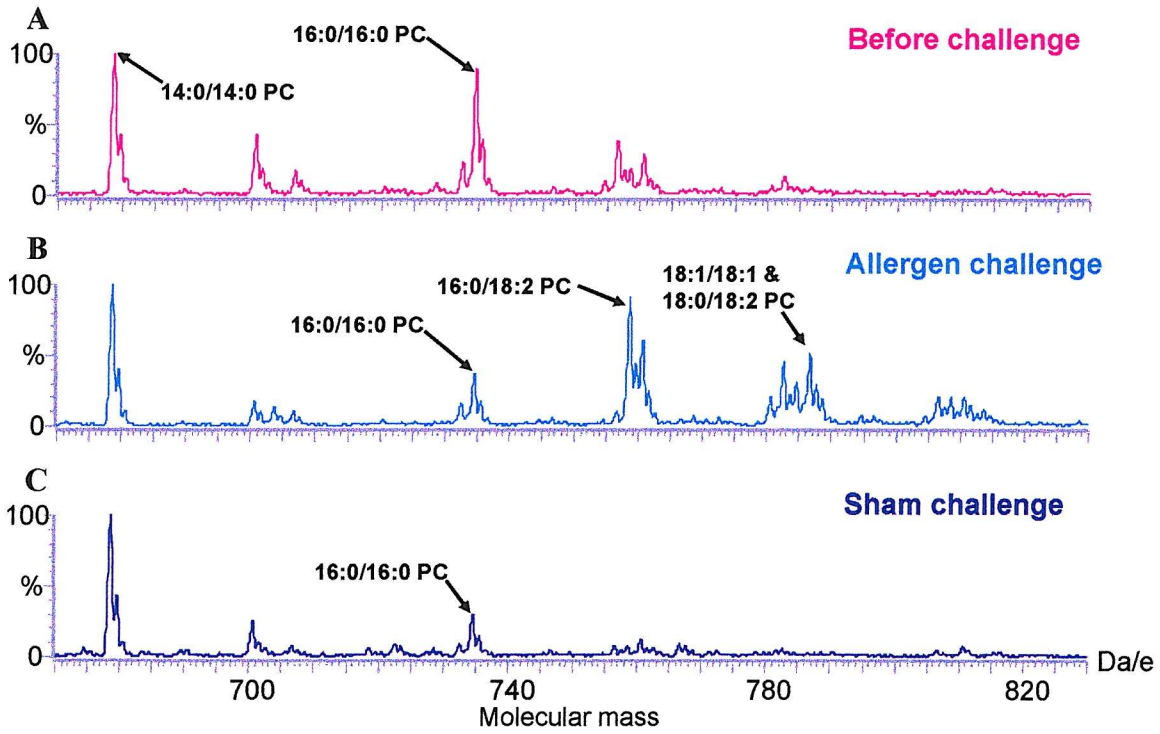


Figure 6.4 Positive ionisation mass spectra of BALF from one patient (A) before challenge, (B) after allergen challenge and (C) after sham challenge.

6.3.4 Use of tandem MS-MS to confirm *m/z* 786 after allergen challenge is mainly 18:0/18:2PC

Tandem MS-MS was used to confirm that the increase in the isobaric *m/z* 786 peak was due to a substantial increase in 18:0/18:2PC. Figures 6.5a and b show the product ions generated by fragmentation of the chloride adducts of 18:1/18:1PC and 18:0/18:2PC under negative ionisation to identify the major molecular species present. Figure 6.5a was from an asthmatic subject after sham challenge and confirms this peak as an approximately equimolar mixture of 18:1/18:1PC and 18:0/18:2PC. Figure 6.5b was from an asthmatic subject after allergen challenge, whose positive ionisation spectrum displayed a large amount of the *m/z* 786 component. The product spectrum showed a substantial increase in the amount of 18:2 fatty acid anion (*m/z* 279) produced upon fragmentation (figure 6.5a) indicating that the major species present after allergen challenge was 18:0/18:2PC with a small amount of 18:1/18:1PC.

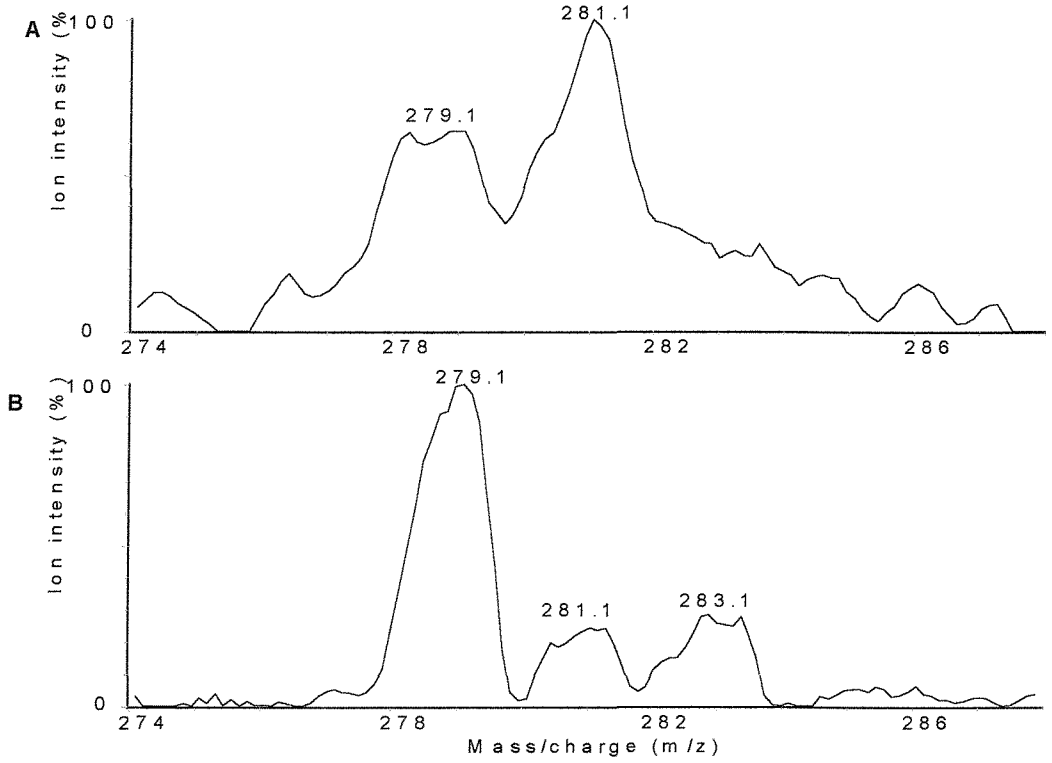


Figure 6.5 Structural assignment of the isobaric PC species18:1/18:1PC and 18:0/18:2PC (m/z 786 in positive ionisation mode) in BALF from an asthmatic subject. Tandem MS-MS was performed on the corresponding chloride adduct (m/z 820) in negative ionisation mode. The fatty acid anions produced from such fragmentation are shown. (A) After sham challenge, the presence of the 18:1 anion (m/z 281) and 18:2 anion (m/z 279) confirmed this ion as an equimolar mixture of 18:1/18:1PC and 18:0/18:2PC. (B) The increased abundance of the 18:2 fatty acid anion (m/z 279) after allergen challenge indicated that this ion was largely sn-118:0/sn-218:2PC.

6.3.5 Possible origins of the increased 18:2 containing species in allergen challenged asthmatic subjects BALF

The possible origin of the increased 18:2 containing PC species in the BALF of the allergen challenged asthmatic subjects was investigated. The mass spectrum of BALF PC from an asthmatic subject challenged with allergen that demonstrated the most extreme changes (figure 6.6a) was compared with the serum sample taken on the same day (figure 6.6b) and a typical neutrophil mass spectrum (figure 6.6c). The PC species present in neutrophils were dominated by 18:1 containing species with the major species being 16:0/18:1PC. In contrast the major PC species present in serum was 16:0/18:2PC, and two of the other major PC species (18:1/18:2 and 18:0/18:2 PC) were characterized as 18:2 containing species. The increase in the 18:2 containing PC species in the BALF of this asthmatic subject after allergen challenge appears to be characteristic of serum PC as opposed to cellular derived PC species, while species typical of surfactant were decreased. For instance the presence of large amounts of 18:2 containing species in both the BALF trace and the serum trace, namely 16:0/18:2PC and 18:0/18:2PC, was consistent with the infiltration of plasma-derived PC into the BALF. Compared to the BALF before allergen challenge (figure 6.4a) 16:0/16:0PC was no longer the major species after challenge.

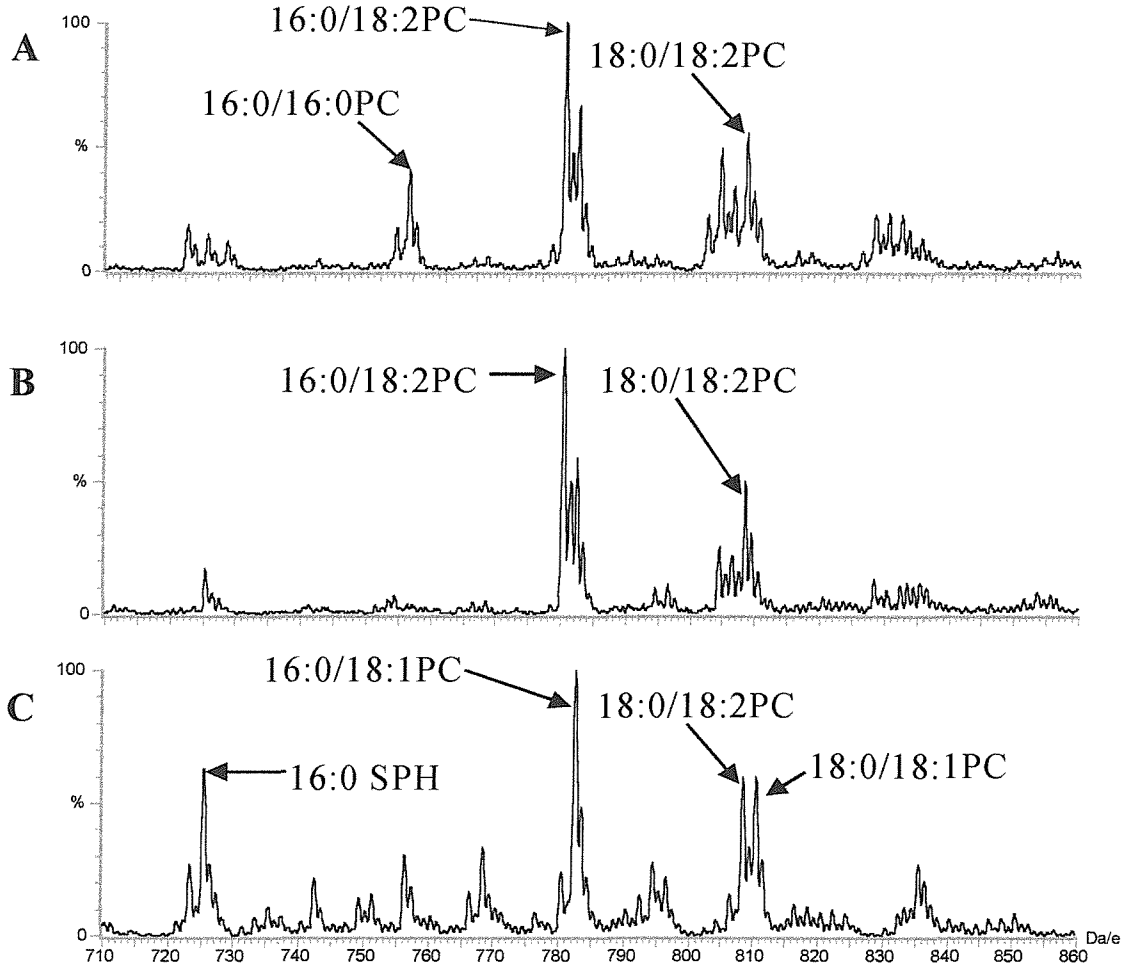


Figure 6.6 Comparison of positive ionisation mass spectra from (A) BALF and (B) plasma from the same asthmatic subject after allergen challenge and (C) a typical neutrophil mass spectrum. The neutrophils used for this particular mass spectrum were obtained from myself prior to separation and lipid extraction.

6.3.6 Serum phosphatidylcholine composition

Serum PC molecular species were characterized in all the subjects before and after challenge (table 6.2). These results illustrate that the major molecular species in serum PC was 16:0/18:2PC, followed by 16:0/18:1PC, 16:0/20:4PC, 18:1/18:2PC and18:0/18:2PC, these molecular species were increased in the BALF of asthmatic subjects after allergen challenge (figure 6.3). There were negligible amounts of 16:0/16:0PC in any serum sample. There were no significant differences to serum PC composition between the asthmatic and control subjects on either day 1 before the segmental challenges, or following challenge on day 2.

Table 6.2 Serum phosphatidylcholine molecular species composition

	Phosphatidylcholine molecular species (% total)			
	Control Subjects		**Asthmatic Subjects**	
PC Species	**Day 1**	**Day 2**	**Day 1**	**Day 2**
14:0/16:0 PC	0.7 (0.4 – 1.0)	0.7 (0.6 – 1.1)	0.79 (0.0 – 1.7)	0.9 (0.0 – 1.4)
16:0/16:1 PC	1.9 (1.7 – 2.5)	2.5 (2.1 – 3.6)	2.2 (1.8 – 3.0)	2.3 (1.8 – 3.0)
16:0/16:0 PC	0.6 (0.5 – 0.8)	0.6 (0.4 – 2.0)	0.7 (0.4 – 1.9)	0.7 (0.4 – 1.3)
16:0/18:2 PC	27.0 (26.9 – 31.0)	27.2 (25.6 – 28.8)	28.6 (22.7 – 32.3)	28.6 (23.7 – 33.3)
16:0/18:1 PC	12.5 (11.7 – 13.1)	13.8 (12.5 – 15.7)	13.5 (10.8 – 19.5)	14.2 (11.7 – 19.0)
16:0/20:4 PC	13.2 (9.9 – 13.6)	11.9 (10.3 – 13.6)	9.6 (7.8 – 13.6)	10.3 (8.0 – 14.3)
18:1/18:2 PC	8.3 (6.0 – 8.7)	7.3 (6.2 – 8.6)	8.3 (6.5 – 9.6)	7.9 (6.2 – 10.0)
18:0/18:2 PC &18:1/18:1 PC	13.3 (12.0 – 17.2)	14.9 (11.2 – 16.9)	14.3 (11.0 – 17.8)	14.3 (11.5 – 18.1)
18:0/18:1 PC	2.5 (2.2 – 2.7)	2.4 (2.1 – 3.5)	2.8 (2.2 – 3.9)	2.7 (1.5 – 3.5)
16:0/22:6 PC	4.6 (3.4 – 6.2)	4.6 (3.7 – 5.8)	5.0 (3.3 – 6.1)	4.2 (3.4 – 8.4)
18:1/20:4 PC	3.2 (3.0 – 4.5)	3.1 (2.8 – 3.4)	2.7 (2.0 – 3.6)	2.7 (2.2 – 4.8)
18:0/20:4 PC	5.9 (5.5 – 6.4)	6.0 (5.4 – 6.5)	5.2 (3.9 – 7.5)	4.7 (3.1 – 7.2)
18:0/20:3 PC	2.5 (2.4 – 3.0)	2.6 (2.0 – 3.3)	2.3 (1.7 – 3.1)	2.5 (0.7 – 4.1)
18:0/22:6 PC	2.2 (1.9 – 3.9)	2.0 (1.7 – 3.2)	2.4 (1.6 – 6.2)	2.3 (1.5 – 8.4)

Serum PC molecular species composition for both asthmatic and control subjects on days 1 and 2 of the study. Day 1 of the study was prior to the allergen challenge and day 2 was 24 hours after challenge. Results are presented as median values with ranges indicated.

6.3.7 Plasma infiltration into BALF

The results suggest that the increased amount of total PC present in the asthmatic subjects after allergen challenge was consistent with infiltration of plasma derived PC and perhaps minor alterations to surfactant PC composition. The possibility that the apparent decrease in the fractional concentration of 16:0/16:0PC in the asthmatic subjects following allergen challenge (figure 6.3) could be due to plasma filtration was investigated further. In figure 6.7 16:0/16:0PC was taken as an index of lung surfactant and compared with 16:0/18:2PC as an index for plasma phospholipid. There was a strong correlation between the relative decrease in %16:0/16:0PC and the increase in %16:0/18:2PC above the baseline value of approximately 10%, the normal mole % of 16:0/18:2PC in lung surfactant. The implication of this analysis is that the decrease in the percentage of 16:0/16:0PC in asthmatic subjects challenged with allergen was probably due to increase in plasma PC from oedema fluid.

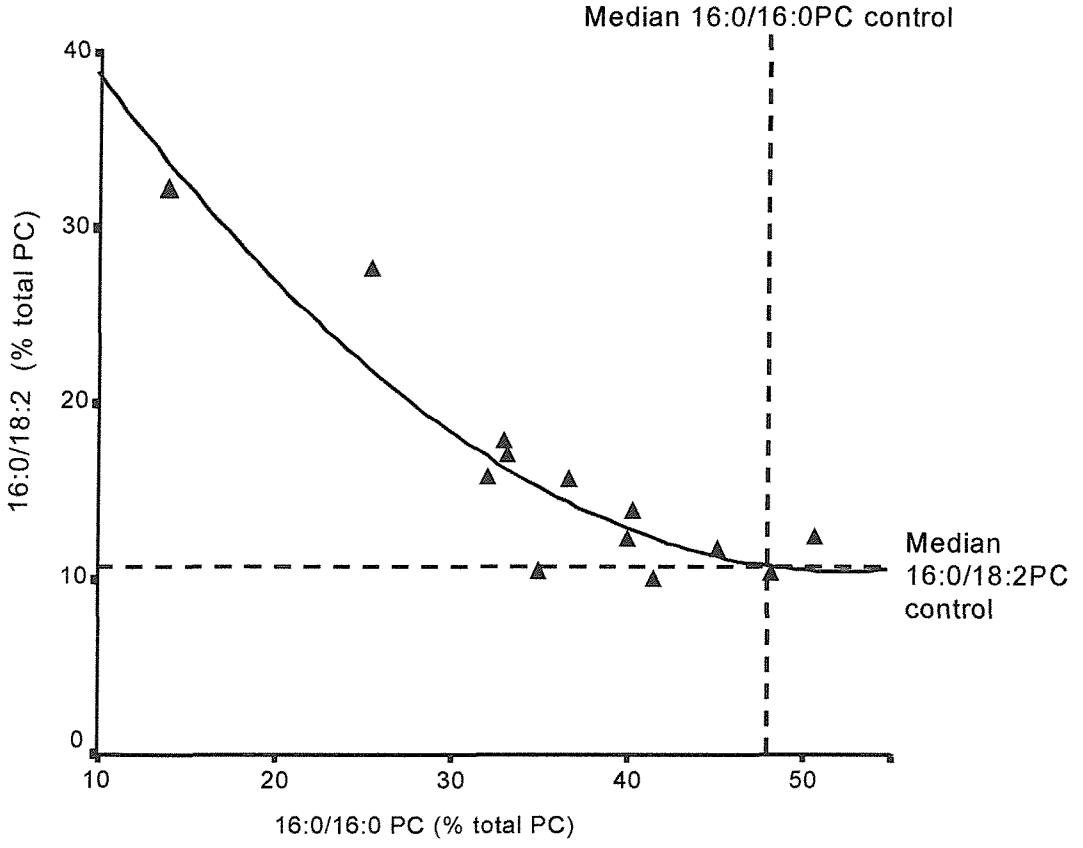


Figure 6.7 Assessment of the extent of plasma phospholipid infiltration in BALF from asthmatic subjects after allergen challenge. The percentage contents of 16:0/16:0PC and 16:0/18:2PC were correlated as markers for surfactant and plasma phospholipid respectively. Dashed lines represent median values from control subjects after allergen challenge.

If the mole % of 16:0/18:2PC increased as a result of plasma infiltration, then the protein concentration in the BALF should also have increased. There was a strong correlation ($p<0.001$) between protein concentration and %16:0/18:2PC in the asthmatic subjects after allergen challenge (figure 6.8). Results already published on the same samples reported that allergen challenge to these asthmatic patients increased the protein concentration and decreased surface activity in BALF 24 hours later [299]. Consequently, the decreased surface activity, indicated by an increase in minimal surface tension (γ_{min}), also had a strong correlation ($p<0.001$) with mole %16:0/18:2PC (figure 6.9). These results suggest strongly that there were negligible changes to surfactant-specific phospholipids following allergen challenge in asthmatic patients. They also confirm that increased 16:0/18:2PC is potentially a sensitive marker for plasma infiltration in the airways of asthmatic subjects.

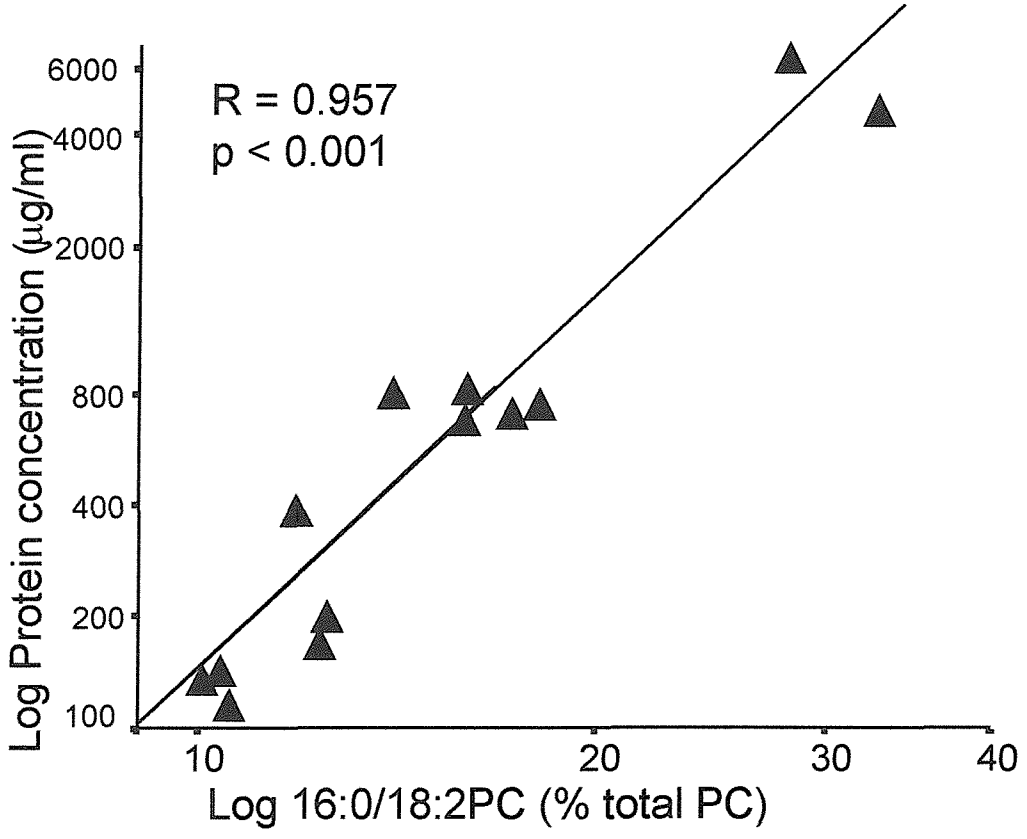


Figure 6.8 The fractional concentration of 16:0/18:2PC in BALF from asthmatic subjects after allergen challenge was correlated with total protein concentration

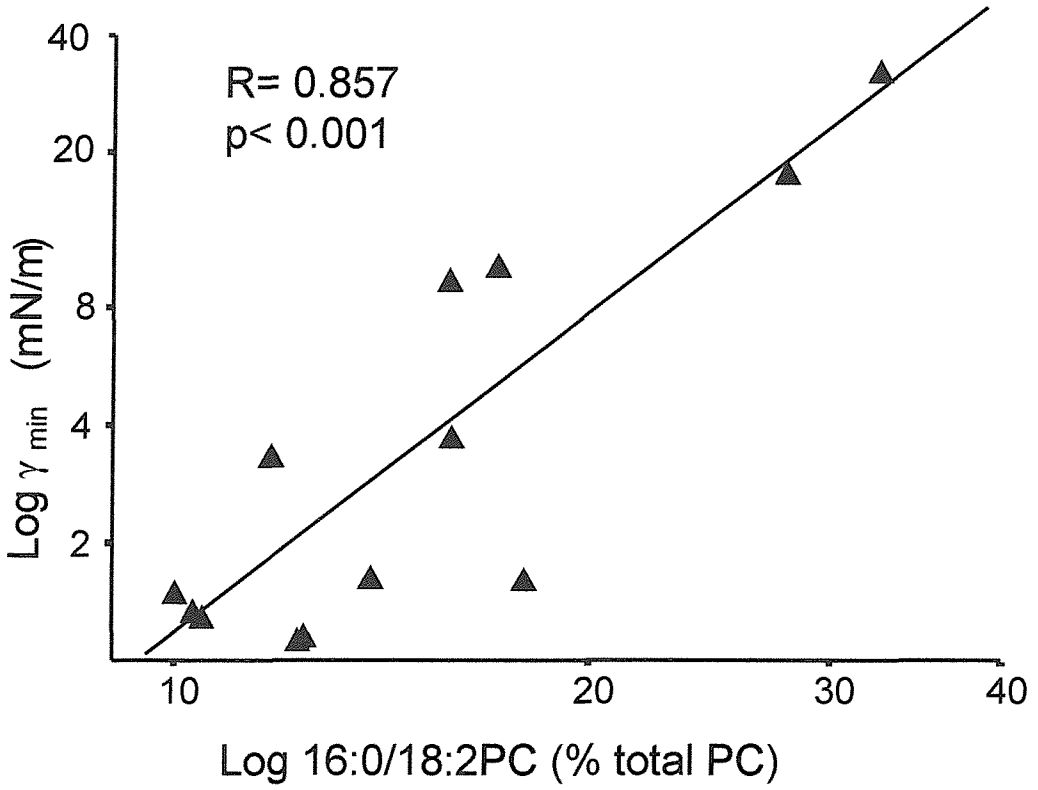


Figure 6.9 Correlation of the fractional concentration of 16:0/18:2PC with the minimum surface tension (an indicator of surface activity) of BALF from allergen challenged asthmatic subjects.

6.3.8 Evidence for lyso-PC in BALF of three extreme allergen challenged asthmatic subjects

Using ESI-MS, 16:0LPC was detected at low concentrations in BALF from 3 asthmatic subjects after allergen challenge. Due to the lack of an internal standard for LPC in these samples it was not possible to quantify the amount present. The positive ionisation mass spectrum of the BALF from one of the three patients is shown before (figure 6.10a) and after allergen challenge (figure 6.10b). LPC was not detected in the BALF of either subject group under baseline conditions or in the other asthmatic subjects after allergen challenge. The 3 samples with detectable LPC also showed the greatest increase in PC molecular species characteristic of plasma. Importantly, 16:0LPC was readily detected in all serum samples, in agreement with previous reports (figure 6.10c) [301,302]. Therefore, 16:0LPC could be present in the BALF due to the infiltration of plasma derived PC species into the airways.

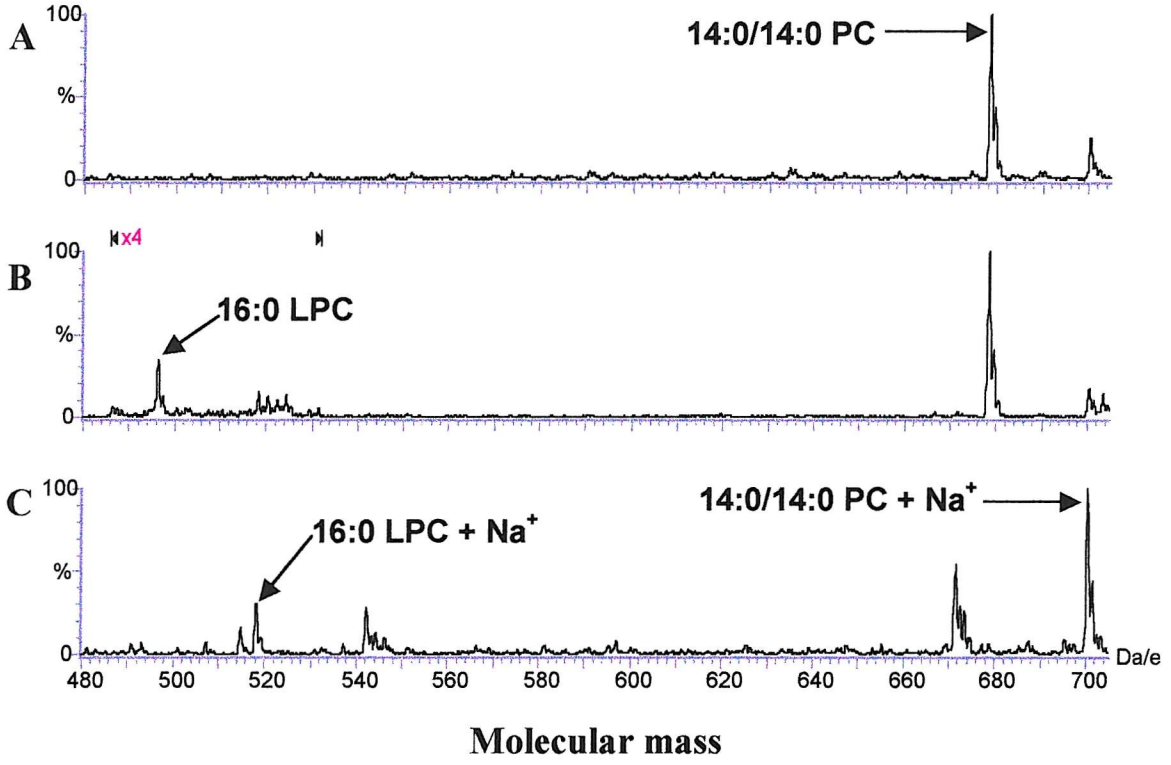


Figure 6.10 Comparison of the positive ionisation spectra over the m/z range 480-705 to identify LPC from one asthmatic subject (A) BALF before challenge (B) BALF after allergen challenge and (C) serum sample

While it most likely that the LPC detected in BALF was derived from the plasma infiltrate, it is also possible that a proportion was released directly into the alveolar space by the action of $sPLA_2$ as part of the lung inflammatory response to the allergen challenge in the asthmatic subject [4]. There were too few subjects with measurable LPC in BALF for these two potential mechanisms to be examined in greater detail. The absence of measurable LPC in BALF in the majority of asthmatic subjects after allergen challenge may have been due to variation in disease severity or rapid metabolism or degradation of the LPC in the alveolar space.

6.4 Discussion

For the first time the molecular species compositions of PC and PG in BALF were measured after local allergen challenge in asthmatic and control subjects. These PC and PG compositions before challenge were identical for both subject groups and were characteristic of purified surfactant. After allergen challenge, the increased

concentrations of unsaturated PC species in the asthmatic group, but not the control group, (figure 6.3) was striking. Comparison of molecular species compositions suggested strongly that this altered PC composition was due to infiltration of plasma phospholipid components (Table 6.2 and figure 6.6). The compositions of both serum PC and the increased BALF PC after allergen challenge were both dominated by 18:2-containing species, principally 16:0/18:2PC. This increased content of 18:2 containing species evidently contributed to the decrease in the percentage concentration of 16:0/16:0PC in asthmatic subjects after local allergen challenge. It is also possible, however, that surfactant PC was altered under these conditions, especially as the decrease in 16:0/16:0PC was greater then the increase in 16:0/18:2PC. This suggestion agrees with results from another study in our laboratory that found a progressive decrease in the fractional concentration of 16:0/16:0PC in the sputum of asthmatic subjects with increasing disease severity. This decrease in mole %16:0/16:0PC also correlated inversely with an increase in mole %16:0/18:2PC [303], which suggests that the infiltration of plasma phospholipids is apparent in the moderate to severe asthmatics while in the stable condition. The presence of increased %16:0/18:2PC in the BALF may contribute to the severity of the asthma.

The results presented in this study after allergen challenge were all obtained 24-hours after allergen instillation. They provide no evidence for any alteration to surfactant phospholipid composition that may have occurred at earlier time points. Indeed, it is possible that any such changes may have resolved by 24 hours. This distinction is important given previous reports of the kinetics of secretory phospholipase A_2 ($sPLA_2$) activation in asthmatic subjects after allergen challenge [4,208]. For instance $sPLA_2$ activity increased significantly 4 hours after allergen challenge and decreased towards baseline levels after 24 hours [208]. Lyso-phospholipids are the products of $sPLA_2$ activity, and lysophosphatidylcholine (LPC) molecular species have been reported to increase 20 hours after allergen challenge [4]. I have also shown LPC in the BALF from three asthmatic subjects 24 hours after allergen challenge. The origin of the 16:0LPC in the BALF cannot solely be attributed to $sPLA_2$ activity on lung surfactant due to the presence of 16:0LPC in the serum of asthmatic and control subjects. However the implications of the presence of LPC in the alveolar space is still significant as LPC has been shown to

exert an injurious effect on the alveolar type I cellular membranes [304], which may also contribute to the epithelial desquamation and sloughing that is typical of asthmatic airways [288].

The observation of phospholipids characteristic of plasma in BALF of mild asthmatic subjects 24 hours after local allergen challenge has potentially important implications for concepts relating inactivation of surfactant to the acute disease process in asthma. Phospholipids are transported in plasma as integral components of lipoproteins, which implies that lung inflammation in asthma causes sufficient disruption of airway epithelial cell junctions to permit infiltration of relatively large lipoprotein particles. This conclusion is important, as lipoprotein components cannot be measured in airway or alveolar fluid from the lungs of healthy people or from many patients with a variety of chronic conditions. In addition to this lipoprotein infiltration being relatively specific to asthma, the absence of plasma phospholipid components in BALF from asthmatic subjects under baseline conditions suggests that such infiltration is a transient component of the acute disease response.

Increased concentration of protein components of plasma, such as fibrin [305], has previously been suggested to contribute to the severity of the asthmatic response by direct inhibition of the surface tension properties of surfactant [57]. In this model, it is envisaged that impaired surfactant function potentiates airway oedema and results in liquid accumulation in the narrowest sections of conducting airways. This in turn could then contribute to the mucus plugging and reduced airflow characteristic of acute asthma. It is also possible that the lipoprotein component of the plasma infiltration of BALF could be an additional factor in this response. This concept is supported by a recent report that described physical interactions between serum lipoproteins and surfactant [306]. Analysis of BALF from a patient with extensive lipoprotein infiltration of the lungs demonstrated the presence of abnormal complexes between surfactant and serum lipoproteins, probably due to the binding of surfactant protein A to lipoprotein. The formation of comparable surfactant:lipoprotein complexes might be expected to contribute to the impaired surfactant function in the acute asthmatic response.

The evidence suggests that significant surfactant alterations occur during the acute asthmatic response of mild asthmatics and one report showed that these alterations are reversible [307]. The study involved obtaining BALF 48 hours after segmental allergen challenge in control and mild asthmatic subjects. Similar to the data from this study they demonstrated the surfactant function from asthmatic subjects to be reduced after allergen challenge in contrast to asthmatic subjects prior to allergen challenge and control subjects. The paper then showed that when the BALF from the challenged allergen segment was washed to remove all the water-soluble inhibitors the surfactant function was restored to normal in most cases. This evidence suggests that the surfactant complex does not undergo a significant amount of hydrolysis during the acute asthmatic response in mild asthmatic subjects. Therefore the alterations in the BALF observed after local allergen challenge of an asthmatic subject can be attributed to the influx of plasma components. The ability of the asthmatic subject to clear these inhibitory components from the alveoli may predetermine the severity of the disease and the recovery time from an acute episode.

Chapter Seven

Acute Respiratory Distress Syndrome

(ARDS)

7.1 Introduction

Acute Respiratory Distress Syndrome (ARDS) is a clinically and pathophysiologically complex disease involving an acute inflammatory reaction in the lung. A variety of different insults have been reported to lead to ARDS, for instance sepsis, burns, pancreatitis or pneumonia [62,308-310]. Although ARDS is initiated by different mechanisms a common final pathway results in alveolar damage. ARDS is characterised by increased vascular permeability, airway obstruction, pulmonary infiltrates and hypoxemia leading to a need for artificial ventilation and removal of alveolar secretions by suctioning [63,308,310]. Despite advances in intensive care medicine ARDS has a mortality rate in excess of 50% [311], and the mechanisms involved in the development of this acute disease are still uncertain.

Alterations to lung surfactant are thought to contribute to the etiology of ARDS [48,62,64,312]. However, it must be emphasised that ARDS is a severe, multifactorial disease in which surfactant is only one facet to the disease process. It was over 30 years ago that Asbaugh and colleagues first proposed that abnormal surfactant function played a role in the pathogenesis of ARDS [313]. They identified an increase in the minimum surface tension of surfactant isolated from autopsy specimens of patients who had died from ARDS and postulated that this abnormal surfactant contributed to the respiratory failure seen in the patients [313]. In later studies, surfactant abnormalities in ARDS patients have been demonstrated both qualitatively and quantitatively [64,77,314-316]. Gregory at al. demonstrated that several of these surfactant alterations already occurred in patients at risk of developing ARDS, suggesting that these abnormalities of surfactant occur early in the disease process [67].

Many studies have looked into the surfactant composition of ARDS patients and tried to identify what causes the surfactant dysfunction. One of the most consistent findings from both human studies and animal models of ARDS is the alteration of phospholipid composition in the BALF from these subjects [64,65,67,77,314,317-319]. The changes observed in all the studies include decreased quantities of disaturated PC and total PG associated with increased amounts of PI, sphingomyelin (Sph) and lysophosphatidylcholine (LPC). These changes in the phospholipid composition occurred early in the course of injury,

before significant pathological abnormalities were evident [67]. The alterations became more pronounced as injury progressed and were accompanied by decreased levels of the surfactant proteins SP-A and SP-B [67,314,320]. A recent report proposes that the surfactant-specific proteins in particular SP-A undergo direct damage in the lungs of patients with ARDS probably by proteolysis by neutrophil elastase and this may account for the decreased levels reported in ARDS patients [321].

The cause of these changes in the phospholipid composition of surfactant from ARDS patients is subject to much speculation. The alveolar type II cells responsible for secreting surfactant are damaged during acute lung injury, which may result in deceased and/or altered surfactant production [322]. As alveoli are flooded with plasma proteins during the early stages of ARDS, endogenous surfactant may be inactivated [62,323-325] or washed away[326]. Some of the changes in the phospholipid composition could result from contamination of the alveolar surfactant with cell debris and oedema fluid. Other reports suggest that PLA_2 is involved possibly by hydrolysing the surfactant phospholipids [114,327] and also in the inflammatory process by generating lipid derived chemical mediators such as eicosanoids, lysophospholipids and platelet activating factor (PAF)[328].

The role of PLA_2 in ARDS is of particular interest as there are reported elevated levels of PLA_2 in these patients. PLA_2 activation and circulatory release have been recognised in clinical disorders that promote systemic inflammation. Patients with sepsis, acute pancreatitis, peritonitis and severe multiple trauma (each of which predispose to ARDS [308,309]) have increased PLA_2 activity in their sera [210,329-332]. The quantitative increases in circulating PLA_2 activity correlates with both the degree of illness [210,332] and the extent of associated pulmonary insufficiency [210 331].

Only one study has shown the presence of PLA_2 in BALF of patients suffering from ARDS and the PLA_2 levels correlated positively with the severity of ARDS [3]. In the study, two forms of PLA_2 were identified with increased activity in the BALF of ARDS patients, a group IIa $sPLA_2$ and a form that was biochemically and immunochemically distinct from group I, group II, and cytosolic PLA_2 [3]. The group IIa $sPLA_2$ is a secretary enzyme that originates from macrophages [333], neutrophils [334], platelets [141] and inflamed

synovial tissue [143](see section 1.7.4.1). Unlike some of the other PLA_2s the group IIa $sPLA_2$ requires a critical concentration of anionic phospholipid in order for the enzyme to bind to the substrate, prior to any significant hydrolysis occurring (see chapter 5)[200].

Human lung surfactant contains a particularly high proportion of PG, approximately 15 mole % (figure 4.10), considering there is a virtual absence of PG in the outer monolayer of eukaryotic cell membranes. The presence of unusually high proportions of PG in lung surfactant could make surfactant susceptible to group IIa $sPLA_2$ mediated hydrolysis. Evidence from chapter 5 concluded that purified lung surfactant could act as a substrate for the enzyme *in vitro,* although hydrolysis required high enzyme concentration and a 3-hour incubation period.

In this chapter, ESI-MS was used to assess any phospholipid changes that occurred in the tracheal aspirate samples from four ARDS patients, by comparison with purified surfactant from control subjects. The samples from the ARDS patients were then analysed for the presence of group IIa $sPLA_2$. Using the information from chapter 5 it was then possible to hypothesise if the changes observed in the surfactant phospholipid composition were compatible with PLA_2 activity. The aim of the chapter was to provide initial observations to support a future substantive investigation as to whether the hydrolysis of surfactant phospholipids by group IIa $sPLA_2$ is part of the disease process in ARDS.

7.2 Methods

The study of surfactant phospholipids from ARDS patients was carried out in Southampton General Hospital intensive care unit (ICU), as a pilot study over a three month time period. Ethical approval was granted from the Southampton and South West Hampshire Local Ethics Committee (submission number 80/97) along with agreement from the ICU consultants to provide information on the status of patients. Patients were selected with clinical and radiographic diagnosis of ARDS, requiring intubation and ventilation. The physiotherapists obtained secretions from the lower airways (tracheal aspirates) by suctioning using a catheter inserted into the bronchus via the endotracheal tube as part of routine care of intubated patients with ARDS. The samples were placed

on ice and then processed as in section 2.2. During the three month time period only four patients reached the criteria required for the study. Therefore due to poor recruitment and the time consuming nature of the work no further studies were carried out.

The phospholipid composition of purified surfactant from control subjects (for preparation see section 2.2.1) was for comparison with that of tracheal aspirates from ARDS patients. BALF was not used as a control, because PI in BALF from control subjects could not be reliably measured. Alterations to surfactant PI composition in ARDS are considered to be significant, therefore the comparison with purified surfactant was important. The use of purified surfactant was supported by results presented in chapter 4 which demonstrated that phospholipid compositions of BALF from healthy rats and rabbits did not differ appreciably to their purified surfactant compositions.

7.3 Results

7.3.1 Total PC, PG and PI in ARDS patients compared to control subjects

Previous studies of BALF from ARDS patients have measured phospholipid classes and not individual molecular species. This study utilised ESI-MS and analysed compositions of individual phospholipid molecular species and, by their addition also the phospholipid classes. Figure 7.1 compares the total PC, PG and PI as a percentage of the sum of the individual phospholipid molecular species measured for both control subjects and ARDS patients. There was a wide range in the disease severity of the 4 ARDS patients, but for the purpose of comparison with a control group they have been grouped together.

The percentage of total PC in the ARDS patients was not significantly different to the controls. However the fraction of total PG was significantly lower in ARDS patients compared to control subjects ($p<0.005$). Decreased PG has been reported previously in ARDS, and in some patients PG has been described to be virtually absent [65,77] The proportion of PI in the total phospholipid was appreciably higher in ARDS patients compared to control subjects ($p<0.005$), another finding that agrees with previous studies [65,67,77].

The PC/PG ratio was significantly higher in the ARDS (9.5, 6.1-14.0) when compared to the control group (4.6, 3.2-6.1) (Mann Whitney test, $p<0.005$). This increase in the PC/PG ratio in the ARDS patients may have resulted from either an increase in the PC or a decrease in the PG. Potential sources of increased PC could be from either infiltration of cellular debris or plasma derived PC as was seen in the BALF of the allergen challenged asthmatic subjects (chapter 6). The decreased PG may arise from group IIa $sPLA_2$ hydrolysis of surfactant phospholipids. As already demonstrated *in vitro* group IIa $sPLA_2$ action on surfactant results in an increase in the PC/PG ratio (see section 5.3.4.4), this was proposed to be due to the enzymes preferential hydrolysis of PG. However, due to the variation in the volume and concentration of phospholipids in the fluid obtained from the ARDS patients it was not appropriate to compare the absolute concentrations to determine what had occurred.

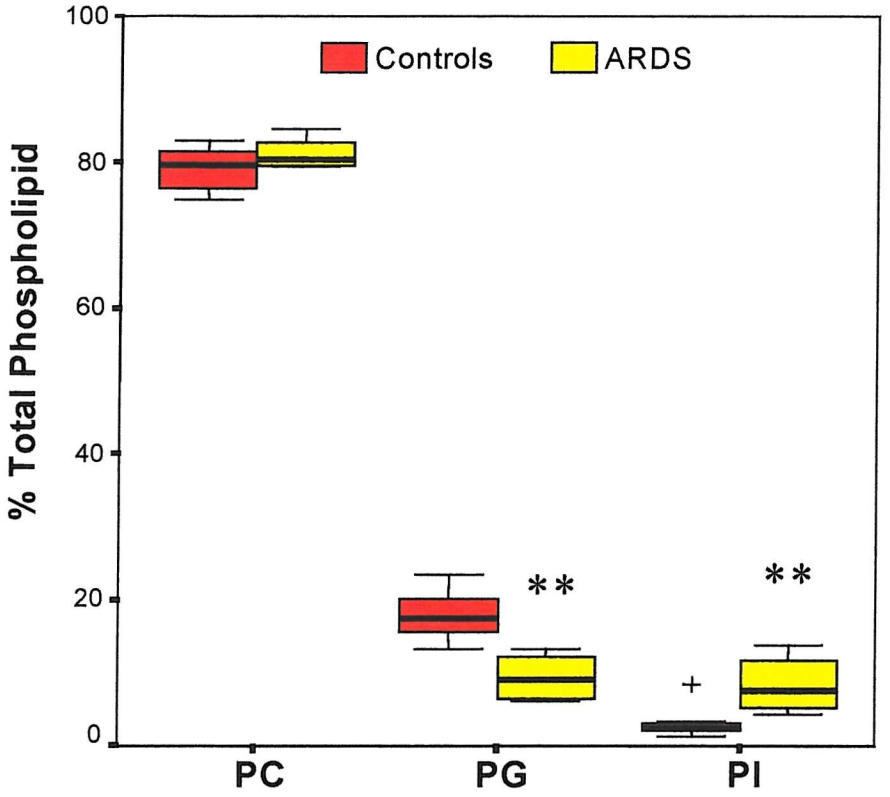


*Figure 7.1 Comparison of the proportions of PC, PG and PI of the total phospholipid measured in the tracheal aspirates of ARDS patients (n=4) compared with purified surfactant from control subjects (n=10). Total concentrations of PC, PG and PI were calculated by the addition of all the individual molecular species. Results are displayed, as median values together with interquartile ranges and 95% confidence limits. Extreme values are indicated by +. Statistical significance was determined by a Mann-Whitney test. ** denotes p<0.005 when compared to the control subjects.*

7.3.2 PC molecular species in ARDS and control subjects

The PC molecular species composition of tracheal aspirates from ARDS patients was very different to that of purified surfactant from control subjects (see figure 7.2). The most notable difference in the PC compositions between these two groups was the dramatic decrease in the percentage of 16:0/16:0PC in the ARDS patients ($p<0.005$). The low proportion of the surface-active component 16:0/16:0PC in the ARDS patients is a potential cause for some of the symptoms of this disease. A reduced proportion of 16:0/16:0PC in surfactant increases surface tension at the air-liquid interface, which results in alveolar collapse, leading to airway obstruction and infiltration of oedema fluid.

The increased presence of PC species such as 16:0/18:2 ($p<0.005$) and the isobaric components 18:1/18:1 and 18:0/18:2 ($p<0.05$), in ARDS patients is evidence for the infiltration of plasma, as these are the major species present in plasma PC (see table 6.2). Another important observation was the significantly higher proportion of the main PC species of neutrophil membranes 16:0/18:1PC in ARDS patients ($p<0.05$). This is likely to indicate an infiltration of cells, probably neutrophils, into the airway. ARDS has been reported to be characterised by increased neutrophils in the BALF when compared to controls [335]. Thus, it is possible that the higher PC/PG ratio that is seen in ARDS may in part be accounted for by increased PC concentration, derived from both cellular and plasma sources.

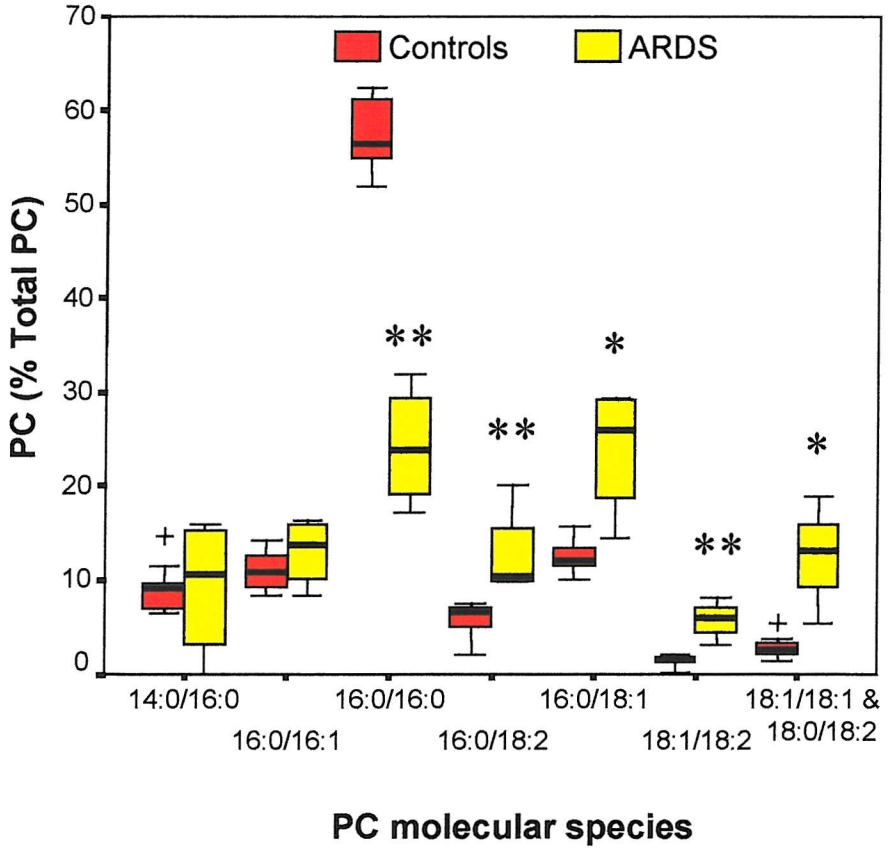


*Figure 7.2 PC molecular species of purified surfactant from control subjects (n=10) and tracheal aspirates from ARDS patients (n=4). Results are displayed, as median values together with interquartile ranges and 95% confidence limits. Extreme values are indicated by +. Statistical significance was determined by a Mann-Whitney test. ** denotes $p<0.005$ and * $p<0.05$ when compared to the control subjects.*

7.3.3 PG molecular species in ARDS and control subjects

Figure 7.3 shows the PG composition of tracheal aspirates from ARDS patients and purified surfactant from control subjects. There was a wide variation in the PG composition of the ARDS patients and this can probably be accounted for due to the differences in the disease severity of these patients. For instance one of the most critically ill patients had virtually no PG present, while the PG composition of another less seriously ill patient resembled that of the control subjects. Despite the variation in PG composition of the ARDS patients, there were many significant differences compared to the control subjects. For instance both 16:0/18:2PG and 18:1/18:2PG were proportionately higher in the ARDS patients ($p<0.005$), while the percentages of 16:0/18:1PG and 18:0/18:1PG were decreased ($p<0.05$). These alterations to the PG composition observed in the ARDS patients are unlikely to have occurred through infiltration of PG from other sources, as PG is not detectable in neutrophil or other eukaryotic cell membranes. PG is however the main constituent of bacterial cell membranes but bacterial sources is unlikely to be the cause of this altered BALF PG compositions. The PG composition of bacterial cells is very different to eukaryotic cells; for instance the gram-positive bacteria *Micrococcus luteus* is composed of 14:0/15:0, 15:0/15:0, 15:0/16:1, 15:0/16:0 and 18:0/15:0 PG species [336]. The molecular masses (therefore *m/z* values) of these PG species are different to those found in the tracheal aspirates from the ARDS patients.

There are two possible explanations for the altered PG composition in ARDS patients. Firstly, there may be differential hydrolysis of the PG species by phospholipases, such as group IIa $sPLA_2$. This enzyme has been reported to be increased in the BALF of ARDS patients [3]. Group IIa $sPLA_2$ has been shown to demonstrate a slight preference for hydrolysing 16:0/18:1PG from surfactant *in vitro* (see section 5.3.4.2), and this might account for the decrease in 16:0/18:1PG in the ARDS patients. Secondly, the type II cells that produce the surfactant phospholipids are probably damaged during the disease process and this may cause an alteration to the type II cell metabolism. The damage may result in the production of PG molecular species in different proportions and hence causing the altered phospholipid composition observed in the ARDS patients.

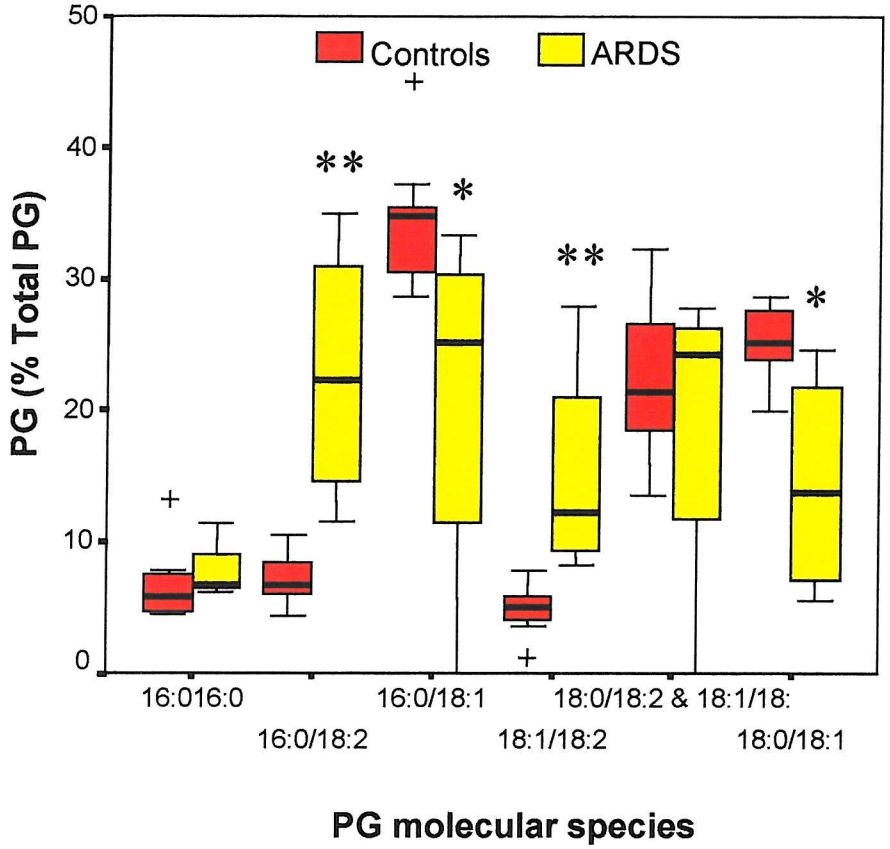


*Figure 7.3 PG molecular species of purified surfactant from control subjects (n=10) and tracheal aspirates from ARDS patients (n=4). Results are displayed, as median values together with interquartile ranges and 95% confidence limits. Extreme values are indicated by +. Statistical significance was determined by a Mann-Whitney test. ** denotes $p<0.005$ and * $p<0.05$ when compared to the control subjects.*

7.3.4 PI molecular species in ARDS and control subjects

The PI compositions of tracheal aspirates from ARDS patients and purified surfactant from control subjects are shown in figure 7.4. The most notable changes were the significantly lower proportion of 18:0/18:1PI ($p<0.005$) and the increased percentage of 18:0/20:4PI ($p<0.05$) in the ARDS patients. The increase in 18:0/20:4PI is likely to be due to infiltration of cells such as neutrophils. 18:0/20:4PI is the main PI species of cell membranes and is probably present due to the infiltration of such cells. Cell membranes are also composed of other PI species such as 18:0/20:3 and the isobaric components 18:0/18:2 and 18:1/18:1 that are also increased in the ARDS patients although not significantly. The reason for the selective decrease of 18:0/18:1PI in ARDS patients is unclear but is probably due to selective hydrolysis or synthesis of this species, as it is only a minor component of cell membrane PI.

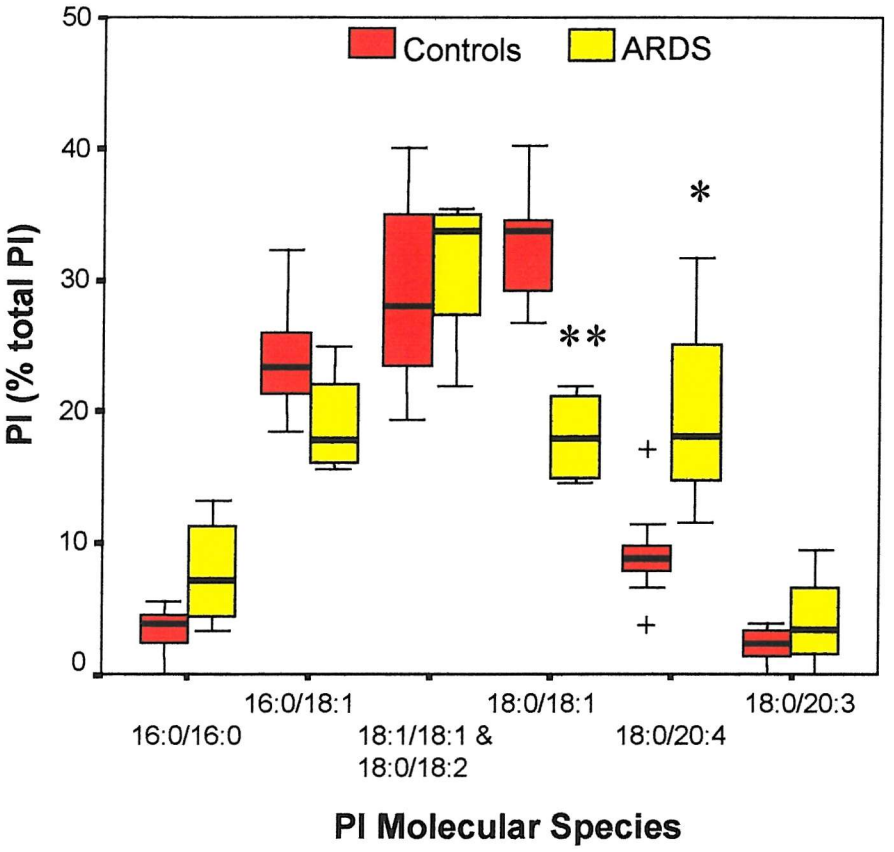


*Figure 7.4 PI molecular species of purified surfactant from control subjects (n=10) and tracheal aspirates from ARDS patients (n=4). Results are displayed, as median values together with interquartile ranges and 95% confidence limits. Extreme values are indicated by +. Statistical significance was determined by a Mann-Whitney test. ** denotes $p<0.005$ and * $p<0.05$ when compared to the control subjects.*

7.3.5 Phospholipase A_2 analysis of ARDS samples

The alterations in the phospholipid composition of the ARDS patients suggest that PLA_2 may be involved at least in part in the disease process. The significantly higher PC/PG ratio in the ARDS patients compared to the controls implies a possible involvement of group IIa $sPLA_2$, by causing a decrease in the PG. There was a wide variation in the PG composition of the ARDS patients that tends to suggest that different processes are occurring in each patient. This is not surprising as ARDS can be caused by many different conditions and in this study the ARDS has been caused by different insults in all of the patients (see table 7.1). However an increase in the amount of PC would also produce such an increase in the PC/PG ratio. The PC composition of the ARDS patients was dominated by PC species characteristically found in cell membranes and plasma, which could increase the amount of total PC in the tracheal aspirates of ARDS patients, resulting in an increased PC/PG ratio.

Table 7.1 Details of the 4 ARDS patients

Patient number	Cause of the disease	Fatal	PL conc (nmoles/ml)	PC/PG ratio	16:0/18:1PG/ 16:0/16:0PC ratio	+ or - $sPLA_2$
1	Acute Pancreatitis	No	111.2	7.0	0.12	+
2	Near Drowning	No	31.5	14.0	0.00	-
3	Leukaemia	Yes	151.8	11.0	0.09	+
4	*	No	279.5	6.1	0.20	-
Control subjects				4.6 (3.21-6.11)	0.13 (0.08-0.18)	

*The median values for the control subjects (n=10) with ranges indicated. PL conc denotes phospholipid concentration. * A more precise diagnosis was unavailable.*

All the ARDS patients have a higher PC/PG ratio than the control group (see table 7.1). This increase in the PC/PG ratio is likely to have occurred by two mechanisms; PG has decreased (possibly due to $sPLA_2$ action) or PC has increased (probably due to cell contamination). The possibility that the surfactant phospholipids were being hydrolysed by $sPLA_2$ was investigated further. The main PG species 16:0/18:1 was taken as an index of surfactant PG and 16:0/16:0PC was taken as an indicator of surfactant derived PC. The ratio of the absolute amounts of 16:0/18:1PG to 16:0/16:0PC in all the ARDS patients was compared to the control group (see table 7.1), the hypothesis being that if the ratio of 16:0/18:1PG to 16:0/16:0PC was lower than the control group then $sPLA_2$ might be responsible for the decrease in 16:0/18:1PG by selectively hydrolysing the surfactant PG species. Alternatively, the amount 16:0/16:0PC may have increased, which could occur due to altered metabolism in the type II cells. If the ratio increases in ARDS then either 16:0/18:1PG increases or 16:0/16:0PC decreases therefore suggesting no involvement of $sPLA_2$ and the changes observed must be due to plasma and cell contamination.

To be able to ascertain if the phospholipid changes that occurred in each ARDS patient were due to the involvement of group IIa $sPLA_2$, the tracheal aspirate from each ARDS patient were analysed for the enzyme by SDS-PAGE and western blotting. The detection

limit of the method was 100ng of enzyme/ml of tracheal aspirate. Figure 7.5 shows one of the blots from patients 1 and 2, where 10 and 20µl of tracheal aspirate from each patient was analysed for group IIa $sPLA_2$. Group IIa $sPLA_2$ was detected in the tracheal aspirate from patient 1 but not in the tracheal aspirate from patient 2. Tracheal aspirates from all of the patients were analysed by the same technique and the presence or absence of the enzyme is noted for each patient in table 7.1.

Figure 7.5 Western blot for group IIa $sPLA_2$. Lane 1 and 10 contain molecular weight markers; lanes 2, 3, 4, and 5 were the group IIa $sPLA_2$ standard at 2ng, 10ng, 20ng and 50ng respectively. 20µl and 10µl of tracheal aspirate from patient number 2 were in lane 6 and 7 respectively, while lanes 8 and 9 contained 20 and 10µl respectively of tracheal aspirate from patient number 1.

Due to the diversity of the phospholipid changes and the presence of $sPLA_2$ in only two of the four tracheal aspirates from these ARDS patients, it is important to look at the individual patient details to try and draw conclusions as to whether the changes in phospholipid compositions were due to $sPLA_2$ activity. These comparisons are examples of the type of analysis and not definitive results due to the low numbers of patients on this study.

Patients 1 and 3 both had a lower ratio of 16:0/18:1PG to 16:0/16:0PC than the controls, although not below the normal range it can be interpreted as suggesting that some of the changes observed are due to the action of $sPLA_2$ on the surfactant phospholipids. The

presence of the enzyme in the tracheal aspirates of both these ARDS patients was confirmed by the western blot analysis. The analysis indicated that in these 2 ARDS patients' the increased $sPLA_2$ was likely to be responsible for some of the phospholipid changes and therefore involved in the disease process. The inactivation of the surfactant by $sPLA_2$ would then lead to oedema fluid seeping into the alveolar space and therefore may also account for the presence of plasma and membrane derived PC and PI species in the airways.

Despite patient 2 having a 16:0/18:1PG to 16:0/16:0PC ratio of zero, no evidence for group IIa $sPLA_2$ was seen on the western blot analysis (see figure 7.5). This however, was probably due to the tracheal aspirate fluid being a very diluted sample from a near drowning victim. The very low phospholipid concentration (31nmoles/ml) of the tracheal aspirate from patient 2 when compared to all the other patients (table 7.1) highlights the watery consistency of the sample and the detection of the enzyme may have been outside the limits of the method used. Figure 7.6 illustrates the absence of PG in this patient by comparing the negative ionisation mass spectrum with a purified surfactant mass spectrum from a control subject. The increased amounts of PI that were present in the tracheal aspirate fluid from this patient are likely to be present due to contamination from cells such as neutrophils and a neutrophil mass spectrum is also shown.

Patient 4 is the only ARDS patient to have a higher ratio of 16:0/18:1PG to 16:0/16:0PC than the control subjects. The alterations to the phospholipid composition in this patient were likely to be solely due to infiltration of cell membrane derived PC. This influx of PC species that are characteristically found in cell membranes is illustrated in figure 7.7. The positive ionisation mass spectrum of the tracheal aspirates from patient number 4 is compared with purified surfactant from a control subject and a typical neutrophil mass spectrum. The figure highlights the increase in 16:0/18:1PC and the isobaric components 18:0/18:2 and 18:1/18:1PC in the ARDS patient suggesting that there is contamination of the tracheal aspirate fluid with membrane derived PC.

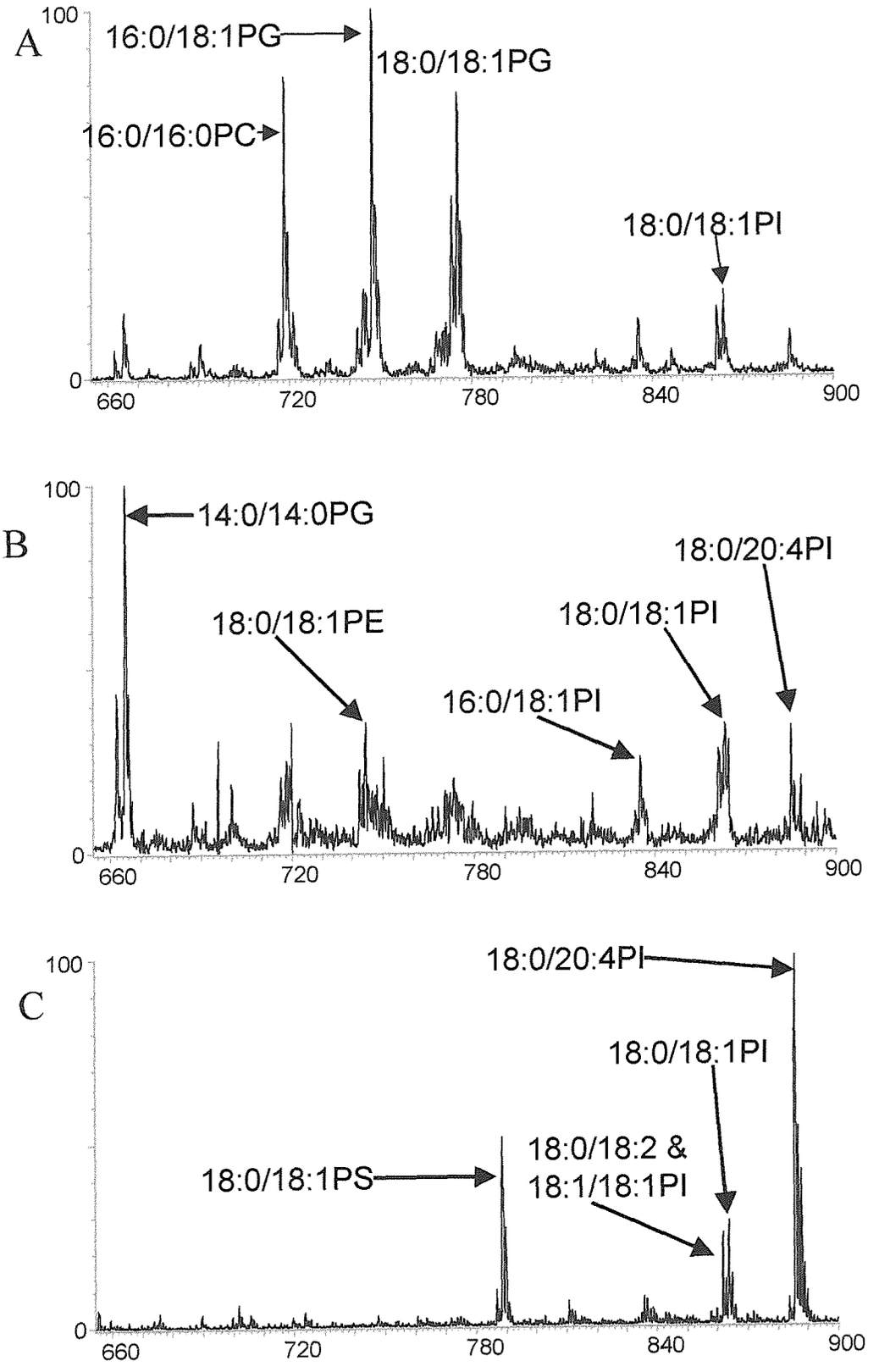


Figure 7.6 Comparison of negative ionisation mass spectra from (A) purified surfactant from a control subject, (B) tracheal aspirate from patient number 2 and (C) a typical neutrophil mass spectrum.

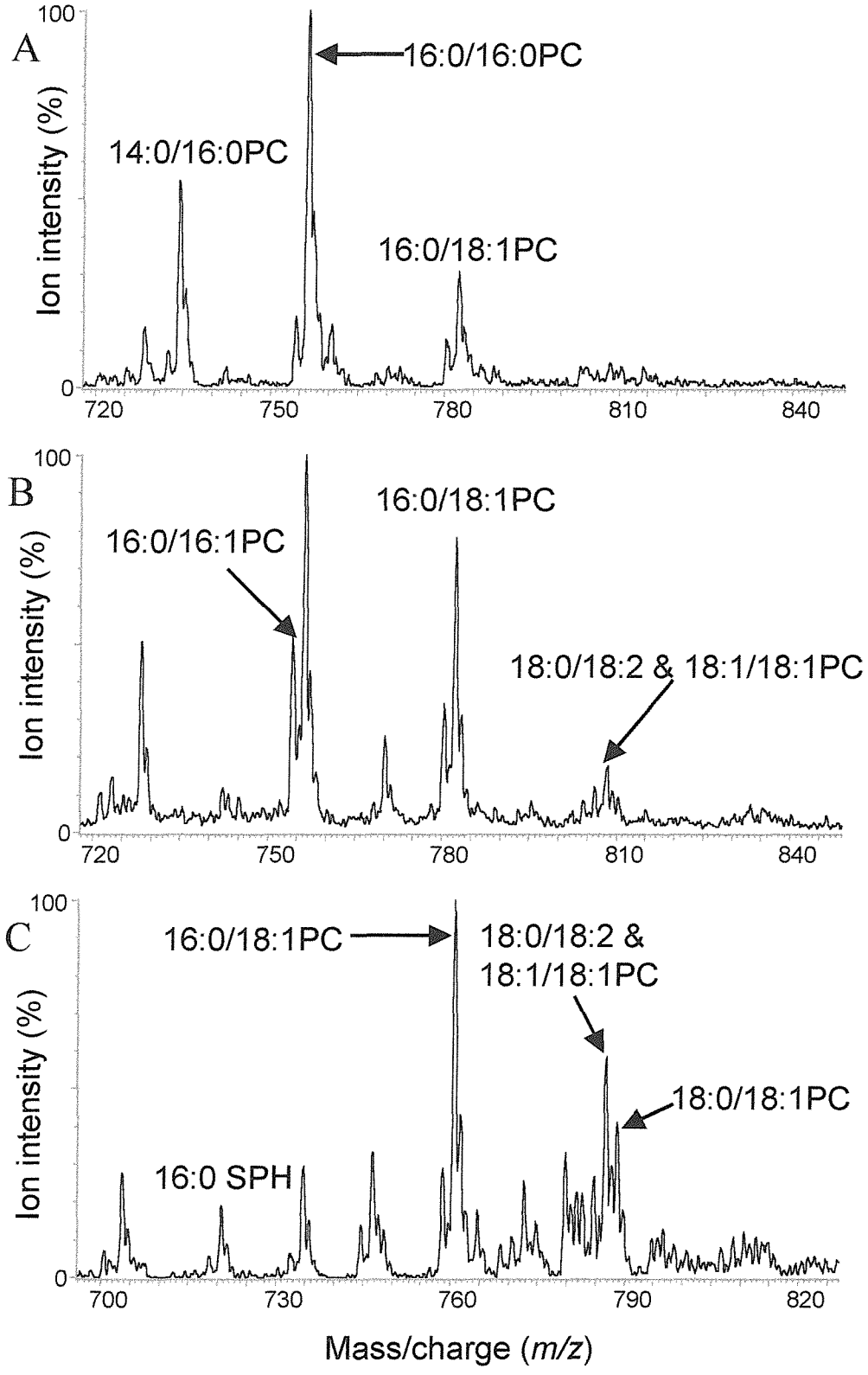


Figure 7.7 Comparison of positive ionisation mass spectra from (A) purified surfactant from control subjects, (B) tracheal aspirate from ARDS patient number 4 and (C) a typical neutrophil mass spectrum.

7.4 Discussion

Results from this small pilot study agreed with previous reports that surfactant is altered in ARDS. The most significant changes were the decrease in the total PG ($p<0.005$) and 16:0/16:0PC ($p<0.005$) along with an increase in total PI (0.005) in the ARDS patients when compared to control subjects. These changes agree with previous reports of decreased saturated PC and total PG along with increased PI, PE and Sph in BALF from ARDS patients (reviewed by Lewis and Jobe 1993 [62]). The use of ESI-MS in this study enabled the identification of the PC, PG and PI molecular species of the tracheal aspirates from the ARDS patients. The molecular species analysis highlighted the increase of PC and PI species such as 16:0/18:1PC ($p<0.05$) and 18:0/20:4PI ($p<0.005$) in the tracheal aspirates from the ARDS patients when compared to the control subjects. These species are characteristically found in cell membranes and confirms that cellular infiltration is part of the disease process.

The main aim of this chapter was to investigate whether the alterations to the phospholipid composition was due to the presence of group IIa $sPLA_2$. The hypothesis was that a lower ratio of 16:0/18:1PG to 16:0/16:0PC, when compared to the control subjects implies the involvement of group IIa $sPLA_2$ at hydrolysing the surfactant phospholipids. The lower ratio was likely to be achieved by group IIa $sPLA_2$ hydrolysing the surfactant PG in preference to the surfactant PC as shown *in vitro* in chapter 5. It is hard to draw conclusions from such a small number of ARDS patients, however it does seem that the hypothesis could be true and further large-scale studies would hopefully confirm this.

This small study does emphasise how the complex nature of ARDS is reflected in the changes observed in each patient with different severity of the disease. In this study the severity of the disease was not assessed, however there is a popular method for assessing the disease severity of ARDS and assigning a "lung injury score" [63]. This score takes into account the extent of roentgenographic densities of the lungs, gas exchange abnormalities expressed as the arterial oxygen tension divided by the fractional concentration of inspired oxygen and the positive end-expiratory pressure required to ventilate the patient. A lung injury score would help address the multifactorial nature of

the disease as even in the ARDS patients who had an increase in the 16:0/18:1PG to 16:0/16:0PC ratio as well as showing the presence of group IIa $sPLA_2$, the increase in the PC/PG ratio was not only due to the action of $sPLA_2$. The presence of membrane-derived phospholipids was also evident in the mass spectra from these patients and this must also have contributed to the increase in the PC/PG ratio. There is the possibility that surfactant was inactivated by $sPLA_2$ leading to infiltration of oedema fluids and cells causing a further increase in the total PC and a worsening of the disease state. However it is probable that the infiltration of cells into the alveolar space is an early disease process, the influx of cells such as alveolar macrophages could then release $sPLA_2$ which would then inactivates the surfactant further and thereby worsening the disease state in a vicious cycle.

In conclusion, these results suggest that the presence of $sPLA_2$ may account for the changes in the phospholipid composition of tracheal aspirates from ARDS patients. ARDS is a complex disease and a proper study into the involvement of $sPLA_2$ in ARDS would require large multi centre study. BALF would need to be collected on the day of admission into ICU then on days 3,5 and10. The severity of the ARDS would need to scored for instance on the Murray lung injury score [63] to then be able correlate any changes in phospholipid composition and PLA_2 concentrations over the course of the disease.

The recognition of the involvement of $sPLA_2$ in the disease process might help to introduce new clinical tools for the treatment of ARDS. The presence of $sPLA_2$ that are capable of hydrolysing surfactant phospholipids might explain why the administration of exogenous surfactant to ARDS patients has failed to improve the clinical symptoms [337]. Therefore the administration of surfactant together with group IIa $sPLA_2$ inhibitors could represent a promising strategy for the treatment of ARDS.

Chapter Eight

General Discussion

8.1 General Discussion

Surfactant phospholipid changes observed in inflammatory lung diseases such as acute asthma and ARDS have been studied in this thesis. The possible involvement of the acute phase protein $sPLA_2$ in these diseases has been previously postulated [4,114,208]. *In vitro* work with purified rabbit surfactant demonstrated that surfactant was susceptible to human group IIa $sPLA_2$ hydrolysis, although this occurred with high concentrations of enzyme and over a three-hour incubation (see chapter 5). The effect of $sPLA_2$-mediated hydrolysis of surfactant phospholipids plays in asthma and ARDS has been discussed.

Inactivation of surfactant has to be part of the asthmatic response, as perfectly functioning surfactant would not permit the mucus plugging and constriction of bronchioles that are characteristic of the acute asthmatic response. The study of BALF phospholipids in the acute asthmatic response revealed the presence of PC species characteristic of plasma derived PC, 24 hours after local allergen challenge in mild asthmatic subjects. The presence of these PC species characteristically found in plasma, in BALF of asthmatic subjects was likely to be due to infiltration of oedema fluid into the airways. The localised acute inflammatory reaction in the bronchioles probably arose through a hyperresponsive reaction to the administered allergen in the asthmatic subjects. This inflammation could have caused the infiltration of oedema fluid and lipoproteins into the airways over a transient period of time. Interactions between surfactant and lipoproteins might impair surfactant function causing the narrowing of the affected bronchioles. Asthma is a disease characterised in the bronchioles and not in the alveoli. Surfactant is secreted from the type II cells in the alveoli and enters the bronchioles via the mucocillary escalator. It is possible that as the inflammation subsides, surfactant from the alveoli may restore airway patency, however some of the plasma derived phospholipids remain during the recovery phase.

From the evidence presented it is unlikely that $sPLA_2$ plays a significant role in the acute asthmatic response in mild asthmatics, possibly due to the enzyme not being present in high enough concentrations. Therefore it is unlikely that the group IIa $sPLA_2$ inhibitor LY311727, would be an effective treatment for acute asthma. However the therapeutic

use of surfactant therapy to treat asthma has not been ruled out and might prove to be beneficial.

ARDS is a multifactorial disease where the alteration to the surfactant phospholipid composition is thought to contribute to the etiology of the disease. It is very likely that group IIa $sPLA_2$ mediated hydrolysis of the surfactant phospholipids contributes at least in part to the disease process. Alveolar macrophages have been shown to be the major pulmonary source of group IIa $sPLA_2$ in an experimental model for ARDS[338]. The inactivation of surfactant causes infiltration of plasma and oedema fluid into the lungs. Plasma from ARDS patients contains increasing levels of $sPLA_2$ that correlate with disease severity. However, the primary cause of the inactivation of surfactant remains unclear. Two major possibilities are firstly $sPLA_2$ released from alveolar macrophages causes the inactivation of surfactant leading to infiltration and then further inactivation in a vicious cycle of events as the patients' lungs fill with fluid and the likelihood of survival decreases. Secondly, surfactant is inactivated by another means and $sPLA_2$ then furthers the inactivation as it infiltrates into the alveolar space. Overall $sPLA_2$ appears to play an important role in ARDS and should be taken into account when designing and administering surfactant therapy.

8.2 Future Work

Over past three years mass spectrometers have developed at a remarkable rate, with dramatic improvements in sensitivity and resolution. For instance, the new Quattro Ultima (Micromass, UK) is an electrospray ionisation triple quadrupole mass spectrometer boasting an improvement in sensitivity of about 80 times over the previous model. These improvements in the machines will enable the method to be enhanced so as to reliably measure phospholipids at even lower concentrations from smaller sample volumes. Tandem MS-MS of the PI species will also be possible. The mass spectrometer may also be used to identify other components in surfactant that may be altered in diseases, such as surfactant proteins and cholesterol. As yet there has not been a study in humans to look at the effect of diet on the surfactant phospholipid and cholesterol composition. Changes in these components might account for the increase in

prevalence in asthma that has occurred with an accompanying fall in the consumption of saturated fat and an increase in the amount of polyunsaturated fat in the diet [87].

Further studies could be carried out *in vitro* into the effect of the group IIa and the newly discovered group V $sPLA_2$ on the surfactant phospholipid composition using ESI-MS. The identification of a standard for lyso-PG would also aid in the quantification of the lyso-PG species produced after hydrolysis. It could prove very interesting to investigate the effect of time on the enzymes activity and to try and determine the rate of the enzymes using the ESI-MS. The effect of surfactant proteins also needs to be investigated and by the removal and addition of surfactant proteins (i.e. SP-A) to study the reported inhibitory effect on group IIa $sPLA_2$ activity.

Perhaps the most interesting and clinically useful investigation would be a multi-centre study into the possible involvement of $sPLA_2$ in the alterations of surfactant observed during ARDS. The study would need to be well co-ordinated and samples (small volume bronchoalveolar lavages) collected at set time periods e.g. 0, 3,5 and 10 days after admission into intensive care. The severity of the patients disease would need to be assessed uniformly by a score such as the Murray score [63]. Samples could then be analysed for surfactant phospholipids and $sPLA_2$ as well as inflammatory markers such as TNF-α. An ideal study would involve uniform treatment of the patients, however this is ethically unsound, as the best possible available treatment should be given to all patients. Therefore the use of an animal model of ARDS such as the rabbit injected with LPS to induce ARDS would be a good model to develop a surfactant therapy giving surfactant with the $sPLA_2$ specific inhibitor LY311727 and possibly surfactant proteins such as SP-A. SP-A has been reported to inhibit group IIa $sPLA_2$ and the degradation of SP-A by neutrophil elastase during ARDS may be another crucial factor in the disease process. The break down of SP-A may leave surfactant more susceptible to $sPLA_2$ mediated hydrolysis.

The development of a surfactant therapy specifically for ARDS patients where the components of surfactant that are altered during the disease process are replaced could prove to be a very useful treatment for such a severe disease. An appropriate surfactant

therapy may enable normal lung function to be restored and then the underlying cause for the respiratory failure such as severe burns, pancreatitis and gun shot wounds could then be treated. The only drawback to developing a surfactant therapy is the extortionate cost of surfactant therapy, for instance Survanta® (a bovine lung surfactant extract) used to treat neonatal RDS is instilled by endobronchial tube at a dose of 100mg of phospholipid/kg and costs £300 per 200mg of phospholipid [339]. The dose may be repeated and the cost of a single dose to an adult weighing 70Kg would be over £10,000. Therefore it would also be necessary to develop a means of manufacturing surfactant with all the extra components in at a reduced cost so the treatment would then be widely available.

In summary, the role of $sPLA_2$s in human physiology remains unclear and hence further detailed studies are required to determine the effect of this type of enzyme on lung surfactant and the role of such effects in inflammatory lung disorders.

Chapter Nine

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Appendices

Appendix 1

A macro was written in visual basic for use in Microsoft excel. Below is part of the macro; section 1 involves inputting a filename to retrieve the data from and then pasting this filename at the top of the column in the results sheet which in this example is "pgres.xls". In section 2 the macro searches for the specified *m/z* value (the first value in this case is 665) and then copies the corresponding intensity and pastes it into the results sheet, section 2 can be repeated as many times as required. Finally section 3 returns the active cell in the results sheet ("pgres.xls") to the start of the next column so the procedure can be repeated.

```
Sub Macro2()
    filename = InputBox( _
        prompt:="Please enter filename", _
        default:=" ")
1   Windows("pgres.xls").Activate
    ActiveCell.FormulaR1C1 = filename
    ActiveCell.Offset(1, 0).Range("A1").Select
    Windows(filename).Activate
    Cells.Find(What:="665", After:=ActiveCell, LookIn:=xlValues, _
        LookAt:=xlPart, SearchOrder:=xlByRows, SearchDirection:=xlNext _
        , MatchCase:=False).Activate
    ActiveCell.Offset(0, 1).Range("A1").Select
2   Selection.Copy
    Windows("pgres.xls").Activate
    ActiveCell.Select
    ActiveSheet.Paste
    Windows(filename).Activate
    ActiveCell.Offset(0, 3).Range("A1").Select
    Cells.Find(What:="719", After:=ActiveCell, LookIn:=xlValues, _
        LookAt:=xlPart, SearchOrder:=xlByRows, SearchDirection:=xlNext _
        , MatchCase:=False).Activate
    ActiveCell.Offset(0, 1).Range("A1").Select
2 repeat
    Selection.Copy
    Windows("pgres.xls").Activate
    ActiveCell.Offset(1, 0).Range("A1").Select
    ActiveSheet.Paste
    Windows(filename).Activate
    ActiveCell.Offset(0, 3).Range("A1").Select
3   Windows("pgres.xls").Activate
    ActiveCell.Offset(-3, 1).Range("A1").Select
```