The Vasoactive Potential of Lysophosphatidylcholine, a Product of Surfactant Degradation in the Lung, on the Pulmonary Vasculature Following Exogenous Surfactant Replacement Therapy for Neonatal Respiratory Distress Syndrome

Jason E. Duncan

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Physiology, Faculty of Medicine
University of Manitoba
Winnipeg, Manitoba

ISBN 0-612-13097-5

Name JASON EDWARD DUNCAN

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

BIOLOGY - PHYSIOLOGY
SUBJECT TERM

0 4 3 3
SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS
Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION
General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS
Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES
American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES
Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES
Pure Sciences
Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences
Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY
General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

THE VASOACTIVE POTENTIAL OF LYSOPHOSPHATIDYLCHOLINE, A PRODUCT OF SURFACTANT DEGRADATION IN THE LUNG, ON THE PULMONARY VASCULATURE FOLLOWING EXOGENOUS SURFACTANT REPLACEMENT THERAPY FOR NEONATAL RESPIRATORY DISTRESS SYNDROME

BY

JASON E. DUNCAN

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Acknowledgements

Completion of this research project would not have been feasible without the assistance of many individuals. I would like to thank my supervisor, **Dr. Jaques Belik**, Department of Physiology and Pediatrics, for providing a well planned research project and the support and guidance to ensure its completion. I would also like to thank **Dr. Grant Hatch**, Department of Internal Medicine, Biochemistry and Molecular Biology, for his keen interest in my project and for allowing me to perform much of the biochemical work herein in his laboratory. **Dr. Newman Stephens**, Department of Physiology, also provided me with the opportunity to complete portions of this project in his laboratory, and I am much obliged for his generosity and kindness in this regard. I am also indebted to Dr. Hatch and Dr. Stephens for fulfilling my seemingly endless requests for reference letters, and for their constructive criticism and critique of this thesis manuscript.

The assistance of members of the Section of Neonatology support staff at the Health Sciences Centre including **Paula Raposo**, **Irene Tatsumi** is greatly appreciated. I am especially grateful for the personal assistance of secretary extraordinaire **Pat MacIntosh**. I would also like to acknowledge **Judy Olfert** and **Gail McIndless**, Department of Physiology, for their exceptional secretarial support.

I must acknowledge the technical assistance of my laboratory coworkers including **David Wilson**, **Xiaoling Su**, **Nadene Shirtliff**, **Jacqie Fischer** and **Viciany Fabris**. Without their technical assistance and expertise much of this research could not have been completed.

I would like to acknowledge **Patti Rempel**, **Shannon Lidfors** and **Heather Jenkins**, Department of Physiotherapy and Pediatrics, for their commitment and dedication in collecting and providing me with all the tracheo-bronchial secretion samples from the Neonatal Intensive Care Unit, Health Sciences Centre. I would also like to acknowledge **Dr. Terry Dick**, Department of Zoology, for providing the facilities and equipment necessary for all histological work performed.

I am indebted to the **Manitoba Lung Association** for the benevolent financial support in the form of a Scholarship over the period of study and **The Childrens Hospital Research Foundation of Winnipeg** for their gracious funding of this project. I am also indebted to the **Faculty of Graduate Studies**, the **Graduate Students Association** and the **Alumni Association of the University of Manitoba** for providing Travel Awards for presentation of this research at numerous International Meetings.

During the period of my program in the Department of Physiology, I have made many friends. These include **Dr. Carla Stephens**, **Danny Stephens**, **He Jiang**, **Gang Liu**, **Xiufei Ma**, **Jizhong Wang**, **Ramamohan Suhrabi**, **Weilong Li**, **Tao Fan** and **Phillip Cheng**. I thank you all for your support and friendship. I thank **Andrew Halayko**, not only for his friendship, but also for his technical wizardry both in the laboratory and on the ice rink. I also thank him for the colourful and stimulating discussions we had over many, many lunch hours. I must also thank the wise and philosophical **Anindo Choudhury**, not only for his friendship but especially for his confidence and belief in me.

I thank my family for their support and understanding of my commitment and dedication to this research. My sisters, **Justine** and **Jennifer**, have been very supportive of my research and very tolerant of my erratic behaviour during this period. I especially thank my parents, **Donald** and **Suzanne Duncan** for their emotional support and unconditional love. My parents have always stressed the importance of education and have provided me, always at great personal sacrifice but without question, the opportunity to pursue my goals and aspirations. I humbly dedicate this thesis to them both.

Table of Contents

Secretory Phospholipase A_2 (PLA_2) Deacylation of Surfactant Phosphatidylcholine (PC)

Secretory Phospholipase A_2 (PLA_2) Activity in Tracheo-Bronchial Secretion (TBS) Samples

List of Figures

List of Tables

Abbreviations

AA - Amino acid
ACh - Acetylcholine
ANOVA - Analysis of variance
ARDS - Adult respiratory distress syndrome
BAL - Bronchoalveolar lavage
CB - Composite body
cc - Cubic centimetre
CDP - Cytidine diphosphate
cGMP - cyclic guanosine monophosphate
cm - centimetre
CMP - Cytidine monophosphate
dd - double distilled
DMSO - Dimethyl sulfoxide
DPPC - Dipalmitoyl phosphatidylcholine
EDNO - Endothelium derived nitric oxide
+Endo - Endothelium intact
-Endo - Endothelium denuded
ER - Endoplasmic reticulum
FIO_2 - Fractional concentration of inspired oxygen
GC - Guanylate cyclase
HMD - Hyaline membrane disease
Hz - Hertz
IP_3 - Inositol 1,4,5-triphosphate
kDa - kiloDalton
LAT - Lysophosphatidylcholine acyltransferase
LB - Lamellar body
L-NMMA - N^G-monomethyl-L-arginine
LPC - Lysophosphatidylcholine
M - Molar
MANOVA - Multiway analysis of variance
MAP - Mean airway pressure
MAS - Meconium aspiration syndrome
MB - Methylene blue
µg - microgram
mg - milligram
min - Minute
mm - millimetre
mN - milliNewton
MVB - Multi vesicular body
NICU - Neonatal intensive care unit
NO - Nitric oxide
NOS - Nitric oxide synthase

NRDS - Neonatal respiratory distress syndrome
PC - Phosphatidylcholine
PDA - Patent ductus arteriosus
PE - Phenylephrine
PG - Phosphatidylglycerol
PLA_2 - Phospholipase A_2
pmole - picomole
PPHN - Persistent pulmonary hypertension of the newborn
PVR - Pulmonary vascular resistance
RDS - Respiratory distress syndrome
RER - Rough endoplasmic reticulum
SEM - Standard error of the mean
sGC - Soluble guanylate cyclase
SP-A - Surfactant protein A
SP-B - Surfactant protein B
SP-C - Surfactant protein C
SP-D - Surfactant protein D
TBS - Tracheal bronchial secretion
TLC - Thin layer chromatography
TM - Tubular myelin
TBS - Tracheo-bronchial secretion

Abstract

Studies were conducted to determine the vasoactive potential of lysophosphatidylcholine (LPC), a product of phospholipase A_2 (PLA_2) catalyzed deacylation of surfactant phosphatidylcholine (PC), to mediate a decrease in pulmonary vascular resistance following exogenous surfactant replacement therapy in neonates with respiratory distress syndrome (NRDS). It is hypothesized that LPC, present in exogenous surfactant and generated in the lung following surfactant administration, crosses the epithelial-endothelial barrier of the premature lung to mediate vasodilation of the pulmonary vasculature accounting for the clinically observed decrease in pulmonary vascular resistance reported following exogenous surfactant replacement therapy.

In vitro smooth muscle mechanics studies have demonstrated that LPC is an equally effective endothelium-dependent vasorelaxant of the pulmonary vasculature of both adult and newborn guinea pigs. LPC induced vasorelaxation is mediated by an increase in intracellular smooth muscle cGMP, a result of both enhanced guanylate cyclase (GC) and nitric oxide synthase (NOS) activity. In the newborn guinea pig, however, LPC induced vasodilation is mediated to a greater extent by the NOS pathway compared to the adult which is more dependent on the GC pathway.

Studies addressing the susceptibility of the PC component of the exogenous surfactant preparations Exosurf® and Survanta® to secretory-type PLA_2 deacylation *in vitro* revealed a pronounced difference. The PC component of the Survanta® preparation was readily deacylated by secretory PLA_2 yielding LPC whereas in the Exosurf® preparation the dipalmitoyl phosphatidylcholine (DPPC) component was completely resistant to degradation. The absence of PLA_2 mediated degradation was due to the presence of tyloxapol, a non-ionic inhibitor of secretory PLA_2, added to the Exosurf® preparation to facilitate rapid dispersion of DPPC in the lung following administration.

Analysis of secretory-type PLA_2 activity in tracheo-bronchial secretion (TBS)

samples from neonates in the intensive care unit revealed that, although there was no significant difference in average protein content between TBS samples from NRDS and Non-NRDS neonates, NRDS infants demonstrated a significantly increased average PLA_2 activity and standardized PLA_2 activity ($p<0.05$). Lung PLA_2 activity was also determined to be significantly increased and a possible early marker for NRDS in severely premature infants (24-29 weeks gestational age).

In vivo lung-perfusion studies on adult guinea pigs demonstrated that intra-vascular administration of LPC mediated vasodilation of the pulmonary vascular bed, confirming the results observed *in vitro*. The intra-tracheal administration of LPC, however, resulted in vasodilation of the pulmonary vasculature and decreased pulmonary arterial pressure once in four preliminary trials conducted. This preliminary observation contradicts the proposed hypothesis that LPC crosses the epithelial-endothelial barrier to mediate pulmonary vasorelaxation but does not negate the possibility that LPC is responsible for the decrease in pulmonary vascular resistance observed in infants following surfactant administration. The lack of sufficient epithelial damage in our adult model, as observed for infants in the clinical arena, as a consequence of the ventilation of surfactant deficient lungs, may have allowed for decreased lung permeability and prevented diffusion of LPC across the epithelial-endothelial barrier.

These studies have established the vasoactive potential of LPC on the pulmonary vasculature, *in vitro* and *in vivo*, as well as the susceptibility of exogenous surfactant PC to be degraded to LPC by secretory PLA_2 activity. A significant increase in secretory PLA_2 activity of TBS samples was observed in neonates with NRDS, the infants most likely to receive exogenous surfactant replacement therapy. Additional trials addressing the effect of intra-tracheal adminstration of LPC on the pulmonary vasculature of a newborn, NRDS deficient animal model with equivalent lung epithelial damage as observed in clinical infants are required to completely satisfy and confirm the aforementioned hypothesis.

Foreword

The introduction of exogenous surfactant therapy as a treatment for neonatal respiratory distress syndrome (NRDS) has led to a revolution in neonatal intensive care (Corbet and Long, 1992). Although a significant reduction in neonatal morbidity and mortality has been noted since its inception, numerous concerns have been raised with regard to which exogenous surfactant preparation should be employed, how it should be administered, its composition and the disparity between measured *in vitro* biophysical activity, as determined by surface tension lowering properties, and reported *in vivo* efficacy. Studies addressing these concerns have revealed numerous differences between exogenous surfactant preparations, such as the rapid onset of action of naturally derived surfactant preparations following adminstration (Horbar *et al.*, 1993). Although once thought to be a beneficial quality, rapid onset of action may be detrimental to the immature neonate by rapidly shunting blood from the brain to the lung (Long, 1993a). As well, there is a growing concern regarding the possible transmission of infectious agents, such as bovine spongiform encephalitis, from naturally derived surfactant preparations to treated individuals (Long, 1993a) as well as the possible development of antibodies to protein components of these preparations, which may later inhibit endogenous surfactant development (Holm and Waring, 1993). In this regard, synthetic surfactants seem the logical choice for the treatment of NRDS, however, they seem to lack the *in vitro* qualities of naturally derived surfactant preparations and may themselves contain potentially harmful components.

Current trends in surfactant research are directed at developing "3rd generation" surfactant preparations, termed designer surfactants, which would combine the most beneficial properties of both naturally derived, "1st generation" surfactant preparations and

synthetic, "2nd generation" surfactant preparations (Holm and Waring, 1993). A comprehensive understanding of the pulmonary surfactant system, particularly the fate of administered exogenous surfactant components, is paramount in the achievement of this goal.

It is the purpose of this research thesis to address the vasoactive potential of lysophosphatidylcholine (LPC), a component of administered exogenous surfactant and a product of surfactant degradation in the lung, following exogenous surfactant administration. The objectives of the present study were to: 1) determine the vasoactive potential of LPC on the pulmonary vasculature *in vitro*, 2) determine the susceptibility of exogenous surfactant to secretory-type phospholipase A_2 (PLA_2) activity *in vitro*, 3) measure and quantify lung PLA_2 activity in tracheo-bronchial secretion samples from premature infants and 4) determine the vasoactive potential of LPC *in vivo* and the potential of LPC to cross the lung epithelial-endothelial barrier to mediate a vasoactive response on the pulmonary vasculature. An understanding of the vasoactive potential of LPC on the pulmonary vasculature following surfactant administration may assist in the development of future designer surfactant preparations, especially those developed for target patient populations such as term newborns with persistent pulmonary hypertension syndrome (PPHN).

Literature Review

Introduction

During respiration the exchange of gas is mediated across an air-liquid interface (Hill and Wyse, 1989). The broncho-alveolar units of the lung represent the only internal site within the mammalian body where this interface is present (King, 1984). There is a great discontinuity in energy at this interface, the free energy of the alveolar surface being much greater than the free energy of the two bordering phases; the liquid film which lines the alveoli and the air in the alveoli itself (King, 1982). Pulmonary surfactant, a lipo-protein complex which forms a monolayer lining the inner surface of the lung, acts to reduce surface tension in the alveoli by reducing the transmural pressure required to keep alveoli inflated, thus mediating the large discontinuity in free energy existing at the alveolar surface (Clements, 1977; King, 1984). Surfactant is a contraction of the term "surface active agent".

Neonatal respiratory distress syndrome (NRDS), a condition affecting 30,000 - 50,000 prematurely born babies in North America yearly and associated with a high degree of morbidity and mortality, is caused by a deficiency or impairment of pulmonary surfactant synthesis and/or secretion resulting in decreased lung compliance and altered pulmonary function (Mines, 1993). NRDS infants must expend tremendous amounts of energy to inflate their stiff lungs, and following inflation the lungs tend to deflate very quickly and to abnormally low volumes (Mines, 1993). As well, alveoli of differing sizes are not stabilized and smaller alveoli tend to collapse and become airless after inspiration

(Mines, 1993), emptying their contents into larger alveoli. NRDS is also associated with the maintenance of an abnormally high pulmonary vascular resistance after birth (Kääpä *et al.*, 1993). Gas diffusion becomes compromised as alveolar membranes become thickened and fibrinous in a condition secondarily referred to as hyaline membrane disease (HMD). As a result of the surfactant deficiency, the infant becomes hypoxic, hypercapnic, acidotic, and exhausted (Mines, 1993).

NRDS occurs primarily as a consequence of lung immaturity as type-II epithelial cells, alveolar epithelial cells of the lung responsible for surfactant production and secretion, have not completely developed and do not adequately synthesize and release sufficient quantities of pulmonary surfactant following birth (Avery and Mead, 1959). NRDS may be secondarily caused by a contamination of the functional surfactant monolayer with serum proteins, such as albumin, which leak into the lung and perturb proper surfactant function (Ikegami *et al.*, 1984).

Surfactant production begins *in utero*, components first appearing on the fetal alveolar surface at 25 weeks gestation (Mines, 1993) and detectable in amniotic fluid samples in amounts which change towards the end of gestation in a manner related to fetal lung maturity (Cosmi and DiRenzo, 1988). Indeed, the incidence of NRDS increases dramatically as an infants gestational age at birth decreases. Infants born at 30-32 weeks gestational age experience a 20% incidence rate of NRDS, whereas infants born at 26-28 weeks gestational age experience a 60-80% rate of incidence (Jobe and Ikegami, 1987).

The mortality of infants with NRDS has decreased dramatically in recent years concomitant with recent advances in fetal monitoring and new therapies for the treatment of neonatal respiratory failure, including the accurate prediction of fetal lung maturity (lecithin/sphingomyelin ratio) (Gluck *et al.*, 1971), improved amniocentesis diagnostics

(Hallman, 1984), and enhancement of fetal surfactant synthesis and secretion via antenatal maternal glucocorticoid therapy (Hawkins and DiRenzo, 1986). No treatment for NRDS, however, has had the impact of exogenous surfactant replacement therapy, first proposed by Enhorning *et al.* (1978) and successfully tested on humans by Fujiwara *et al.* (1980). Since its inception, exogenous surfactant therapy has revolutionized neonatal intensive care and the treatment of premature infants and is regarded as the single greatest development in neonatology (Dunn, 1994). Following the inception of exogenous surfactant replacement therapy for treatment of NRDS in 1990, the United States observed the largest decrease in infant mortality ever recorded (Long, 1993b).

Pulmonary Surfactant Secretion and Clearance

The normal metabolism, secretion and clearance of pulmonary surfactant has been detailed in several exceptional reviews (Ballard, 1989; Batenburg, 1992; Jobe, 1994; Possmayer, 1989; Stevens *et al.*, 1989; Van Golde, 1976; Wright, 1990; Wright and Clements, 1987; Wright and Hawgood, 1989; Wright and Dobbs, 1991). Epithelial type-II cells (granular pneumocytes) of the alveoli are responsible for the synthesis, storage, secretion and clearance of pulmonary surfactant (Batenburg and Van Golde, 1979; Van Golde, 1976) (Figure 1). Type II cells are cuboidal epithelial cells which account for 15% of all cells of the lung (40 cell types) (Crapo *et al.*, 1983), yet cover <5% of the alveolar surface area (Dobbs, 1990). Surfactant lipids and proteins are synthesized in the rough endoplasmic reticulum (RER) and assembled in the Golgi apparatus. Lipids are specifically packaged into small lamellar bodies (LB) (Chevalier and Collet, 1972) and proteins into multivesicular bodies (MVB), where they are both transported to growing LB's. The mature LB's contain phospholipids, surfactant proteins A (OReilly *et al.*, 1988;

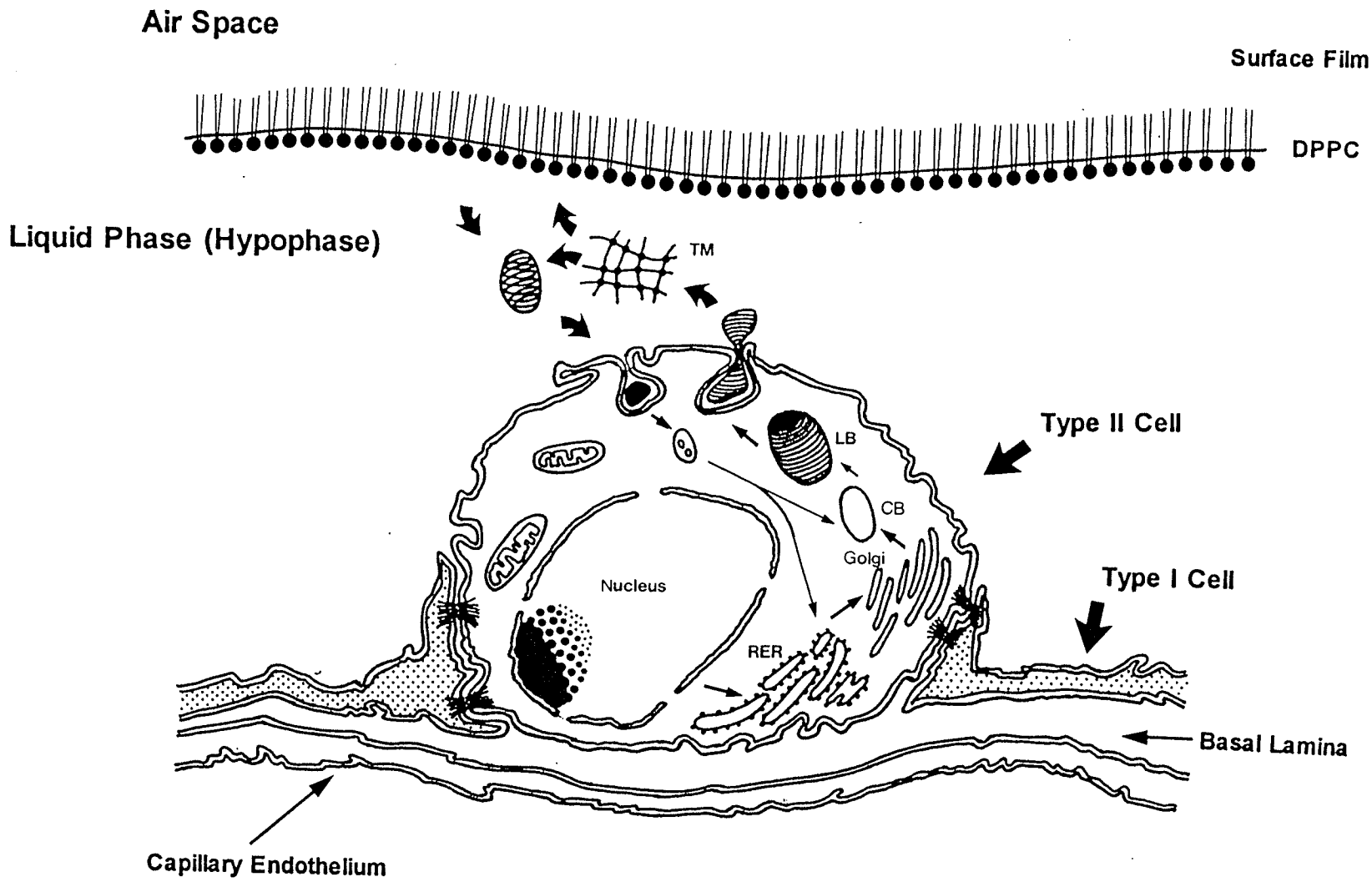

Figure 1. Secretion and Clearance of Pulmonary Surfactant. Schematic diagram of the synthesis, secretion and clearance of pulmonary surfactant components by type II epithelial cells of the alveoli. Surfactant components are synthesized in the rough endoplasmic reticulum (RER) and packaged in the Golgi apparatus and secreted as lamellar bodies (LB) by exocytosis. In the alveolar microenvironment, lamellar bodies undergo an extracellular conversion to tubular myelin (TM) the form which contributes to the generation of the surfactant monolayer. Expended surfactant can be internalized and degraded and reutilized or recycled to the surfactant monolayer (after Wright and Clements, 1987; Van De Graaff and Fox, 1989).

Walker *et al.*, 1986), B (Weaver and Whitsett, 1989) and hydrolytic enzymes (Chander and Fisher, 1990) in an acidic environment (Chander *et al.*, 1986). The hydrolytic enzymes are isolated to the cylindrical core of the LB (Hook and Gilmore, 1982) from which phospholipid lamellae originate and appear to wrap around (Scarpelli, 1990). The presecretory LB's also act as temporary storage vesicles for lipid and protein material and are eventually extruded by exocytosis into the extracellular fluid of the alveolar microenvironment. The secretion of surfactant by type-II cells is regulated locally by increased tidal volumes, effecting a mechanical or shear stress on the epithelial cell layer, and by increased ventilation rates, possibly altering intracellular pH (alkalosis) (Chander and Fisher, 1990). Secretion is also stimulated by agonists for β-adrenergic, purinoceptors and vasopressin receptors (Chander and Fisher, 1990). Through an extracellular conversion process in the alveolar subphase, the contents of the LB's once extruded expand to form tubular myelin (TM) (Williams, 1977), a liquid crystal lattice-like structure which is the contributing form to the generation of a surfactant monolayer (Gil and Reiss, 1973). The mechanism which initiates this conversion presently remains unclear, but observations *in vitro* suggest the requirement for calcium (Gil and Reiss, 1973; Sanders *et al.*, 1980). The processes which mediate the transformation of PC from TM to the surfactant monolayer also remain unclear, but it appears that phospholipids other than dipalmitoyl phosphatidylcholine (DPPC) and phosphatidylglycerol (PG) are excluded.

Surfactant is potentially cleared from the alveolar microenvironment by uptake into epithelial cells, including type-I, type-II or tracheal epithelial cells, alveolar macrophages and diffusion into the blood through the epithelial-endothelial barrier (Wright, 1990). The uptake of surfactant by type II epithelial cells constitutes the major route of surfactant

clearance (Wright and Clements, 1987). Surfactant internalized by type-II cells via endocytosis is either degraded and reutilized for synthesis of new lipids or recycled intact by incorporation into lamellar bodies for resecretion (Hallman *et al.*, 1981). Factors which determine the rate of surfactant internalization by type-II cells are not completely clarified but include the surfactant proteins (Wright, 1990), lipids (Chander *et al.*, 1983), and known stimuli of surfactant secretion including increased tidal volume and ventilation rates (Oyarzún *et al.*, 1980).

Composition of Pulmonary Surfactant

The general chemical composition of natural pulmonary surfactant is quite similar in all mammalian models studied, being comprised of approximately 2% carbohydrate, 8% protein and 90% lipid (Harwood *et al.*, 1975; Possmayer, 1984).

Lipid Component

Approximately 80-90% of the mass of pulmonary surfactant is composed of lipids including phospholipids and neutral lipids (King and Clements, 1972). Most of the phospholipid component is comprised of disaturated phosphatidylcholine (70-80%) with smaller amounts of PG, phosphatidylethanolamine, phoshatidylinositol, phosphatidylserine, and sphingomyelin present (Possmayer, 1984). Of the PC component, 50-70% by weight consists of DPPC, the major surface active component of pulmonary surfactant. DPPC consists of a small, polar, hydrophilic phosphocholine head and a nonpolar, hydrophobic tail of two saturated palmitate residues bound to a glycerol backbone (Figure 2). PG, the second most abundant phospholipid in surfactant accounting for 10% of the total lipid (Rooney *et al.*, 1974) acts to facilitate clearance of DPPC from the airspace (Oyarzún *et*

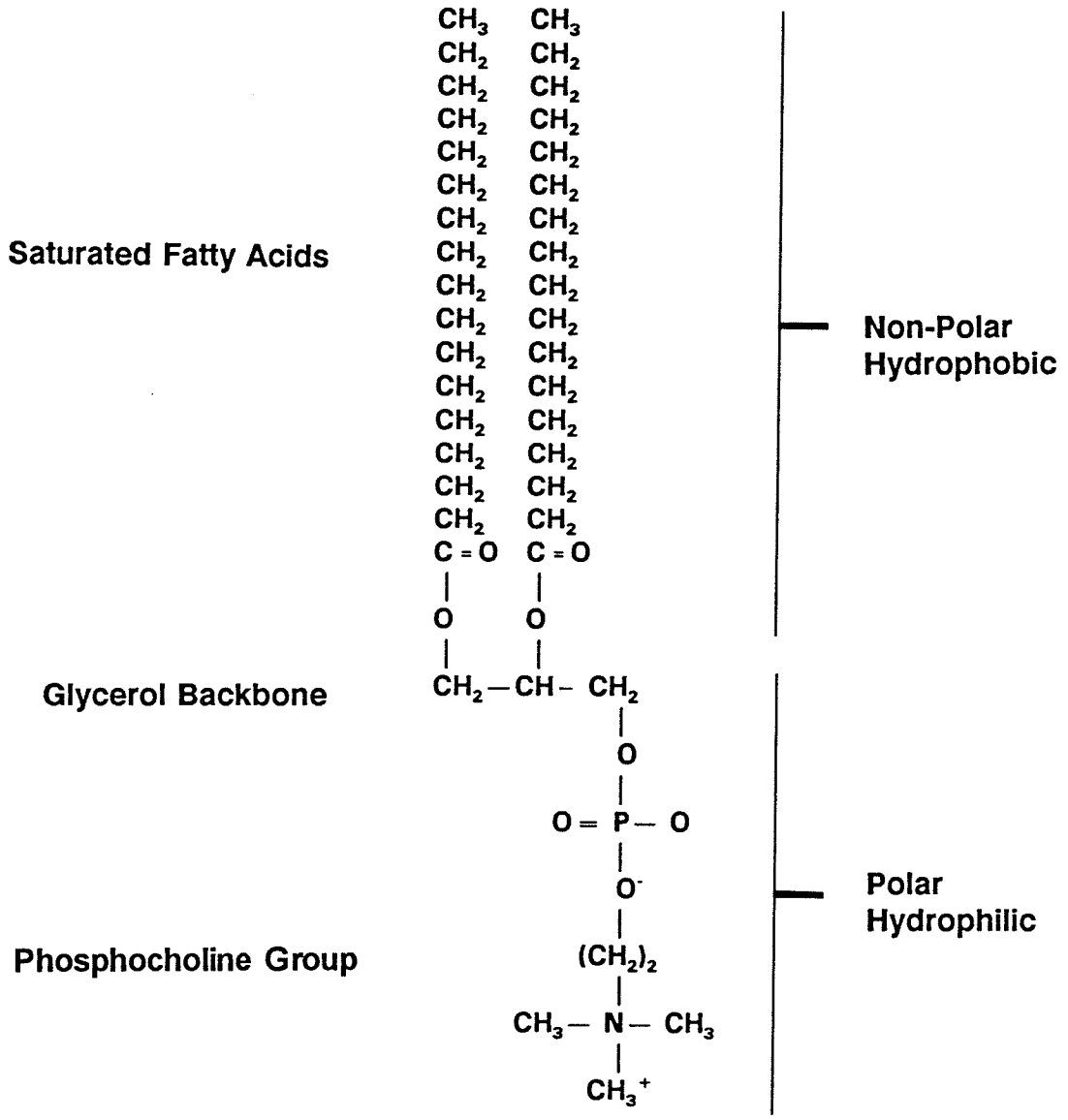

Figure 2. Structure of Dipalmitoyl Phosphatidylcholine (DPPC). DPPC consists of a glycerol backbone to which is esterified 2 saturated, oily, water insoluble palmitate residues and a charged, water soluble, hydrophilic phosphocholine group (after Mines AH, 1993).

al., 1980) by stimulating lipid uptake by type-II cells. Neutral lipid components include cholesterol which accounts for less than 5% of surfactant mass and diacylglycerol which accounts for less than 1% (King and Clements, 1972).

Synthesis of Dipalmitoyl Phosphatidylcholine (DPPC)

PC, the most investigated surfactant phospholipid, is synthesized *de novo* almost exclusively by the incorporation of choline and phosphatidic acid in the cytidine diphosphate (CDP)-choline pathway (Ballard, 1989; Possmayer, 1989) (Figure 3). Choline is phosphorylated by choline kinase (EC 2.7.1.32) and activated via attachment of cytidine 5'-diphosphate (CDP) in a process mediated by choline-phosphate cytidylyl transferase (EC 2.7.7.15) yielding CDP-choline (Batenburg, 1992). Phosphatidic acid is hydrolysed to diacylglycerol by the action of phosphatidate phosphatase (EC 3.1.3.4). PC is synthesized from both diacylglycerol and CDP-choline in a reaction catalyzed by choline phosphotransferase (EC 2.7.8.2). Investigators addressing observed differences in the rate of incorporation of radiolabeled palmitate residues into disaturated diacylglycerol and DPPC suggested that not all surfactant DPPC is formed entirely *de novo*, as described above, but is a product of PC remodelling as well (Post *et al.*, 1983). PC remodelling begins with the deacylation of an unsaturated fatty acid residue at the 2-position of a PC molecule containing a palmitoyl moiety at the 1-position by PLA_2 (EC 3.1.1.4) yielding 1-palmitoyl LPC. Reacylation of the 2-position is achieved with palmitoyl-CoA in a reaction catalyzed by acyl Coenzyme A:LPC acyltransferase (LAT) (EC 2.3.1.23) or by transacylation of two 1-palmitoyl LPC molecules in a reaction catalyzed by LPC:LPC acyltransferase (Batenburg, 1992). Type-II cells are highly enriched in LAT and the LAT pathway is postulated to be the most important in the remodelling of PC (Van Heusden

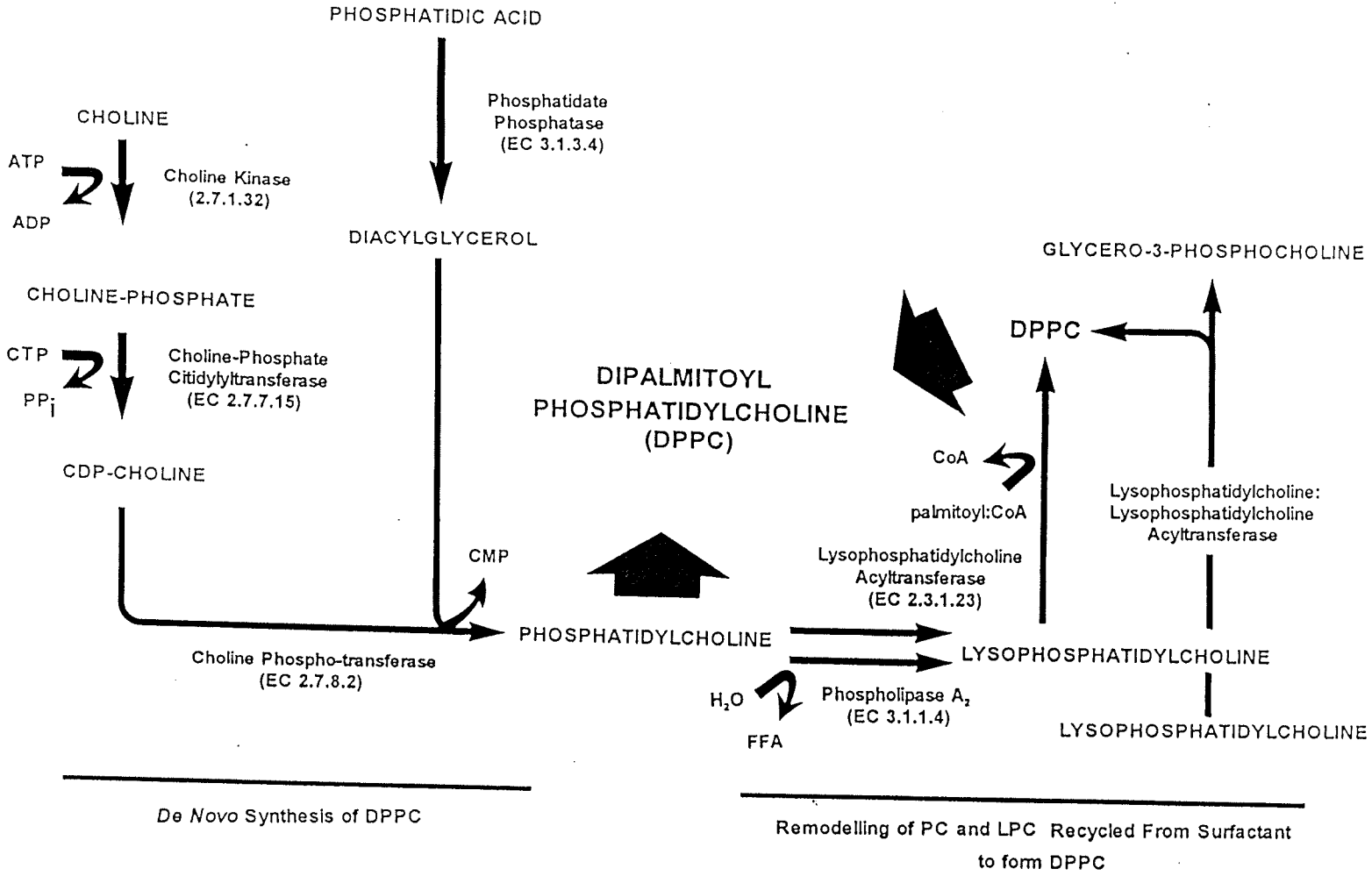

Figure 3. Synthesis of Dipalmitoyl Phosphatidylcholine (DPPC) by Type II Epithelial Cells. The synthesis of DPPC by type II epithelial cells occurs either *de novo* through the cytidine diphosphate (CDP)-choline pathway or by the remodelling of unsaturated phosphatidylcholine (PC) synthesized *de novo* and recycled from PC and lysophosphatidylcholine recycled by type II cells. *De novo* synthesis of DPPC accounts for 45% of secreted DPPC and remodelling accounts for the remaining 55% (after Batenburg, 1992).

et al., 1980). The *de novo* pathway of DPPC synthesis accounts for 45% of secreted surfactant DPPC whereas remodelling accounts for 55% (Den Breejen *et al.*, 1989).

Protein Component

Proteins account for approximately 10% of the mass of pulmonary surfactant and include both serum and non-serum proteins (Whitsett, 1994). It is not clear if serum proteins are secreted by LB's or are present in alveoli as a result of leakage into air spaces. Four non-serum proteins, however, are unique surfactant components and play an essential role in the metabolism and dynamics of pulmonary surfactant (Van Golde *et al.*, 1994). These surfactant proteins have been the subject of several recent reviews (Hawgood, 1989; Possmayer, 1988; Weaver, 1988; Weaver and Whitsett, 1991).

Surfactant Protein-A

The hydrophilic glycoprotein surfactant protein A (SP-A) (King, 1974; King and Clements, 1972; King *et al.*, 1973), the most abundant of the surfactant proteins, consists of 18 monomers (26-36 kDa) arranged as a sulfhydryl dependent oligomer of six triple helices (650 kDa) (Whitsett, 1994). Similar SP-A products are encoded by two different mRNAs (Floros *et al.*, 1985) and undergo considerable post-translational modifications in the RER including siacylation (Katyal and Singh, 1984), acetylation (Floros *et al.*, 1985), sulfation (Weaver *et al.*, 1982), glutamate carboxylation (Rannels *et al.*, 1987) and glycoslation (Whitsett *et al.*, 1985). SP-A has been localized to the Golgi apparatus, RER and LB's of epithelial type-II cells, and is also produced and secreted by epithelial Clara cells (Walker *et al.*, 1986; Wright and Clements, 1987).

In the alveolar microenvironment SP-A is postulated to function in feedback

inhibition of surfactant phospholipid metabolism, inhibiting the secretion of DPPC from type-II cells (Dobbs et al., 1987; Kuroki *et al.*, 1988) and enhancing the uptake of surfactant liposomes by type-II cells (Rice *et al.*, 1989; Wright *et al.*, 1987). SP-A has also been localized by immunocytochemistry at or near the corners of the TM lattice suggesting a role for SP-A in the organization of phospholipid in the alveolar microenvironment (Walker *et al.*, 1986). In the presence of Ca^{2+}, SP-A potentiates the effect of SP-B and SP-C, increasing the adsorption rate of TM PC in the subphase to the surfactant monolayer (Hawgood *et al.*, 1985; Hawgood *et al.*, 1987). SP-A is also proposed to play a role in host defense. SP-A belongs to a group of proteins called collagenous C-type lectins (collectins), which include congluttin and mannose binding protein, both which have remarkable structural homology (amphipathic helical domains) and functional similarities with SP-A and are implicated to play a role in non-antibody mediated host defense against microbes (Whitsett, 1988). Indeed, SP-A has been demonstrated to enhance the phagocytotic activity of alveolar macrophages (Van Iwaarden et al., 1990).

Surfactant Protein-B

The hydrophobic surfactant protein B (SP-B) is a basic peptide of 79 AA (Curstedt *et al.*, 1988; Olafson *et al.*, 1987) which in non-reducing conditions exists as a sulfhydryl dependent homodimer (18 kDa). The precursor form of SP-B (40-42 kDa) (Jacobs *et al.*, 1987) has been localized to the RER, Golgi apparatus, and MVB's of type-II cells, whereas the mature form has been isolated to the MVB's and LB's where it is co-secreted with phospholipids into the alveoli (Voorhout *et al.*, 1992). SP-B is highly surface active and acts in cooperation with SP-A to increase the rate of adsorption of PC from TM in the subphase to the surfactant monolayer (Hawgood *et al.*, 1987). SP-B also enhances PC

uptake by type-II cells (Rice *et al.*, 1989). SP-B is critical to lung function post-parturition (Nogee *et al.*, 1993) and its presence in amniotic fluid predicts normal lung function in premature neonates (Pryhuber *et al.*, 1991).

Surfactant Protein-C

The hydrophobic surfactant protein C (SP-C) exists as a disulfide dependent homodimer. The precursor form of SP-C (20-22 kDa) is co-localized in type-II cells with precursor SP-B. Mature SP-C (5 kDa), which contains 1 or 2 palmitoyl groups linked to cysteine residues at the NH_2 terminal, is highly localized in the LB's and co-secreted into the alveoli with SP-B and phospholipid (Whitsett, 1994). SP-C, like SP-B, also acts to increase the rate of PC adsorption from TM to the surfactant monolayer, but not as rapidly as does SP-B. SP-C also enhances PC uptake by type-II cells (Rice *et al.*, 1989).

Surfactant Protein-D

The hydrophilic glycoprotein surfactant protein D (SP-D), recently identified in rat bronchoalveolar lavage (BAL) (Persson *et al.*, 1988; Persson *et al.*, 1989), is a multimer of disulfide bonded trimers composed of 12, 43 kDa polypeptides. Like SP-A, SP-D contains a collagen-like domain at its amino terminal and is a member of the calcium-dependent lectin protein family believed to play an important role in non-antibody mediated host defense mechanisms (Scarpelli, 1990).

Biophysical Mechanism of Action

Ventilation is influenced by three properties of the lung: 1. compliance, or distensibility of the lungs, 2. elasticity, or tendency of the lung to resist deformation and

3. surface tension (Leff and Schumacker, 1993). Surface tension occurs at the air-surface interface of the liquid lining the alveoli as a consequence of the mutual attraction between lateral liquid molecules at the liquid surface. Surface tension and tissue elasticity in the lung produce an inward directed force which is directly proportional to surface tension but inversely proportional to the radius of the alveoli (Figure 4). According to the Law of LaPlace, alveoli of differing radii would thus have differing surface tensions, the smaller the alveoli the greater the surface tension. Therefore smaller alveoli would have the tendency to collapse and empty their contents into larger alveoli with a lower surface tension. It is the primary role of pulmonary surfactant to ensure that this does not occur. DPPC, the major surface active component of surfactant (King, 1974), possesses the biophysical structural properties to facilitate the disruption of surface tension. Its hydrophobic and hydrophilic properties orient this molecule perpendicular to the surface of a liquid with its hydrophilic polar head dissolved and its hydrophobic nonpolar tail extending above the liquid surface. In this orientation, DPPC facilitates the disruption of the lateral mutual attraction between water molecules at the liquid surface and consequently decreases alveolar surface tension. Pulmonary surfactant not only lowers surface tension in the alveoli, but its surface tension lowering properties become improved as the radius of each alveoli becomes smaller during expiration (Figure 5). DPPC, a solid at normal body temperature, resists surface compression (Van Golde *et al.*, 1994). Consequently, as the alveoli become smaller during expiration and the surface tension of the lung is expected to increase, the surface area of the lung becomes smaller and more fluid unsaturated lipid molecules in the surfactant monolayer get "squeezed out" and the DPPC film at the surface becomes more refined. The extent to which "squeeze out" occurs is determined by the presence of acyl chain unsaturation and also by the rate of

Law of LaPlace

$$P = \frac{2 \times T}{r}$$

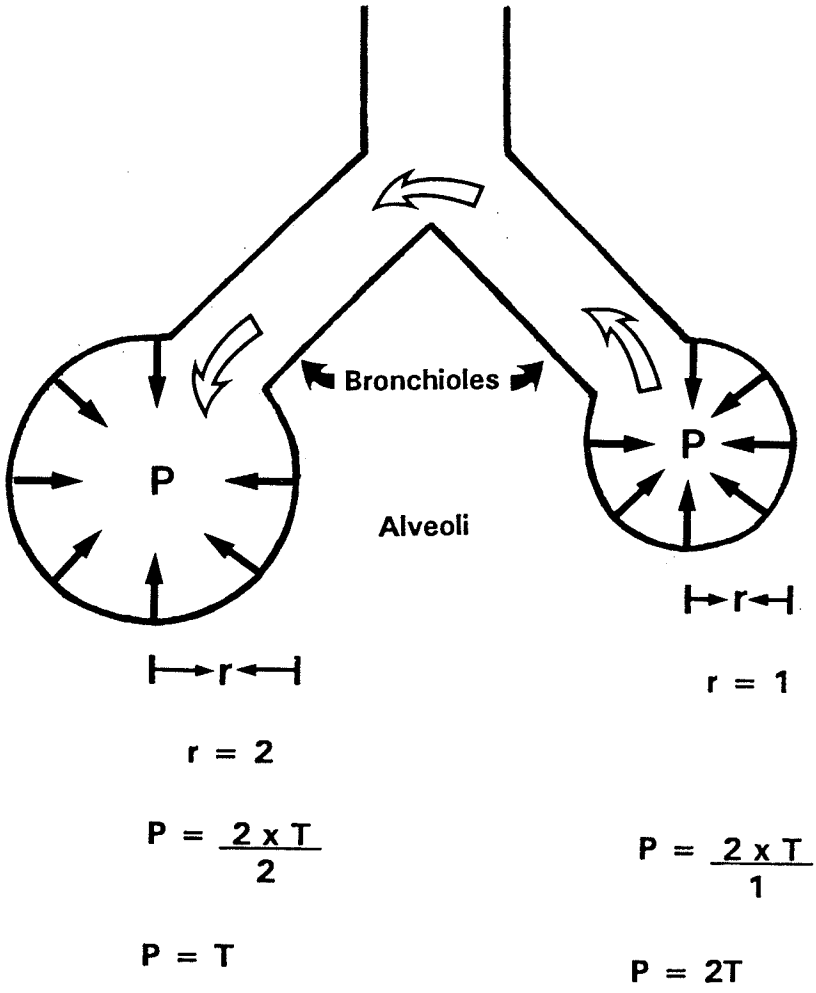

Figure 4. The Law of LaPlace Applied to Lung Alveoli. Surface tension in the alveoli creates an inward force tending to collapse alveoli. The transmural distending pressure (P) required to maintain an alveolus at a constant volume, assuming spherical geometry, is equivalent to twice the surface tension divided by the radius of the sphere. Therefore, when considering two connected alveoli of different radii, a greater transmural distending pressure is required to prevent the smaller alveoli from collapsing, and emptying into the larger one (after Van De Graaff and Fox, 1989).

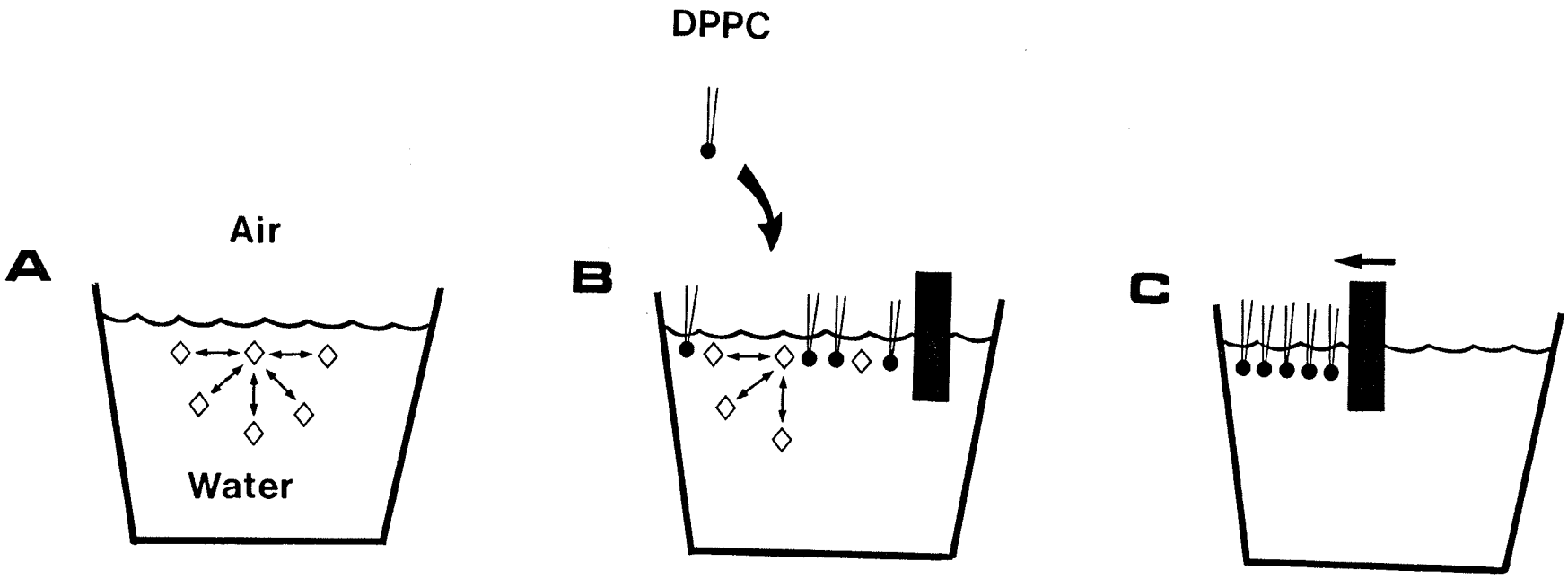

Figure 5. Effect of Dipalmitoyl Phosphatidylcholine (DPPC) on Surface Tension. **A)** Water molecules (◊) have a mutual attraction to other water molecules in all directions. This lateral hydrogen bonding between water molecules at the water surface creates a surface tension at the air water interface. **B)** Addition of DPPC, which due to its hydrophobic tail and hydrophilic head preferentially occupies the area at the air water interface, disrupts the polar attraction of surface water molecules thus lowering surface tension. **C)** As the DPPC film and the DPPC molecules therein become compressed there is an increasing disruption of this attraction and increased surface tension lowering capabilities (after Mines AH, 1993).

surface film compression (Pastrana-Rios *et al.*, 1994). The greater the decrease in surface area of the lung, the greater the refinement of the monolayer and concomitantly the greater the surface tension lowering properties of the surfactant monolayer. As the DPPC resists surface compression, it also builds a film pressure which counteracts the surface tension and tissue elasticity in the alveoli (Mines, 1993).

Surfactant thus prevents alveoli from collapsing due to surface tension and tissue elasticity. Even upon complete expiration the alveoli remain open with a residual volume of air inside, thus decreasing the surface tension which has to be overcome during inspiration. Therefore, the stabilization of the terminal airspaces becomes independent of alveolar size (Mines, 1993). Recruitment of mixed film components into the surfactant monolayer occurs as the surface area of the lung increases during inspiration (Wright and Hawgood, 1989).

Surfactant is also proposed to be critical in the prevention of pulmonary edema (Gil, 1985; Pattle, 1958) by decreasing the driving force for edema formation.

Efficacy of Exogenous Surfactant as a Treatment for Neonatal Respiratory Distress Syndrome (NRDS)

Exogenous surfactants utilized in the treatment of NRDS have been classified into 4 general categories depending on their origin; natural surfactants, modified natural surfactants, artificial surfactants and synthetic natural surfactants (Jobe and Ikegami, 1987). Exogenous surfactant treatment may be preventive through prophylactic administration, which ensures early treatment, or through a rescue treatment protocol following diagnosis of NRDS. In recent years there has been a considerable number of randomized controlled clinical investigations determining the benefit of single (Corbet *et al*, 1991a; Corbet *et al.*, 1991b; Horbar *et al.*, 1989; Horbar *et al.*, 1990; Kendig *et al.*,

1988; Kwong *et al.*, 1985; Soll *et al.*, 1990; Stevenson *et al.*, 1992), and multiple (Auten *et al.*, 1991; Hoekstra *et al.*, 1991; Liechty *et al.*, 1991; Long *et al.*, 1991) administration regimens of synthetic and natural exogenous surfactants for the treatment of NRDS. As well, trials comparing single vs. multiple regimens (Dunn *et al.*, 1990; Speer *et al.*, 1992) have also been completed. Comparison of these trials is extremely difficult and is further complicated by the dosing protocol used (prophylactic or rescue), the surfactant type incorporated (natural or synthetic), and the differing gestational ages and birth weights and resultant degree of NRDS of enrolled infants. In general, an improvement in lung function and oxygenation was observed in the early period following a single dose administration of exogenous surfactant (Corbet *et al.*, 1991a; Corbet *et al.*, 1991b; Soll *et al.*, 1990; Kendig *et al.*, 1988; Kwong *et al.*, 1985). A decrease in mortality was observed in a few of these trials (Corbet *et al.*, 1991a; Soll *et al.*, 1990). It was suggested in one single dose study that the benefits of multiple post-ventilatory doses of exogenous surfactant would be required for optimal therapy (Kendig *et al.*, 1988). Multiple dosing studies seemed to demonstrate a greater improvement in lung function and oxygenation parameters of infants and a much lower incidence of NRDS during the first 72 hours following administration (Kendig *et al.*, 1989). It was confirmed that treatment with multiple doses of surfactant was more effective than single-dose treatment in sustaining improved oxygenation and reducing overall mortality (Dunn *et al.*, 1990; Hoekstra *et al.*, 1991; Merrit *et al.*, 1986a; Speer *et al.*, 1992; Ten Centre Study Group, 1987).

There has also recently been an interest in comparing the efficacy of synthetic surfactant preparations with naturally derived surfactant preparations. There have been 3 clinical trials comparing the naturally derived surfactant preparation Survanta® with the synthetically derived Exosurf® (Horbar *et al.*, 1993; Mondalou *et al.*, 1994; Vermont Oxford

Trials Network, 1994). Although results indicate that one preparation was not superior to the other in the overall reduction in infant mortality, it appeared that the initial response to treatment with naturally derived exogenous surfactant preparations, as measured by FIO_2 and MAP, was quicker and more profound (Horbar *et al.*, 1993). It has been suggested that this rapid response may be a consequence of the presence of the hydrophobic surfactant proteins SP-B and SP-C in the naturally derived preparations (Dunn, 1994). In general, studies of naturally derived preparations vs. synthetic preparations indicated that infants treated with natural surfactants consistently required less ventilatory support over the first few days of life (Dunn, 1994), but no differences in the decrease of overall mortality were observed.

Effect of Surfactant on Pulmonary Hemodynamics

A decrease in pulmonary vascular resistance (PVR) following exogenous surfactant administration has been reported in human neonates (Kääpä *et al.*, 1992; Kääpä *et al.*, 1993). In an immature baboon model of NRDS, a transient increase in lung blood flow was observed following surfactant treatment and returned to pretreatment levels after 7 hours (Vidyasagar *et al.*, 1985). As well, a significant increase in the left-to-right shunt of blood across the patent ductus arteriosus (PDA) was observed following surfactant administration in an preterm lamb model of NRDS (Clyman *et al.*, 1982). The PDA acts as a large communication corridor between the aorta, which exits the left ventricle, and the pulmonary artery, which exits the right ventricle, in the neonate. Aortic and pulmonary pressures are relatively equal across the PDA and any shunting of blood across the PDA, be it left-to-right or right-to-left, is a consequence of changes of the vascular resistance of the pulmonary or systemic vasculature (Rudolph, 1974). An increase in left-to-right

shunting could therefore be due to increased systemic vascular resistance or decreased PVR. The left-to-right shunting observed following exogenous surfactant administration has been confirmed to be a result of decreased PVR (Clyman *et al.*, 1982).

Although the underlying cause of the decrease in PVR following exogenous surfactant replacement therapy presently remains unclear, numerous explanations for this observation have been proposed. The reported decrease in PVR could strictly be a result of extra-alveolar vessels becoming "uncollapsed" as lung mechanics improve following surfactant administration. The recruitment of alveoli following exogenous surfactant administration leads to an increase in alveolar volume which tends to stretch and distort these collapsed vessels augmenting their diameter and minimizing their resistance (Leff and Schumacker, 1993). Another plausible explanation is that exogenous surfactant administration improves lung mechanics and oxygenation which concomitantly reverses hypoxic pulmonary vasoconstriction (Leff and Schumacker, 1993). This improved availability of oxygen may lead to oxygen-mediated pulmonary vasodilation and a resultant decrease in PVR. It has also been suggested that an improvement in respiratory acidosis as a result of improved lung mechanics results in increased blood flow to the lungs (Clyman *et al.*, 1982). These explanations for the decrease in PVR following exogenous surfactant administration are based on the biophysical improvements in lung mechanics afforded by surfactant.

Hypothesis

It is the purpose of this thesis and the research reported herein to attempt to address, on a pharmacological level, a third plausible explanation for the observed decrease in PVR following exogenous surfactant administration. This thesis addresses the hypothesis that surfactant replacement therapy in NRDS significantly reduces PVR through endothelium-dependent LPC induced vasorelaxation of pulmonary arterial smooth muscle. Potentially elevated secretory PLA_2 activity associated with NRDS and lung inflammation may lead to deacylation of DPPC, present in exogenous surfactant, and accumulation of LPC in the lung which diffuses across the epithelial-endothelial barrier and mediates relaxation of the pulmonary vasculature.

Background and Rationale

The rationale for the testing of this hypothesis is based on the following background information regarding the potential role of biochemical agents, including PLA_2 and LPC, in NRDS.

Phospholipase Enzymes

Phospholipases constitute a diverse family of enzymes that catalyze the hydrolysis of membrane phospholipids (Kaiser *et al.*, 1990). PLA_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) specifically hydrolyses the 2-acyl position of a glycerophospholipid yielding a lysophospholipid and a free fatty acid (Vadas and Pruzanski, 1986). Two distinct forms of PLA_2 occur in the lung: a secretory-type PLA_2 and a cytoplasmic-type PLA_2. There are two distinct forms of cytoplasmic-type PLA_2 limited to the cytosolic compartment in association with the plasma membrane or within organelles of the vacuolar system

(Kramer *et al.*, 1990): 1) a Ca^{2+}-independent, acidic pH optimum form identified in lamellar bodies, the organelle for surfactant secretion and storage (Heath and Jacobson, 1976) and 2) a Ca^{2+}-dependent, alkaline pH optimum form associated with the lung microsomal or mitochondrial fraction (Filgueiras and Possmayer, 1990; Chander *et al.*, 1982). The secretory-type PLA_2, immunologically related to pancreatic PLA_2, is Ca^{2+}-dependent (in mM range) with optimal activity at an alkaline pH (8.5) (Bennet *et al.*, 1990).

Lung phospholipases, especially the cytoplasmic-type PLA_2, play an important role in the remodelling and recycling of phospholipid components of pulmonary surfactant. However, elevated or skewed levels of secretory-type PLA_2, could potentially hydrolyse the surfactant phospholipid component yielding increased concentrations of LPC and free fatty acids as by-products in the lung, and potentially inactivate surfactant biophysical activity. Elevated levels of PLA_2 activity in serum and in lung lavage has been reported in adult respiratory distress syndrome (ARDS) (Romaschin *et al.*, 1992) and in a rat model of ARDS (Von Wichert *et al.*, 1981). As well, the composition of pulmonary surfactant from individuals with ARDS consistently demonstrates a decrease in phospholipid concentration concomitant with an increase in LPC concentration, a trend which is accordant with increased secretory PLA_2 activity in the lung (Gregory *et al.*, 1991; Hallman *et al.*, 1982; Petty *et al.*, 1977). The deleterious effect of PLA_2 upon surfactant is further supported by a 42% decrease in DPPC, associated with a 5-fold increase in PLA_2 activity, observed in lung lavage samples from dogs with induced pancreatitis (Das *et al.*, 1987; Morgan *et al.*, 1968).

Although attempts have been made to characterize lung secretory PLA_2 activity in individuals with ARDS, limited data regarding the lung content and activity of secretory PLA_2 in newborns with NRDS are available. NRDS is a condition primarily caused by

deficiency of surfactant, but symptomatic and radiologic similarities with ARDS (Hallman *et al.*, 1982) suggest the potential for increased PLA_2 activity in NRDS individuals as well.

Most, if not all, infants with NRDS are maintained on respiratory support. Ventilation of surfactant-deficient lungs is associated with necrosis and desquamation of airway epithelium within minutes of ventilation causing epithelial lesions and triggering an inflammatory response (Robertson, 1988). Secretory PLA_2 is a mediator of inflammatory eicosanoid production, and plays an important role in the initiation and propagation of inflammatory processes (Pfeilschifter, 1995; Vadas and Pruzanski, 1990).

Lysophosphatidylcholine (LPC) Mediated Vasorelaxation

PLA_2 catalyzes the deacylation of a variety of PC's resulting in the formation of various forms of LPC, collectively known as lysolecithins. LPC's have been shown to be endothelium-mediated vasorelaxants of the pulmonary (Bing and Saeed, 1987; Bing *et al.*, 1988; Saito *et al.*, 1988; Wolf *et al.*, 1991) and systemic vasculature (Menon and Bing, 1991; Dudek *et al.*, 1993) both *in vitro* and *in vivo*. In general, the degree of vasorelaxation is dependent on LPC aliphatic chain length, those LPC's with the longest aliphatic chains possessing the greatest relaxing activity (Saito *et al.*, 1988). Palmitoyl LPC, for example, has been shown to be a particularly effective systemic vasorelaxant *in vitro* (Saito *et al.*, 1988). This LPC species is formed via PLA_2 catalyzed deacylation of DPPC, the most widely used PC in artificial surfactant for the treatment of NRDS. It is also the principal surface-active ingredient of natural surfactant (Klaus *et al.*, 1961).

Research Protocol Outline

The hypothesis that exogenous surfactant replacement therapy in NRDS leads to a significant reduction in PVR through an endothelium dependent LPC induced vasorelaxation of pulmonary arterial smooth muscle was tested with four independent research protocols. LPC may be present in the exogenous surfactant itself, or may be generated in the lung by increased levels of secretory PLA_2.

1. *In vitro* analysis of the vasoactive effects of LPC on pulmonary arterial preparations from newborn and adult guinea pigs. This study addressed the potential age related vasoactive differences to LPC and the role of the endothelium in this response. As well, the role of nitric oxide synthase (NOS) and guanylate cyclase (GC) activity in the mediation of a vasoactive response to LPC was investigated in both adults and newborns.

2. Determination of the secretory PLA_2 deacylation profile of the PC pool present in two commercially available exogenous surfactant preparations, the synthetic Exosurf® and the naturally derived Survanta®. The LPC which mediates the vasorelaxation of the pulmonary vasculature following exogenous surfactant administration may be present in the surfactant as part of its composition or may be generated in the lung by increased levels of secretory PLA_2. This study addressed the potential susceptibility of two exogenous surfactant preparations, currently employed in Canadian hospitals for the treatment of NRDS, to secretory PLA_2 activity; the artificial surfactant Exosurf® neonatal (Burroughs Wellcome) and the modified natural surfactant Survanta® beractant (Ross Laboratories).

3. Determination of lung secretory PLA_2 activity in pre-term infants suffering from NRDS. Following exogenous surfactant treatment, greater amounts of LPC than present in exogenous surfactant may be generated in the lung by secretory PLA_2, compounding the vasoactive potential of LPC. This study measured the relative levels of secretory PLA_2 activity from tracheo-bronchial secretions (TBS) samples obtained from pre-term infants with NRDS in the neonatal intensive care unit (NICU) at the University of Manitoba Health Sciences Centre (HSC) to determine any correlation between degree of prematurity and the levels of secretory PLA_2 activity.

4. Determination of the effects of LPC on pulmonary vascular resistance following *in vivo* administration into the pulmonary vasculature and tracheal instillation in lung ventilation/perfusion studies. This study attempted to confirm *in vivo* the effects of LPC on the pulmonary vasculature previously observed *in vitro*. As well, this study attempted to determine the ability of for intra-tracheally administered LPC to cross the epithelial-endothelial barrier and mediate a vasoactive response within the pulmonary vascular bed.

In vitro Analysis of Lysophosphatidylcholine (LPC) on Pulmonary Arterial Preparations

Introduction

LPC, a product of PLA_2 (EC 3.1.1.4) hydrolysis of the acyl moiety at the 2-position of PC, is an endothelium-dependent relaxant of precontracted systemic (Bing and Saeed, 1987; Bing *et al.*, 1988; Saito *et al.*, 1988; Wolf *et al.*, 1991) and pulmonary (Dudek *et al.*, 1993; Menon and Bing, 1991) arteries. This was determined following the observation that melittin, an activator of PLA_2, and thimerosal, an inhibitor of acyl-coenzyme A:lysolecithin acyltransferase (EC 2.3.1.23), both induced endothelium-dependent vasorelaxation in precontracted vascular preparations (Förstermann *et al.*, 1986) (Figure 6). LPC, via its detergent properties, perturbs cell membrane architecture influencing membrane receptor function and the activity of membrane associated enzymes (Bing *et al.*, 1993; Saito *et al.*, 1988). LPC modulates the activity of many enzymes (Kirschbaum and Bosmann, 1973; Kirschbaum and Bosmann, 1974; Mookerjea and Yung, 1974a; Mookerjea and Yung, 1974b; Oishi *et al.*, 1988; Sandermann, 1978; Shier and Trotter, 1976) including guanylate cyclase (GTP pyrophosphate-lyase (cyclizing)) (EC 4.6.1.2) (Menon *et al.*, 1989; Shier *et al.*, 1976; White and Lad, 1975; Zwiller *et al.*, 1976). LPC induced vasorelaxation of precontracted smooth muscle preparations is accompanied by an observed rise in cyclic guanosine 3',5'-monophosphate (cGMP) (Menon *et al.*, 1989), a well established intracellular mediator of vasodilation (Murad, 1986). Endothelium-dependent LPC induced vasorelaxation is also mediated by endothelial derived nitric oxide (Dudek *et al.*, 1993). Recently, LPC has been shown to enhance the enzymatic activity of nitric oxide synthase (NOS) (EC 1.14.13.39) from cultured aortic endothelial cells (Ohashi *et al.*, 1993). LPC-induced vasorelaxation, therefore, is likely mediated by the

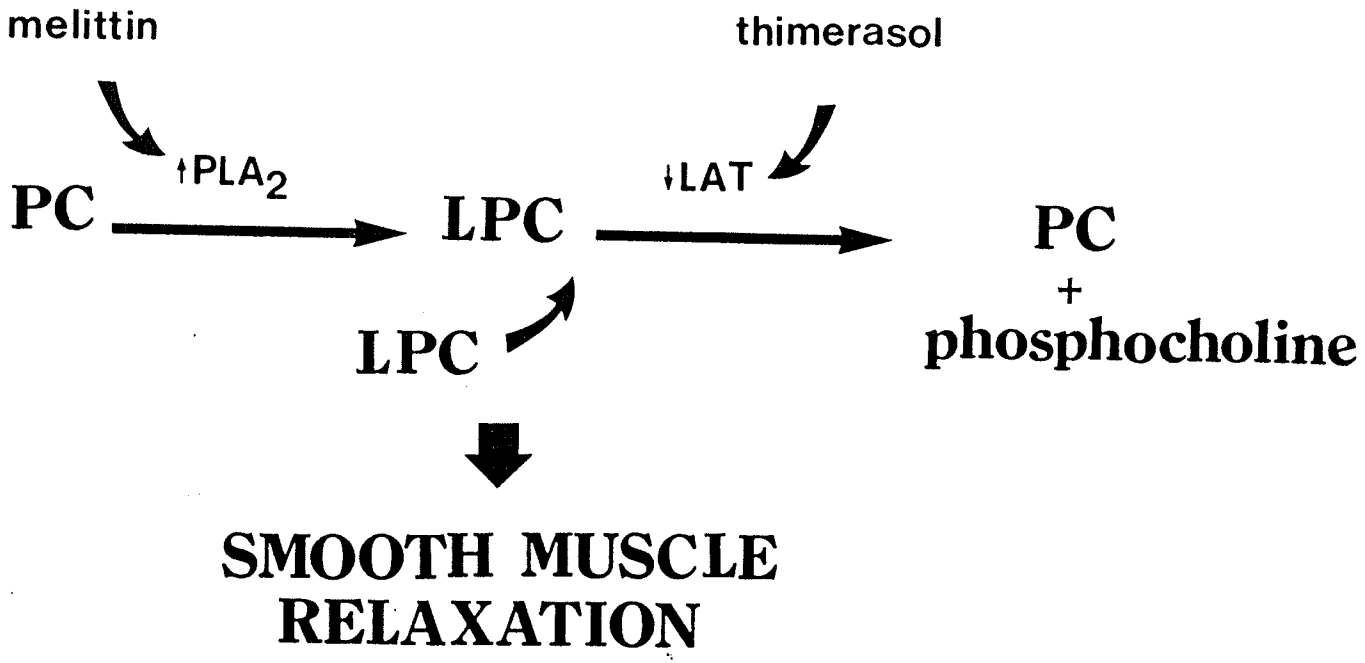

Figure 6. Melittin and Thimerasol Mediated Smooth Muscle Vasorelaxation. The addition of melittin, an activator of PLA_2 activity, or thimerasol, an inhibitor of acyl-coenzyme A:lysolecithin acyltransferase (LAT) activity, to precontracted arterial preparations facilitates the accumulation of LPC and results in smooth muscle vasorelaxation.

direct activation of guanylate cyclase (GC) leading to increased intracellular cGMP production and smooth muscle relaxation or via alteration of NOS activity leading to endothelium derived nitric oxide (EDNO) mediated increase in soluble guanylate cyclase activity, increased intracellular cGMP production and smooth muscle relaxation (Figure 7). Although unclear, it appears that increased cytoplasmic cGMP promotes the reduction of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through activation of cGMP-dependent protein kinases which in turn phosphorylates phospholamban and cytoskeletal proteins which upregulate Ca^{2+}-ATPase activity on the sarcoplasmic reticulum and plasma membrane lowering $[Ca^{2+}]_i$ and promoting smooth muscle relaxation (Lincoln and Cornwell, 1993). It has also been suggested that elevated cytosolic cGMP may inhibit inositol-1,4,5-triphosphate (IP_3) formation and IP_3 receptor activity, inhibiting the release of Ca^{2+} from the sarcoplasmic reticulum, and that cGMP decreases the sensitivity of contractile proteins to increased $[Ca^{2+}]_i$ (Lincoln *et al.*, 1994).

Although LPC-mediated vasorelaxation has been observed in precontracted pulmonary arterial preparations from adult animal models there has been no attempt to characterize the vasoactive potential of LPC in newborn pulmonary arterial preparations. Evidence suggests that aging is associated with thinning and loss of endothelium (Bar, 1978; Stewart *et al.*, 1987) and that endothelium-dependent vasorelaxation in the rat is impaired in an age-related manner (Hongo *et al.*, 1988; Moritoki *et al.*, 1986; Soltis, 1988). The vasoactive potential of LPC on the pulmonary vasculature of the newborn is of particular interest given the recent increase in usage and acceptance of exogenous surfactant as a therapy for the treatment of NRDS. Exogenous surfactant preparations contain tremendous amounts of PC in the form of DPPC, approximately 13 mg/ml of surfactant. Individuals may be administered up to 100 mg of phospholipids/kg birth weight which is susceptible to PLA_2 degradation. Naturally derived exogenous surfactant

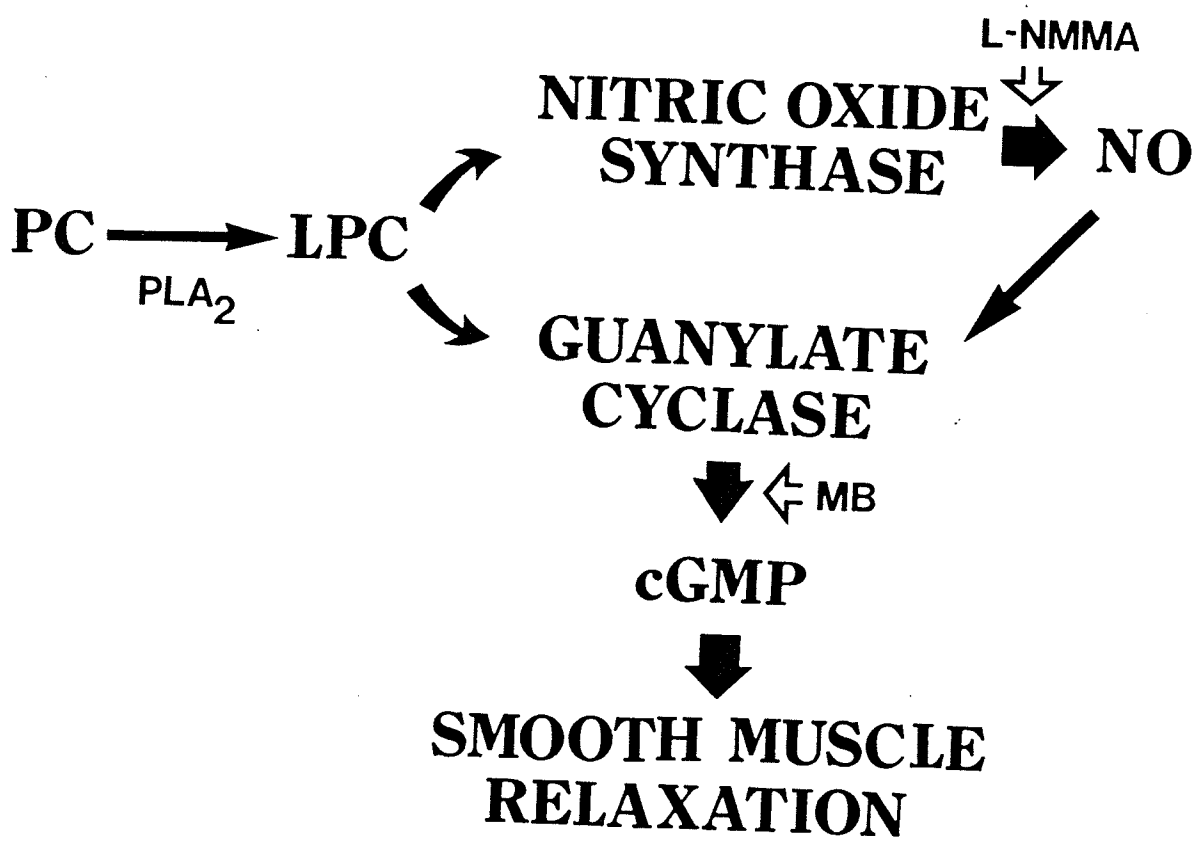

Figure 7. Potential Role of Nitric Oxide Synthase (NOS) and Guanylate Cyclase (GC) in Lysophosphatidylcholine (LPC) Mediated Smooth Muscle Vasorelaxation. LPC has been demonstrated to increase the activity of both endothelial NOS and GC activity. LPC mediated smooth muscle vasorelaxation is associated with an increase in cyclic guanosine monophosphate (cGMP), a well established intracellular mediator of vasorelaxation, suggesting two potential pathways for its action. N^G-monomethyl-L-arginine (L-NMMA) and methylene blue (MB) are inhibitors of NOS and GC respectively.

preparations, such as Survanta®, already contain LPC as part of their composition (Duncan *et al.*, 1995).

The purpose of this study was to investigate, *in vitro*, potential differences in vascular reactivity between precontracted newborn and adult guinea pig pulmonary arterial preparations to LPC. Given that pulmonary endothelial NO production is developmentally regulated (Shaul *et al.*, 1993), we also investigated the role of NOS and GC in the response of each.

Materials and Methods

Materials

L-α-LPC (derived from egg yolk), L-phenylephrine hydrochloride, acetylcholine chloride (ACh), propranolol HCl, potassium chloride, sodium bicarbonate, sodium chloride, dimethyl sulfoxide, N^G-monomethyl-L-arginine and methylene blue were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium phosphate monobasic, magnesium sulfate, calcium chloride, and D-glucose anhydrous were purchased from Mallinckrodt division of Anachemia Science (Winnipeg, MB). Ketalean® (ketamine hydrochloride) was purchased from MTC Pharmaceuticals (Cambridge, ON) and Rompun® (xylazine) obtained from Bayvet Division, Chemagro Limited (Etobicoke, ON). Pentobarbitone sodium was purchased from BDH Inc. (Toronto, ON).

Pulmonary Arterial Preparations

Female adult (800-1000 grams, >2 months of age) and newborn (90-110 grams, <3 days of age) Hartley guinea pigs were studied under a protocol approved by the University of Manitoba Ethics Committee on animal experimentation. Animals were sedated with intramuscular injections of Rompun® (xylazine) (5 mg/kg) and Ketalean®

(ketamine hydrochloride) (20 mg/kg) prior to being anaesthetized with an intraperitoneal injection of sodium pentobarbitone (30 mg/kg). Following sternotomy, the heart and lungs were removed and immediately immersed in Krebs-Henseleit solution (115 mM NaCl, 25 mM $NaHCO_3$, 1.38 mM $NaHPO_4$, 4.51 mM KCl, 2.46 mM $MgSO_4 7H_2O$, 1.91 mM $CaCl_2$, and 5.56 mM D-glucose anhydrous) at 37°C, saturated with 95% O_2/5% CO_2. Under a dissecting microscope the pulmonary trunk was carefully freed of adhering adventitial connective tissue, removed, and cut into transverse rings 1.5-2.5 mm in width. Care was taken to ensure that the arterial rings were obtained from the medial portion of the pulmonary trunk in order to avoid a preparation which contained the vestigial ductus arteriosus in newborns and ligamentum arteriosum in adults. Arterial ring preparations were immediately suspended from the magnesium arm of an electromagnetic isometric force transducer by 7-0 braided silk (Ethicon Ltd), from a wire triangle to a fixed wire post in a muscle bath chamber of 40 ml. The muscle bath was filled with Krebs-Henseleit solution maintained at 37°C and constantly perfused with 95% O_2/5% CO_2. The preparations were suspended at a resting tension of 2 mN for a one hour equilibration period prior to experimentation.

Pharmacologic Smooth Muscle Mechanical Studies

Following equilibration, immediately prior to experimentation, the vessel bath medium was renewed. All preparations were tested isometrically at their optimal length which was determined by 2 supramaximal electric field stimulations (18.8V, 12s, 60Hz) of each preparation, 5 minutes apart, at 2 mN incremental preloads. The major function of vascular smooth muscle is the regulation of vascular tone and resistance in which shortening parameters are the important ones to study. The smooth muscle mechanical studies were conducted under isometric conditions, however, because study of isotonic

relaxation is problematic in that there is lack of a valid index which should be independent of the load on the muscle and the muscles initial contractile element length which both affect isotonic relaxation.

The optimal length (L_o) of each preparation was determined when the increase in force generation to electrical stimulation at each increased preload was less than 10% at the previous preload. The force measured at L_o was considered to be maximal active tension (Po). Vessel width, circumference, and weight were measured immediately following the experimental protocol. Stress was normalized to the vessel segment cross sectional area (weight/resting length) assuming a wall vessel density of 1.06 g/cm^3.

Newborn and adult preparations were both tested under four experimental conditions: 1) with endothelium intact (+Endo), 2) with endothelium denuded (-Endo), 3) with endothelium intact and NOS chemically inhibited with N^G-monomethyl-L-arginine (+Endo(L-NMMA)), and 4) with endothelium intact and GC activity chemically inhibited with methylene blue (+Endo(MB)). -Endo preparations had the endothelium physically removed by gentle mechanical abrasion of the intimal surface with a tapered wooden applicator stick. All preparations were precontracted with a concentration of phenylephrine (PE) which was determined to induce a 50% maximal PE contraction ($3x10^{-6}$ M). To facilitate the maintenance of a steady tension plateau with PE, the β-blocker L-propranolol ($2x10^{-5}$ M) was added to the muscle chamber 30 minutes prior to PE precontraction to eliminate non-specific β-adrenergic vasorelaxation. Chemical blockade of NOS activity in +Endo(L-NMMA) preparations was achieved by pre-exposing preparations to the L-arginine analogue L-NMMA ($1x10^{-3}$ M) 30 minutes prior to PE precontraction. Blockade of GC activity in +Endo(MB) preparations was achieved by pre-exposing preparations to methylene blue (MB) (10^{-10}-10^{-4} M) prior to PE precontraction. When a steady tension plateau was achieved following PE addition, cumulative doses of ACh (10^{-8}-10^{-3} M) were

added to the muscle chamber as a means of confirming the integrity of the endothelium. Following confirmation of endothelial integrity the preparation was washed, allowed to re-equilibrate, precontracted, and exposed to cumulative doses of LPC (10^{-8}-10^{-4} M). The LPC used was derived from egg yolk and contained primarily palmitic (67.1%), stearic (28.6%), and oleic (1.8%) moieties of LPC. The LPC was dissolved in 0.1 ml of dimethyl-sulfoxide (DMSO) and 0.9 ml of Krebs-Henseleit, prior to being diluted into a graded series, according to the method of Bing and Saeed (1987). Addition of DMSO to the muscle bath did not alter the achieved force tension plateau. Changes in smooth muscle tension (P_o) and length (L_o) were continuously monitored at a frequency of 5Hz and recorded over the 35 minute duration of each experiment by means of a customized data acquisition program (AT Lever Data Capture Program - Cunningham Engineering).

Smooth muscle pharmacological responses to ACh and LPC are reported as percent relaxation or contraction from the precontracted steady force-tension plateau achieved following addition of the α-adrenergic stimulant PE (3×10^{-6} M).

Histological Analysis

Following experimentation, vessel preparations were allowed to re-equilibrate and fixed in 10% buffered formalin (Fisher's solution) and embedded in TissuePrep© (56°C) (Fisher Scientific) to allow transverse sectioning of each preparation. Sections (5μm) were stained with Ehrlich's haematoxylin and counterstained with eosin to allow visualization of endothelium (Humason, 1972).

Statistical Analysis

Results are reported as mean ± SEM. The pulmonary arterial vessel characteristics and smooth muscle mechanics were analyzed by analysis of variance

(ANOVA). Dose response data were analyzed by ANOVA for repeated measures. Individual means were compared using multiple comparison by Newman-Keuls test. Significance was accepted at $p<0.05$.

Results

Vessel Properties

The vessel characteristics and smooth muscle mechanical properties of the newborn and adult guinea pig pulmonary arterial preparations are described in Table 1. There were no significant differences observed between the measured vessel parameters of newborn +Endo and -Endo preparations or adult +Endo and -Endo preparations. All adult preparations (+Endo and -Endo) differed significantly from all newborn preparations (+Endo and -Endo) ($p<0.05$).

Determination of a 50% Maximal Phenylephrine (PE) Contraction

Both newborn and adult guinea pig pulmonary arterial preparations were exposed to cumulative doses of PE to determine the concentration which elicited a 50% maximal contraction (Figure 8A, Figure 9A). Figure 8A and 9A represents a plot of the percentage of maximal PE contraction against concentration of PE. Figures 8B and 9B represents a plot of the percentage of maximal PE contraction, as determined from the regression equation of plot 8A and 9A, against logarithmic concentration PE. A plot of the percent maximal PE response against logarithmic concentration of PE revealed that in the newborn guinea pig (Figure 8B) a 50% maximal PE contraction was elicited with $1x10^{-6}$ M PE. In the adult guinea pig (Figure 9B), a 50% maximal contraction was obtained with $4x10^{-5}$ M PE. A concentration of $3x10^{-6}$ M PE was utilized to effect an approximate 50% maximal PE contraction in both adult and newborn preparations.

Table 1. Vessel characteristics and smooth muscle mechanical properties †

	Adult		* Newborn	
	+Endo	-Endo	+Endo	-Endo
Number of Vessels	17	7	14	7
Width (mm)	2.05±0.08	1.96±0.12	1.39±0.06	1.43±0.18
Circumference (cm)	1.00±0.04	0.98±0.05	0.52±0.02	0.50±0.03
Weight (mg)	12.15±1.86	12.77±2.25	2.27±0.16	2.40±0.40
Resting Length (mm)	6.16±0.13	6.20±0.63	2.97±0.11	2.93±0.18
Cross Sectional Area (mg/mm)	2.05±0.35	2.03±0.32	0.77±0.05	0.88±0.18
Maximum Force (mN)	15.92±0.98	17.38±1.75	4.60±0.44	5.06±1.02
Stress (mN/mm^2)				
Electrical	10.11±1.09	9.43±1.04	6.29±0.70	6.45±1.02
Phenylepherine ($3x10^{-6}$ M)	16.97±1.60	14.90±1.15	7.75±0.75	7.44±0.83

† Values are mean ± S.E.
* $p<0.05$ as compared to all adult preparations

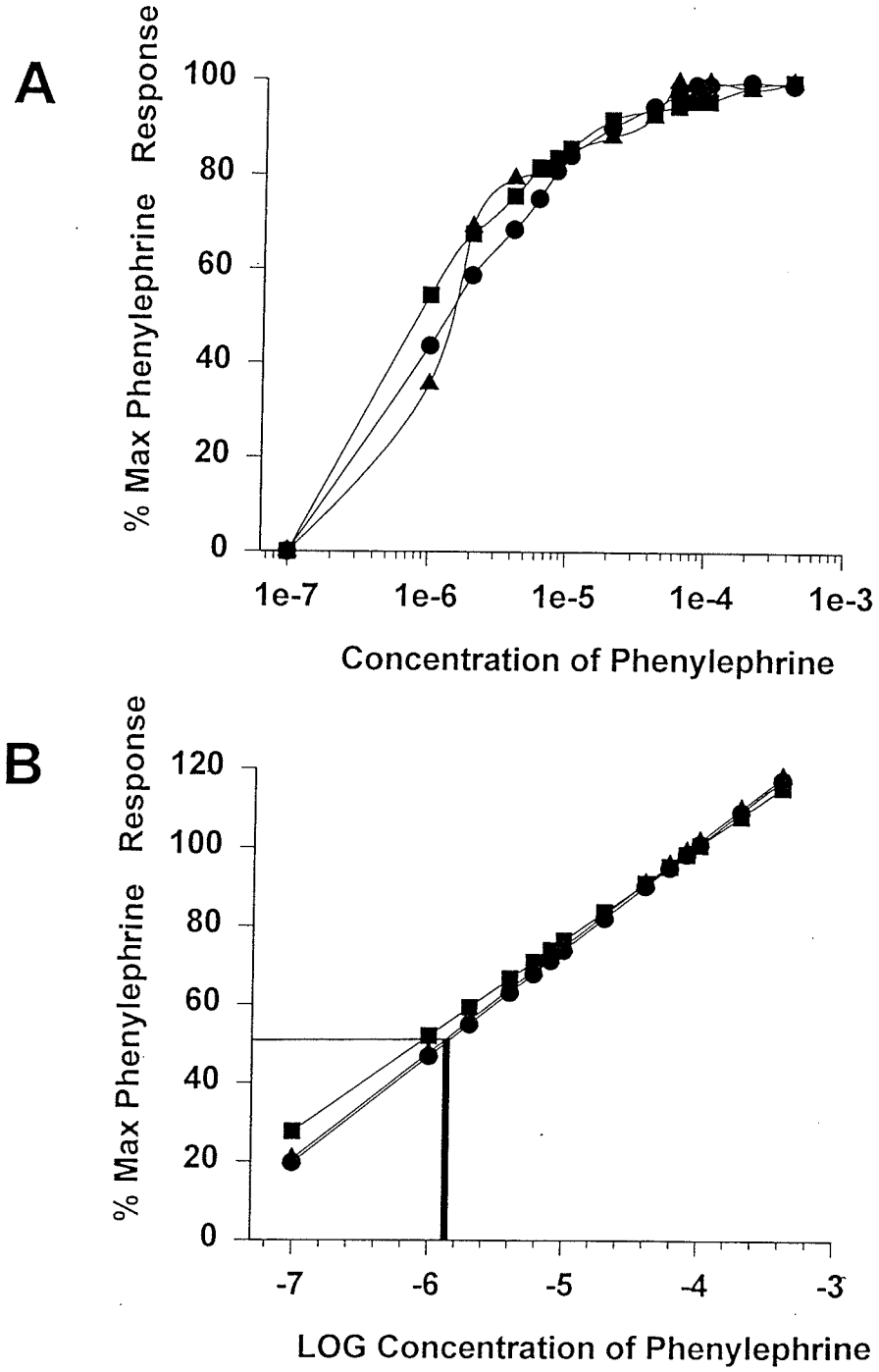

Figure 8. Dose Response Curve of Newborn Guinea Pig Main Pulmonary Arterial Preparations to Phenylephrine (PE). (A) Three newborn pulmonary arterial preparations were exposed to increasing cumulative doses of PE and the increase in force measured at each concentration was plotted as a percentage of the maximal PE contraction observed. **(B)** A plot of the LOG concentration of PE revealed that a 50% maximal PE contraction was observed at $2x10^{-6}M$.

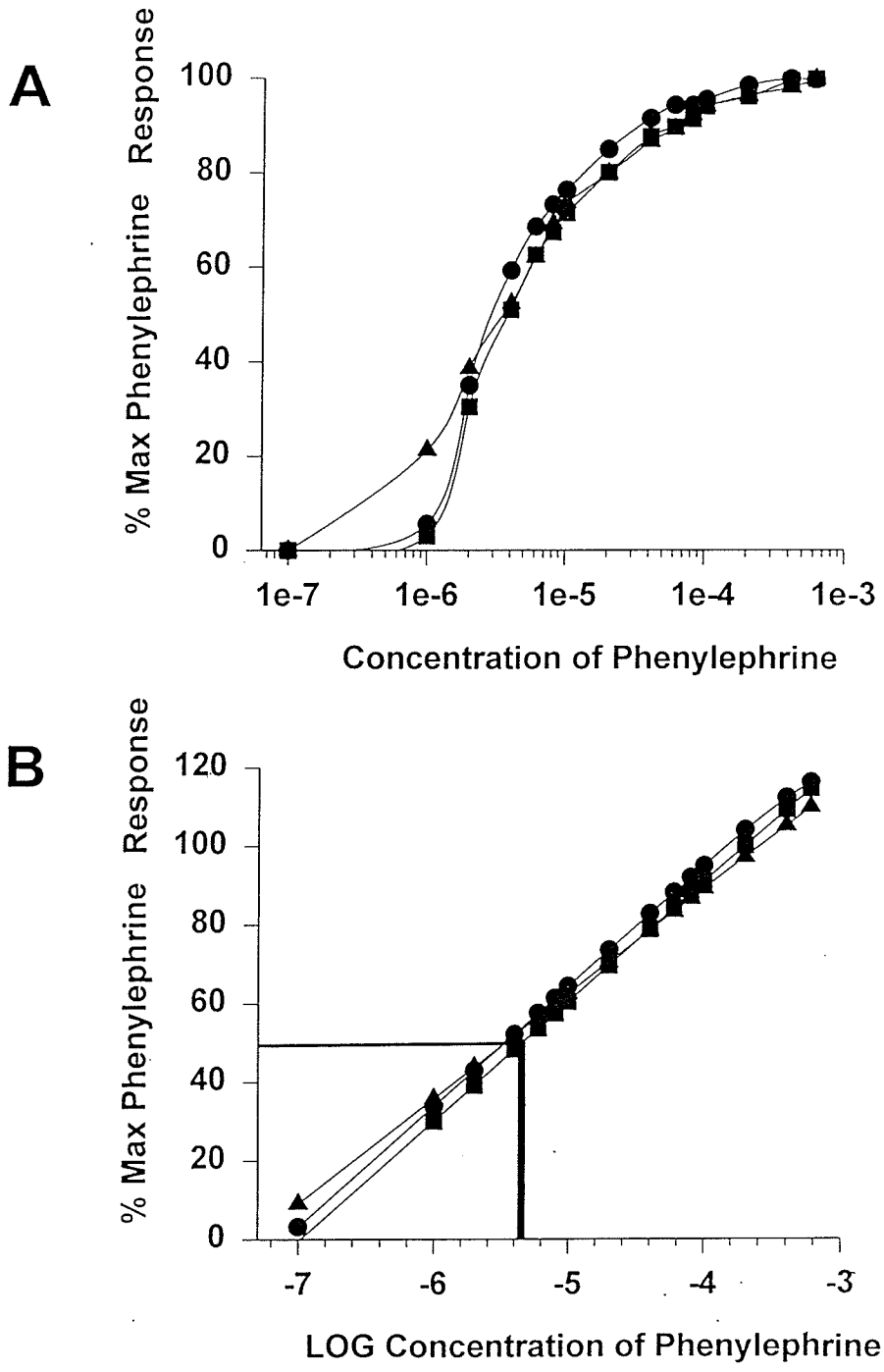

Figure 9. Dose Response Curve of Adult Guinea Pig Main Pulmonary Arterial Preparations to Phenylephrine (PE). (A) Three adult pulmonary arterial preparations were exposed to increasing cumulative doses of PE and the increase in force measured at each concentration was plotted as a percentage of the maximal PE contraction observed. **(B)** A plot of the LOG concentration of PE revealed that a 50% maximal PE contraction was observed at $6x10^{-6}$M.

Confirmation of Endothelial Integrity

The confirmation of endothelium maintenance or denudation following dissection was verified by the addition of cumulative molar doses of ACh to each precontracted pulmonary arterial preparation and by histological analysis (Figure 10). Endothelial presence was confirmed in both adult and newborn preparations by an observed relaxation to ACh. A maximal relaxation of 23.8 ± 6.8 % was observed in adult(+Endo) preparations at 10^{-5} M ACh, whereas in newborn(+Endo) preparations a maximal relaxation of 26.7 ± 4.7 % was observed at 10^{-6} M ACh ($p<0.05$) (Figure 11). Endothelial denudation abolished ACh mediated relaxation at these concentrations (Figure 10), yielding contractions of 21.6 ± 3.2 % in adult(-Endo) ($p<0.05$) preparations and 11.0 ± 1.2 % in newborn(-Endo) ($p<0.05$) preparations. Maximal contraction for both adult and newborn preparations was observed at 10^{-3} M ACh. A significant difference was observed in maximal ACh contraction between adult(+Endo) (9.4 ± 4.2 %) and (-Endo) (27.9 ± 3.0 %) preparations ($p<0.05$). This difference was not observed between newborn(+Endo) (18.7 ± 3.3 %) and (-Endo)(15.0 ± 1.6 %) preparations ($p<0.05$).

Lysophosphatidylcholine (LPC) Induced Smooth Muscle Vasorelaxation

Precontracted adult and newborn pulmonary arterial preparations with endothelium intact or denuded were exposed to cumulative molar doses of LPC (Figure 12). There was no significant difference between adult(+Endo) and newborn(+Endo) preparations in response to LPC ($p<0.05$) (Figure 13). A maximal relaxation of 66.5 ± 4.3 % for adult(+Endo) and 74.7 ± 6.1 % for newborn(+Endo) preparations was observed at 10^{-4} M (Figure 13). Endothelial denudation significantly suppressed, but never completely inhibited, LPC induced relaxation at this concentration (Figure 12) in both adult(-Endo) (14.9 ± 4.2 %) ($p<0.05$) and newborn(-Endo) (15.2 ± 4.8 %) ($p<0.05$) preparations (Figure

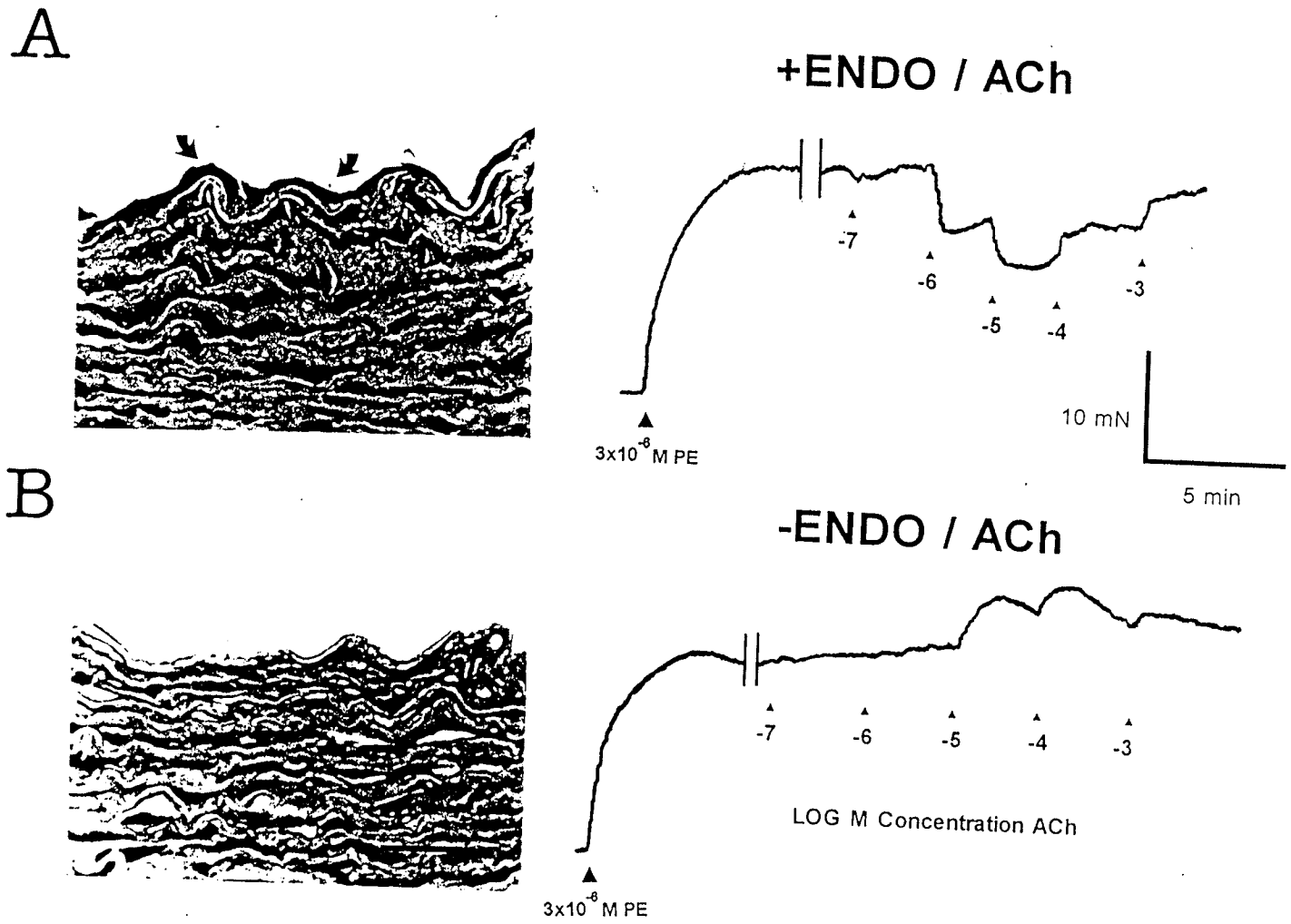

Figure 10. Confirmation of Endothelial Integrity In Pulmonary Arterial Preparations. The confirmation of the presence or removal of endothelium was achieved by exposing preparations to cumulative doses of the endothelial-dependent vasorelaxant acetylcholine (ACh) and by histological analysis. Adult arterial preparations with endothelium intact **(A)** (arrows) demonstrated a relaxation to ACh at 10^{-7}-10^{-5}M concentrations. Preparations denuded of endothelium **(B)** demonstrated no relaxation to ACh at 10^{-7}-10^{-6}M concentrations. Similar observations were made in preparations from newborns. Scale = 20μm.

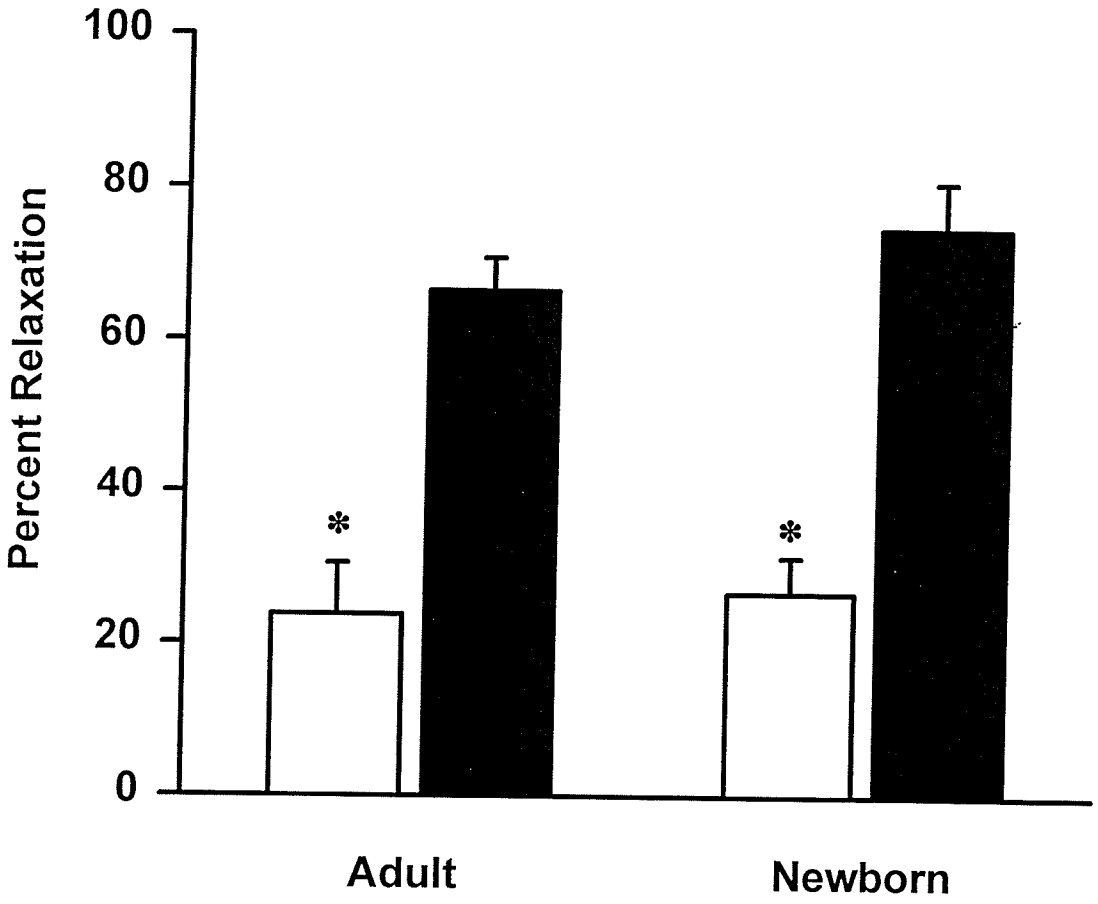

Figure 11. Comparison of Maximal Vasorelaxation of Adult and Newborn Guinea Pig Pulmonary Arterial Preparations in Response to Acetylcholine (ACh) and Lysophosphatidylcholine (LPC). Maximal ACh (□) mediated relaxation in pulmonary arterial preparations with endothelium intact (+Endo) was observed at 10^{-6}M in adults and 10^{-5}M in newborns. Maximal LPC (■) relaxation was observed at 10^{-4}M in both adults and newborns and was 2-fold greater than maximal ACh mediated relaxation (*$p<0.05$).

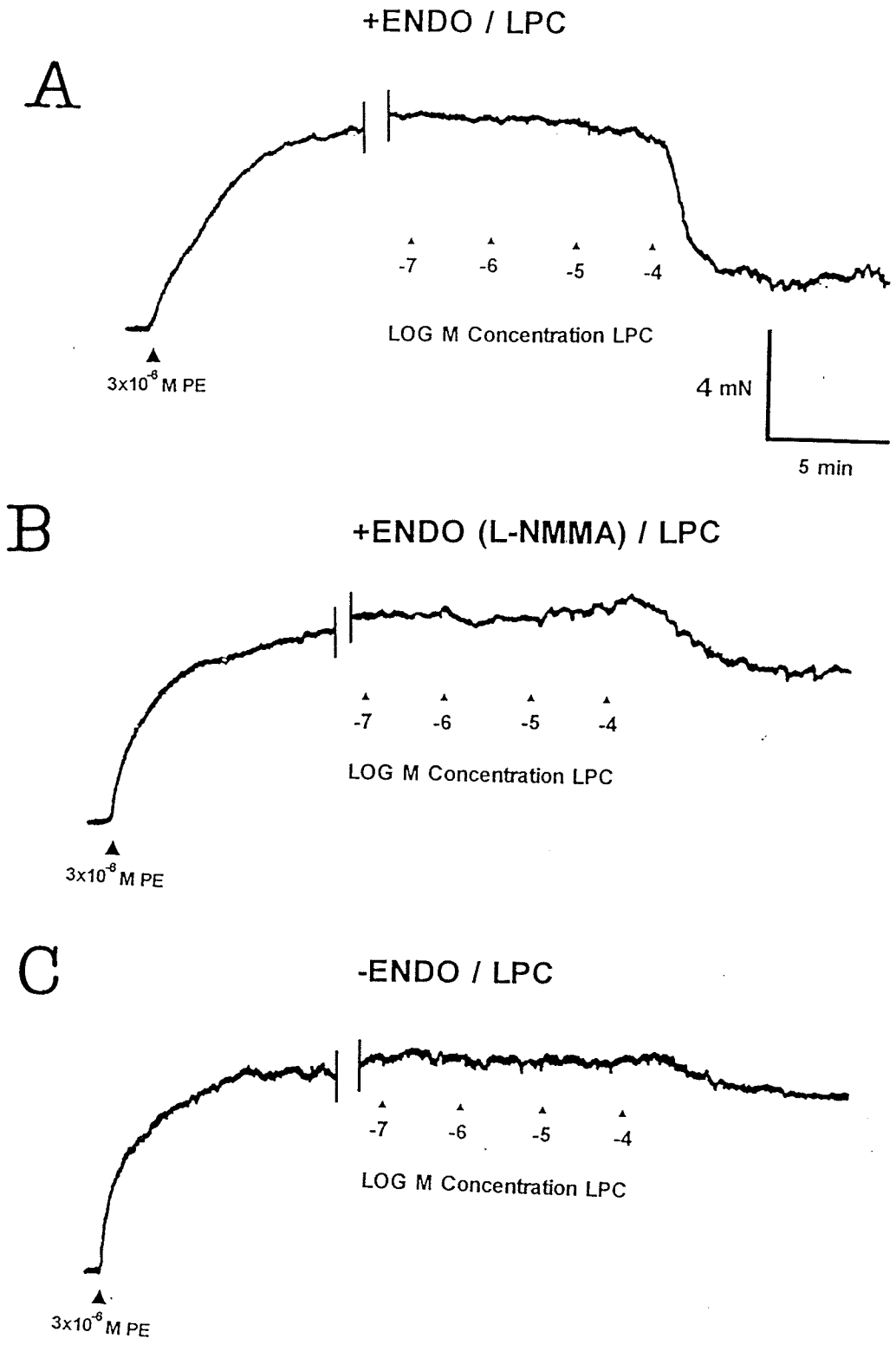

Figure 12. Contractile Changes of Guinea Pig Pulmonary Arterial Preparations to Lysophosphatidylcholine (LPC). Representative responses of phenylephrine precontracted newborn guinea pig pulmonary arterial preparations to LPC with **(A)** endothelium intact (+Endo), **(B)** endothelium intact and nitric oxide synthase blocked (+Endo(L-NMMA)), and **(C)** endothelium denuded (-Endo). Similar observations were made in adult preparations.

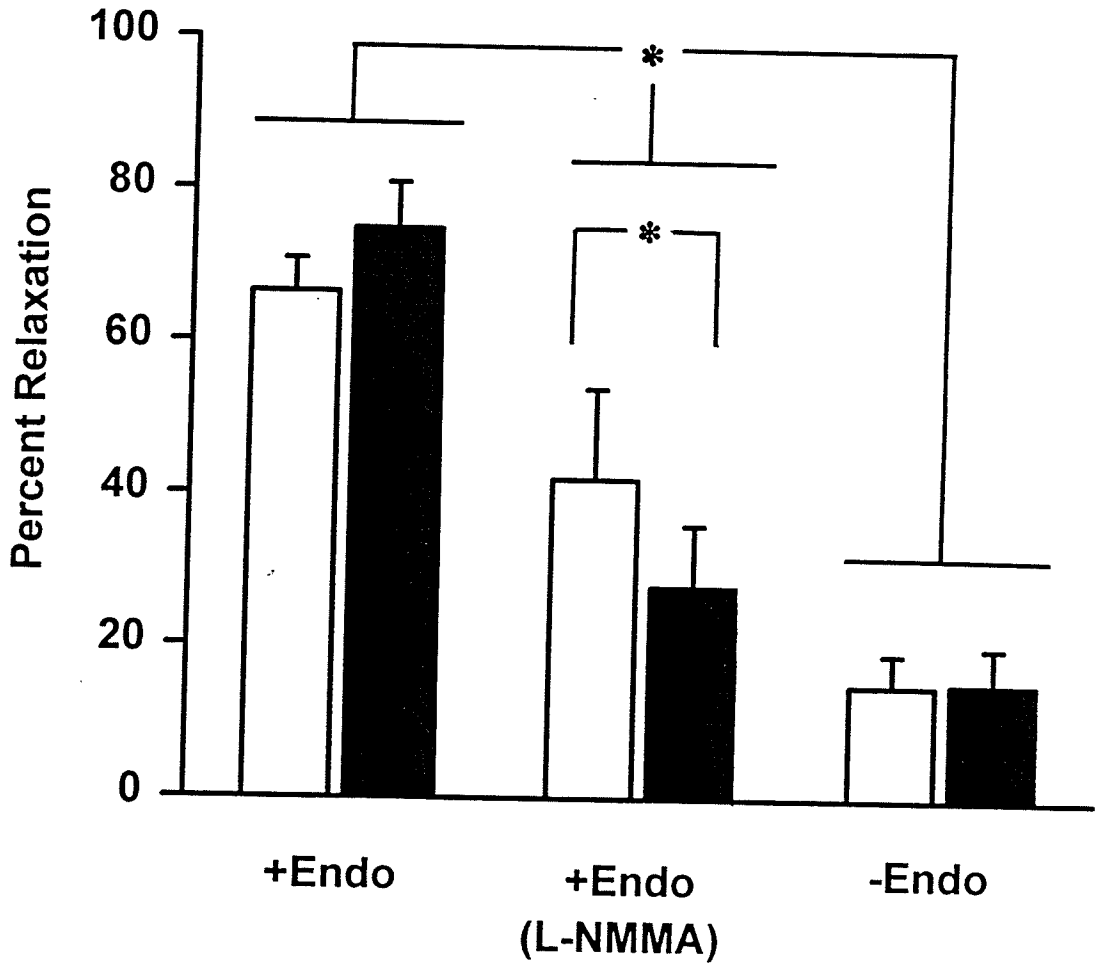

Figure 13. Maximal Vasorelaxation of Adult and Newborn Guinea Pig Pulmonary Arterial Preparations in Response to Lysophosphatidylcholine (LPC). Maximal LPC (100μm) induced vasorelaxation of phenylephrine precontracted adult (□) and newborn (■) guinea pig pulmonary arterial preparations with endothelium intact (+Endo), endothelium intact and nitric oxide synthase blocked (+Endo(L-NMMA)) and endothelium denuded (-Endo). Significant differences were observed between +Endo, +Endo(L-NMMA) and -Endo pulmonary arterial preparations between adult and newborn guinea pigs. A significant difference was also observed between adult(+Endo(L-NMMA)) and newborn(+Endo(L-NMMA)) pulmonary arterial preparations (* $p<0.01$).

13). Response to LPC following endothelial denudation was not significantly different between adult (-Endo) and newborn (-Endo) preparations.

Inhibition of Nitric Oxide Synthase (NOS) Activity

Precontracted adult(+Endo(L-NMMA)) and newborn(+Endo(L-NMMA)) pulmonary arterial preparations pre-incubated 30 minutes with 10^{-3} M L-NMMA, an inhibitor of NOS activity, were exposed to cumulative molar doses of ACh and LPC. This concentration of L-NMMA was observed to completely inhibit NO mediated ACh induced smooth muscle relaxation in both adult(+Endo(L-NMMA)) and newborn(+Endo(L-NMMA)) preparations (Figure 14). In the adult(+Endo(L-NMMA)) ACh induced vasorelaxation was inhibited in the presence of 10^{-3} M L-NMMA, yielding a 24.5 ± 3.8 % contraction at 10^{-5} M ACh. This was not significantly different from the 21.6 ± 3.2 % contraction observed at 10^{-5} M ACH in adult(-Endo) preparations ($p<0.05$).

Inhibition of NOS, however, did not completely suppress LPC mediated relaxation at 10^{-4} M, but significantly attenuated relaxation as compared with results observed in adult(+Endo) and newborn(+Endo) preparations (Figure 12). A maximal relaxation of 42.5 ± 12.0 % was observed at 10^{-4} M LPC in adult(+Endo(L-NMMA)) preparations ($p<0.05$) (Figure 13). At 10^{-4} M LPC, a maximal relaxation of 27.8 ± 8.2 % was observed in newborn(+Endo(L-NMMA)) preparations ($p<0.05$) (Figure 13). Attenuation in relaxation to LPC under NOS blockade was not as great, but significantly different between adult(-Endo) ($p<0.05$) and newborn(-Endo) ($p<0.05$) preparations. There was a significant difference observed in response to LPC at 10^{-4} M between adult(+Endo(L-NMMA)) and newborn(+Endo(L-NMMA)) preparations ($p<0.05$) (Figure 13).

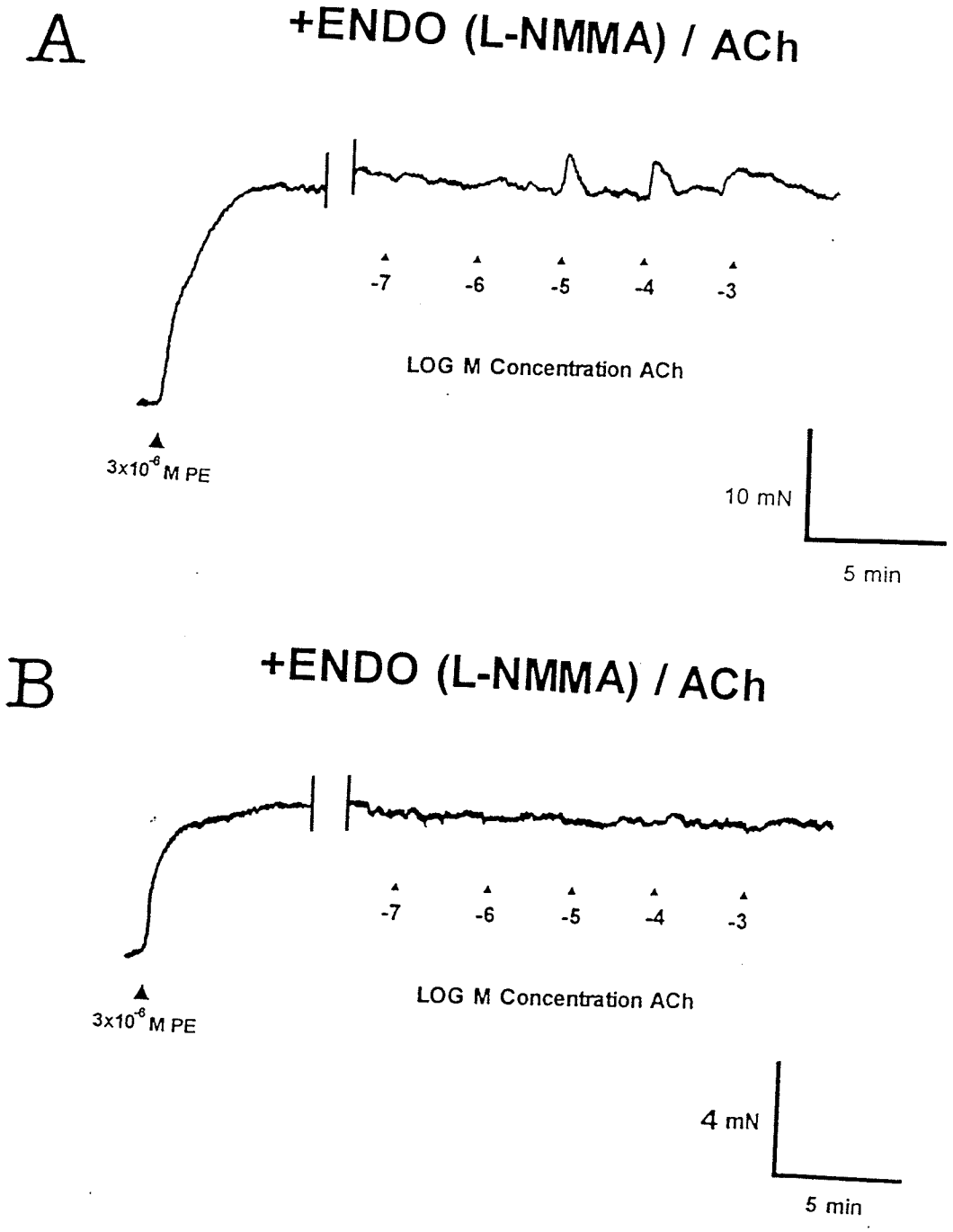

Figure 14. Inhibitory Effect of N^G-monomethyl-L-arginine (L-NMMA) on Acetylcholine (ACh) Mediated Vasorelaxation of Newborn and Adult Guinea Pig Pulmonary Arterial Preparations. L-NMMA (100μM) is a potent inhibitor of nitric oxide synthase activity, completely suppressing endothelium-dependent nitric oxide mediated ACh smooth muscle relaxation in both adult **(A)** and newborn **(B)** guinea pig pulmonary arterial preparations.

Inhibition of Guanylate Cyclase (GC) Activity

Precontracted adult(+Endo(MB)) and newborn(+Endo(MB)) pulmonary arterial preparations pre-incubated 20 minutes with varying concentrations (10^{-10}-10^{-M}) of MB, an inhibitor of GC activity, were exposed to 10^{-M} LPC. There was no significant difference in maximal LPC mediated relaxation in adult(+Endo) and newborn(+Endo) preparations in comparison to LPC relaxation observed in adult(+Endo(MB)) and newborn(+Endo(MB)) in the presence of 10^{-10} and 10^{-8}M MB (Figure 15). Attenuation of LPC relaxation to 10^{-M} LPC was equivalent in both adult(+Endo(MB)) and newborn(+Endo(MB)) preparations in the presence of 10^{-6} and 10^{-M} MB (Figure 15).

DISCUSSION

We have demonstrated that LPC is an effective vasorelaxant of both adult(+Endo) and newborn(+Endo) guinea pig pulmonary arterial preparations with maximal relaxation observed at 10^{-4} M (Figure 12, Figure 13). Significant inhibition in maximal LPC relaxation was observed in both adult and newborn preparations in the presence of 10^{-3} M L-NMMA ($p<0.05$) and following endothelial denudation ($p<0.05$) (Figure 12, Figure 13). There was no significant difference in maximal LPC relaxation between adult(+Endo) and newborn(+Endo) preparations or adult(-Endo) and newborn(-Endo) preparations ($p<0.05$) (Figure 13). However, significant age related differences were observed in degree of inhibition of LPC relaxation in the presence of 10^{-3} M L-NMMA between adult(+Endo(L-NMMA)) and newborn(+Endo(L-NMMA)) preparations ($p<0.05$) (Figure 13). Maximal LPC relaxation (10^{-M}) was attenuated equally in both adult(+Endo(MB)) and newborn(+Endo(MB)) preparations in the presence of increased concentrations (10^{-10}-10^{-M}) of MB (Figure 15). Accordingly, the most parsimonious explanation of these observations is that LPC is an equally effective vasodilator of the pulmonary vasculature in both adult

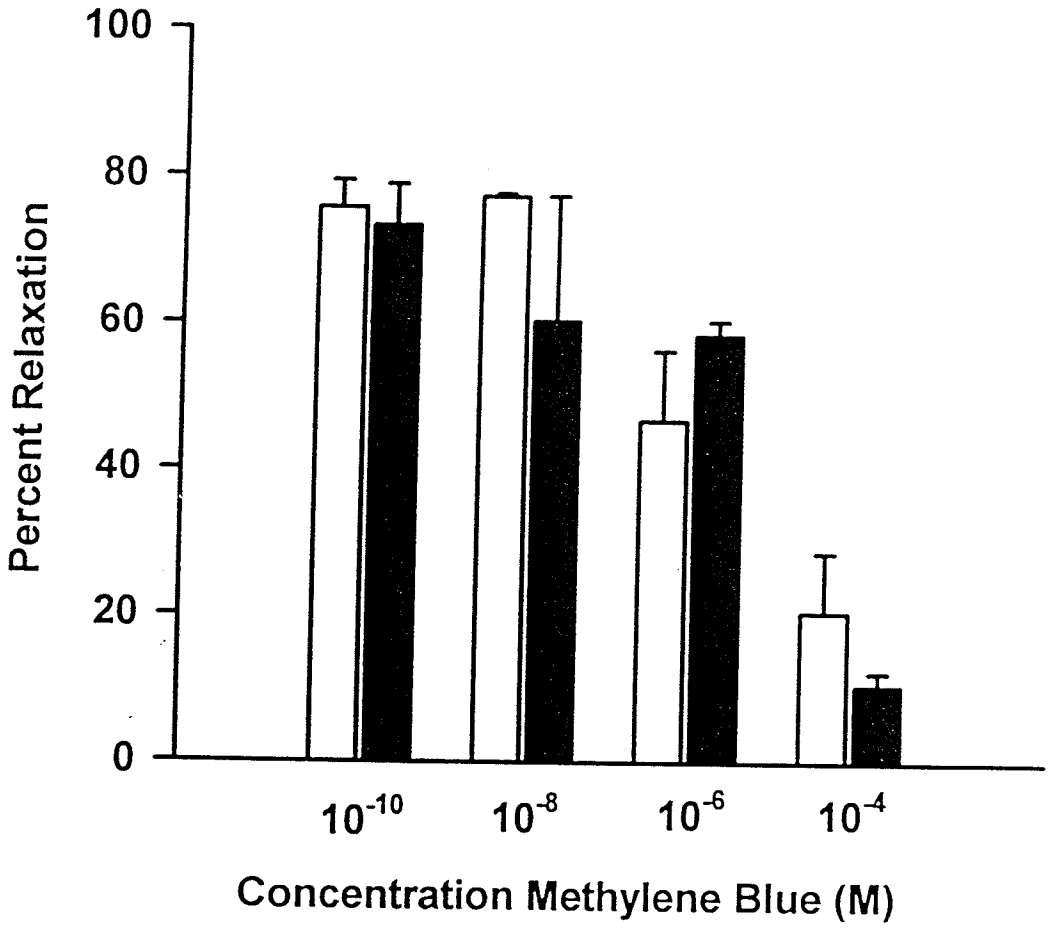

Figure 15. Inhibitory Effect of Methylene Blue (MB) on Maximal Lysophosphatidylcholine (LPC) Mediated Vasorelaxation in Newborn and Adult Guinea Pig Pulmonary Arterial Preparations. Comparison of maximal LPC mediated vasorelaxation of adult (□) and newborn (■) guinea pig pulmonary arterial preparations in the presence of varying concentrations of the guanylate cyclase inhibitor MB (+Endo(MB)).

and newborn guinea pigs. LPC induced vasodilation is mediated by both GC and NOS activity, however, in the newborn guinea pig the response is mediated to a greater extent by the NOS pathway compared to the adult which is more dependent on the GC pathway. Both act via cGMP and the observed difference may be a reflection of developmental differences in smooth muscle Ca^{2+}-ATPase activity between adult and newborn pulmonary arterial preparations or differences in the direct activation of Ca^{2+}-dependent K^+ channels by NO (Bolotina *et al.*, 1994).

At high concentrations LPC has a cytotoxic effect on cell membranes (Weltzien, 1979) causing electrophysiological perturbations (Kinnaird *et al.*, 1988) and cardiac arrhythmias (Corr *et al.*, 1979; Corr *et al.*, 1984). We exposed our preparations to maximal LPC concentrations of LPC at 10^{-4} M, a concentration previously demonstrated to decrease vascular resistance *in vivo* without producing arrhythmias or haemolysis (Bing *et al.*, 1988) This concentration of LPC was also previously demonstrated to maximally stimulate CDP-choline cholinephosphotransferase activity in rat liver microsomes (Parthasarathy and Bauman, 1979). Naturally derived surfactant preparations may contain as much as 500 nmoles LPC/ml of administered surfactant (Duncan *et al.*, 1995). Aside from containing quantities of LPC, the PC component of naturally derived surfactants is susceptible to PLA_2 deacylation yielding additional LPC (Duncan *et al.*, 1995). Although the fate of LPC in the pre-term lung following exogenous surfactant administration has not been completely clarified, it is possible that some may reach the pulmonary vasculature.

Histological examination confirmed the presence or denudation of endothelium in all preparations (Figure 10). However, as observed in arterial preparations from similar pharmacological experiments (Furchgott and Zawadzki, 1980), unrubbed preparations retained only 80-85% of their endothelial cells at the end of experimentation suggesting minimal loss of endothelium during dissection, experimentation or fixation.

It has been previously demonstrated in the adult guinea pig main pulmonary artery with endothelium intact that ACh (10^{-6} M) mediated a vasorelaxation of 60% in norepinephrine (10^{-6} M) precontracted preparations (Sakuma *et al.*, 1988). In the presence of 0.25 mM L-NMMA this relaxation was inhibited approximately 62% yielding a suppressed relaxation of 24% (Sakuma *et al.*, 1988). In our adult preparations with endothelium intact maximal relaxation to ACh (10^{-5} M) yielded a relaxation of 23.8 ± 6.8% following PE ($3x10^{-6}$ M) precontraction. In the presence of 10^{-3} M L-NMMA this relaxation was inhibited and a contraction of 24.5 ± 4.5% was observed. In the presence of 1mM L-NMMA, LPC mediated relaxation at 10^{-4} M was inhibited 66.0 ± 7.4% in the newborn(+Endo(L-NMMA)) preparation while yielding a 36.7 ± 16.2% inhibition in the adult(+Endo(L-NMMA)) preparation (Figure 13). These data would indicate that the vasoactive response of the pulmonary vasculature to LPC in the newborn preparations is more dependent on NOS activity compared with the adult.

Age related differences to vasoactive agents have been previously reported (Charpie *et al.*, 1994; Moritoki *et al.*, 1986). Age related differences in the endothelium dependency of the vasoactive potential of serotonin and endothelin-1 have been observed in human infant and adult vertebral arteries (Charpie *et al.*, 1994). As well, an age related decrease in vasodilator response to histamine has been reported in rat mesenteric arteries (Moritoki *et al.*, 1986).

Secretory Phospholipase A_2 (PLA_2) Deacylation of Surfactant Phosphatidylcholine (PC)

Introduction

In recent years NRDS has been treated successfully with exogenous surfactant replacement therapy (Jobe and Ikegami, 1987). Two surfactants currently approved for clinical use in Canadian hospitals and employed in replacement therapy are Exosurf® and Survanta®. Exosurf® is a completely synthetic surfactant comprised of 85% DPPC, 9% hexadecanol (cetyl alcohol), and 6% tyloxapol by weight (Phibbs *et al.*, 1991; Tooley *et al.*, 1987). DPPC is the major active component of natural surfactant (King, 1974; King and Clements, 1972; Klaus *et al.*, 1961) responsible for lowering surface tension at the air-alveolar interface. Hexadecanol acts as a spreading agent for DPPC whereas tyloxapol, a non-ionic surfactant, facilitates the dispersion of both DPPC and hexadecanol in the lung. Survanta®, a modified natural bovine surfactant, is comprised of phospholipid, neutral lipids, fatty acids and surfactant associated proteins (SP-B, SP-C) to which DPPC, palmitic acid, and tripalmitin have been added (Survanta® intratracheal suspension package insert).

The clinical effectiveness, frequency and minimal required number of doses of exogenous surfactant administration to preterm infants with NRDS have not been completely clarified and some important differences between synthetic and animal derived preparations have been reported. In preterm infants with NRDS, a single prophylactic dose of Exosurf® resulted in reduced oxygen supplementation requirements over 72 hours (Stevenson *et al.*, 1992) whereas a much shorter duration of action has been observed for animal-derived surfactant products (Kendig *et al.*, 1988; Merrit *et al.*, 1986a). In the case of calf lung surfactant extract, although a significant reduction in the need for oxygen

supplementation and ventilatory support was observed, such improvement was only evident 24 hours post-administration (Kendig *et al.*, 1988). In trials conducted with human amniotic fluid extract (Merrit *et al.*, 1986a), 71% of the treated infants required supplemental doses during the first 48 hours after birth due to increasing ventilatory requirements. In contrast to this apparent superiority of Exosurf® to animal-derived products, administration of this preparation in a preterm lamb model of NRDS was virtually without effect upon lung compliance, oxygenation indices and mortality as compared to the highly effective responses observed with Survanta® (Cummings *et al.*, 1992). The factors accounting for the discrepancies in duration of action and efficacy are probably complex and presently poorly understood yet may be related to differences in the surfactant preparation composition, catabolism and inactivation within the lung.

Recent reports suggest that activation and/or inactivation of exogenous surfactant preparations may be critical for their physiological efficacy. Recovery of the lung lavage material 5 hours post-Exosurf® and Survanta® administration to a preterm lamb revealed a much superior physiologic efficacy when readministered in a preterm rabbit model of surfactant deficiency than the original exogenous surfactant used to treat the lambs (Ikegami *et al.*, 1993). These observations suggest either the presence of inhibitors or absence of catalysts acting upon exogenously administered surfactant.

Many factors have been considered to play a role in the inactivation of exogenous surfactant, including pH (Merrit *et al.*, 1993) and proteins (Cockshutt and Possmayer, 1991; Enhorning *et al.*, 1992; Fuchimukai *et al.*, 1987; Holm and Waring, 1993; Holm *et al.*, 1988; Holm *et al.*, 1991; Ikegami *et al.*, 1984). *In vitro* addition of hypochlorous acid, which may be increased in some inflammatory lung diseases, to Survanta® and Exosurf® results in inactivation of surface tension lowering properties more so in the former

preparation (Merrit *et al.*, 1993). In a lamb model of NRDS, Jobe *et al.* (1988) demonstrated prolonged survival of lambs receiving exogenous, naturally derived surfactant. The improvement in ventilation, however, was short lived due to the inactivation of the surfactant by protein inhibitors present in the airways (Ikegami *et al.*, 1984).

Among the proteins that may alter the biophysical properties of exogenously administered surfactants, phospholipases are of great clinical relevance. PLA_2 hydrolyses PC at the 2-acyl position yielding a single free fatty acid and a LPC moiety. The inhibition of pulmonary surfactant biophysical activity by PLA_2 and its by-products have been demonstrated *in vitro* (Cockshutt and Possmayer, 1991; Enhorning *et al.*, 1992; Holm *et al.*, 1991). In addition, there is evidence that natural surfactant inactivation by increased alveolar levels of plasma PLA_2 may be involved in the pathogenesis of ARDS (Vadas, 1984; Vadas and Pruzanski, 1986). In humans, plasma and lung broncho-alveolar fluid from patients with ARDS have a high degree of PLA_2 activity (Offenstadt *et al.*, 1981; Romaschin *et al.*, 1992). This is further corroborated by observations from animal studies. In dogs, "ARDS like symptoms" were observed following intra-venous administration of PLA_2 (Morgan *et al.*, 1968). Experimentally induced acute pancreatitis in dogs lead to a 5-fold increase in PLA_2 activity from lung lavage samples and an associated decrease in lung DPPC (Das *et al.*, 1987). Increased lung levels of PLA_2 were also reported in rats following induced septic shock and respiratory failure (Von Wichert *et al.*, 1981).

In contrast, limited data on PLA_2 activity in the lung of newborns with NRDS and the possible inactivation of exogenous surfactant by PLA_2 are available. Data from our laboratory indicate that PLA_2 is present in the tracheal bronchial secretions of preterm infants with RDS during the first days of life (Belik *et al.*, 1994). We also have evidence

that indicates PLA_2 is increased nearly 3-fold in infants with NRDS. Yet little is known about the susceptibility of the different exogenous surfactant preparations, utilized in the treatment of these infants, to PLA_2 activity.

The purpose of this study was to evaluate the effect of a secretory-type, Ca^{2+}-dependent, alkaline pH optimum form of PLA_2 on the rate of PC degradation, as measured by LPC generation, in two exogenous surfactant preparations, the synthetic Exosurf® and naturally derived Survanta®. The effect of additions of components of Exosurf®, 6% tyloxapol and/or 9% hexadecanol, on PLA_2 mediated LPC generation was also investigated in preparations of Survanta® and pure synthetic DPPC.

Materials and Methods

Materials

PLA_2 (Type-III from bovine pancreas), cetyl alcohol (1-Hexadecanol), tyloxapol, L-α-DPPC, L-α-LPC, Tris (hydroxymethyl) aminomethane, calcium chloride, potassium chloride, ammonium molybdate, and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Chloroform, methanol, acetic acid (glacial), hydrochloric acid, perchloric acid and potassium phosphate were purchased from Fisher Scientific (Edmonton, AB). Thin layer chromatography (TLC) plates (Whatman K6 silica gel 60 Å, 250μm layer thickness) were purchased from CanLab Division of Baxter Co. (Winnipeg, MB). Survanta® (beractant) was obtained from Ross Laboratories (Columbus, OH) and Exosurf® neonatal (colfosceril palmitate) was obtained from Burroughs Wellcome Inc. (Kirkland, PQ).

Preparation of Phospholipase A_2 (PLA_2)

PLA_2 (Type-III, bovine pancreatic) was prepared by resuspending 25 mg of lyophilized PLA_2 into 500 µl of 50 mM Tris-HCl, pH 8.0, 3 mM $CaCl_2$ buffer. From this a 50 µl aliquot was removed and diluted in 450 µl of the same buffer. A 56 µl aliquot of this preparation was used in each assay to yield 3.36 Units PLA_2 activity/assay (0.672 Units/ml).

Assays for Phospholipase A_2 (PLA_2) Mediated Lysophosphatidylcholine (LPC) Generation in Exosurf® and Survanta®

The material from 3 new vials of Exosurf® or Survanta® were flash frozen in liquid nitrogen, lyophilized and resuspended in 5 ml of 50 mM Tris-HCl, pH 8.0, 3 mM $CaCl_2$ at a concentration of 10 mg/ml. Complete resuspension of the samples into mixed micelles was achieved by bath sonication for 45 minutes. Care was taken to ensure the samples were not exposed to temperatures exceeding 37°C during this period. The samples were pre-incubated at 37°C for a period of ten minutes prior to administration of 3.36 Units PLA_2 which initiated the start of the assay. Immediately following PLA_2 addition and at various intervals thereafter a 1 ml sample was removed and immediately placed into a test tube with 3 ml of chloroform:methanol (2:1 v/v) and vortex-mixed to terminate the reaction. The incubation was maintained at 37°C in an oscillating bath for the duration of the experiment. A 500 µl aliquot of 1% KCl was added to each isolated sample in chloroform:methanol to effect phase separation. Tubes were vortex-mixed and centrifuged at 2000 x *g* for 5 minutes. The upper aqueous phase was removed by aspiration and the organic phase washed twice with another 500 µl of 1% KCl. The remaining isolated organic phase was dried under nitrogen gas.

Determination of the Effects of Tyloxapol and Hexadecanol on Phospholipase A_2 (PLA_2) Activity

To determine the effects of tyloxapol and hexadecanol on PLA_2 activity in preparations of synthetic DPPC and Survanta®, LPC production over time was measured in the presence of tyloxapol, hexadecanol or a combination of the two according to the following protocol. Four new vials of Survanta® were lyophilized, and the powder obtained was pooled and mixed. 75 mg of Survanta® or synthetic DPPC were resuspended in 5 ml of 50 mM Tris-HCl, pH 8.0, 3 mM $CaCl_2$ buffer. Preparations were also resuspended in the presence of 5.29 mg of tyloxapol, 7.01 mg of hexadecanol, or a combination of 5.29 mg tyloxapol and 7.01 mg hexadecanol. These values of tyloxapol and hexadecanol comprised 6% and 9% of the dry weight of the preparations respectively, approximately the same percentage as reported for the synthetic Exosurf® (Phibbs *et al.*, 1991). The assays for all preparations were conducted as described above for Exosurf® and Survanta®.

Isolation of Lysophosphatidylcholine (LPC)

The dried organic phase for all samples was resuspended in 100 µl chloroform/methanol (2:1 v/v). A 25 µl aliquot of the organic phase of each sample was spotted on a 2.5 cm x 20 cm lane of a silica gel TLC plate, which had been cooled to room temperature following heat activation over a 3 hour incubation period (145°C) to remove moisture from the silica gel. A lane containing LPC standard was added to each plate for identification. The plates were immediately developed in a solvent system containing chloroform:methanol:acetic acid:water (50:30:8:3 v/v/v/v) for separation of LPC. LPC was completely resolved from PC and migrated with an R*f* value ≈ 0.09-0.10. After

complete drying, the plates were stained in an iodine chamber for lipid visualization. An equal area of silica gel corresponding to LPC for each lane was removed and the phosphorus mass measured.

Quantification of Lysophosphatidylcholine (LPC)

LPC content was quantified by a modification of the sensitive lipid phosphorus analysis of Rouser *et al.* (1969). In brief, silica gel corresponding to LPC for each sample was completely removed from the plate and collected into disposable 10 ml borosilicate glass culture tubes. Potassium phosphate standards (range 10 to 200 nmoles) were prepared. 450 µl of perchloric acid was added to each tube, covered with teflon tape to prevent loss of perchloric acid fumes, and the lipid digested by heating at 180°C for 60 minutes. Following complete cooling, the reagents added, in order, to each sample were: 2.5 ml of double distilled H_2O, 500 µl of 2.5% ammonium molybdate, and 500 µl of 10% ascorbic acid. The tubes were vortex-mixed immediately following the addition of each reagent. All preparations were subsequently incubated in hot water (95°C) for 15 minutes. After complete cooling and a brief centrifugation period for sedimentation of the adsorbent (5 minutes at 500 x *g*), the absorbance of all samples was read on a spectrophotometer at a wavelength of 820 nm.

Statistical Analysis

Results are reported as mean ± SEM. Data were analyzed by ANOVA for repeated measures and multiple comparison by Fisher's least significant difference test. LPC generation rates were compared using Student's t test for unpaired measures. Significance was accepted at $p < 0.05$.

Results

Phospholipase A_2 (PLA_2) Mediated Lysophosphatidylcholine (LPC) Generation in Preparations of Exosurf® and Survanta®

Preparations of Exosurf® and Survanta® (10 mg/ml) were incubated with PLA_2 for up to 120 minutes. Generation of LPC in the Survanta® preparations was non-linear with a breakpoint at 30 minutes. This is consistent with previous observations of PLA_2 mediated LPC generation (Gregory *et al.*, 1991). LPC generation increased at a rate of 7.6 ± 2.3 nmol/min in the first 30 minutes and decreased to a rate of 1.6 ± 0.2 nmol/min for the remainder of the incubation period ($p<0.05$) (Figure 16). A maximum value of 376 ± 84 nmoles of LPC was generated by 120 minutes. In striking contrast, there was negligible LPC generated over the entire duration of the experiment in the Exosurf® preparations.

Effects of Tyloxapol and Hexadecanol on Phospholipase A_2 (PLA_2) Mediated Lysophosphatidylcholine (LPC) Generation

Preparations of synthetic DPPC (15 mg/ml) were incubated with PLA_2 for up to 120 minutes in the absence or presence of 6% tyloxapol, 9% hexadecanol or both and the generation of LPC determined by TLC (Figure 17). The generation of LPC from synthetic DPPC preparations was non-linear, however, with a breakpoint at 15 minutes. LPC generation increased at a rate of 46.9 ± 16.3 nmol/min during the first 15 minutes and diminished to a rate of 10.6 ± 2.2 nmol/min for the duration of the assay ($p<0.05$) (Figure 17, Figure 18). A maximum of 1818 ± 247 nmoles of LPC was generated by 120 minutes. This represented a 15.5 ± 2.5% hydrolysis of the initial concentration of DPPC present.

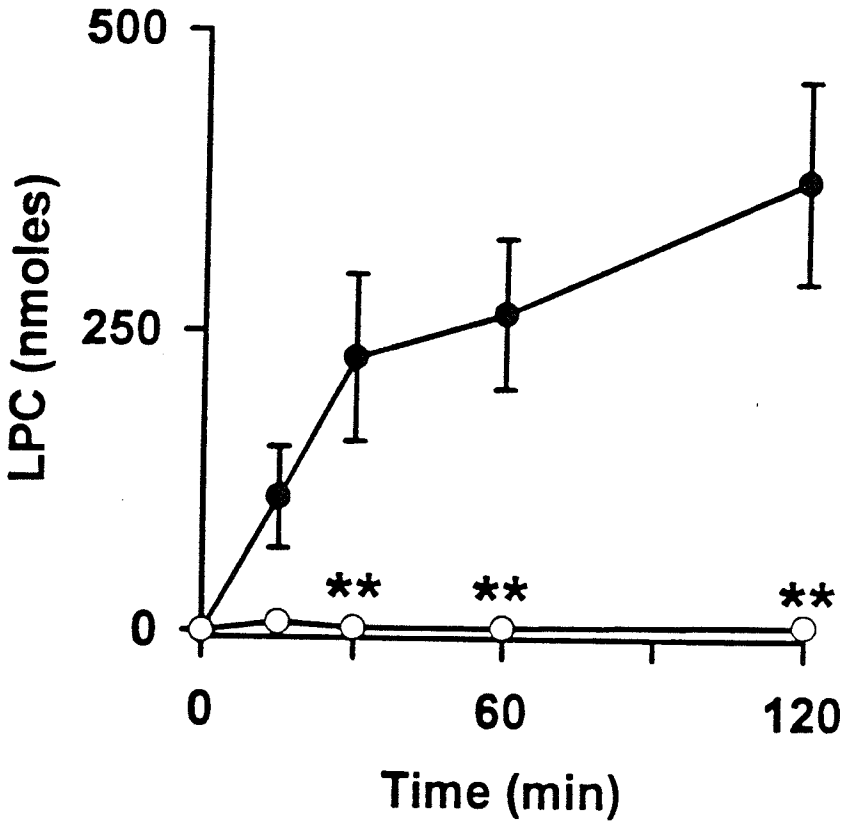

Figure 16. Phospholipase A_2 (PLA_2) Mediated Lysophosphatidylcholine (LPC) Generation in Preparations of Exosurf® and Survanta®. Generation of LPC by bovine pancreatic PLA_2 in preparations of Exosurf® (O) and Survanta® (●) (10 mg/ml) (**p<0.01). Note that there is negligible LPC generation in the Exosurf® preparation.

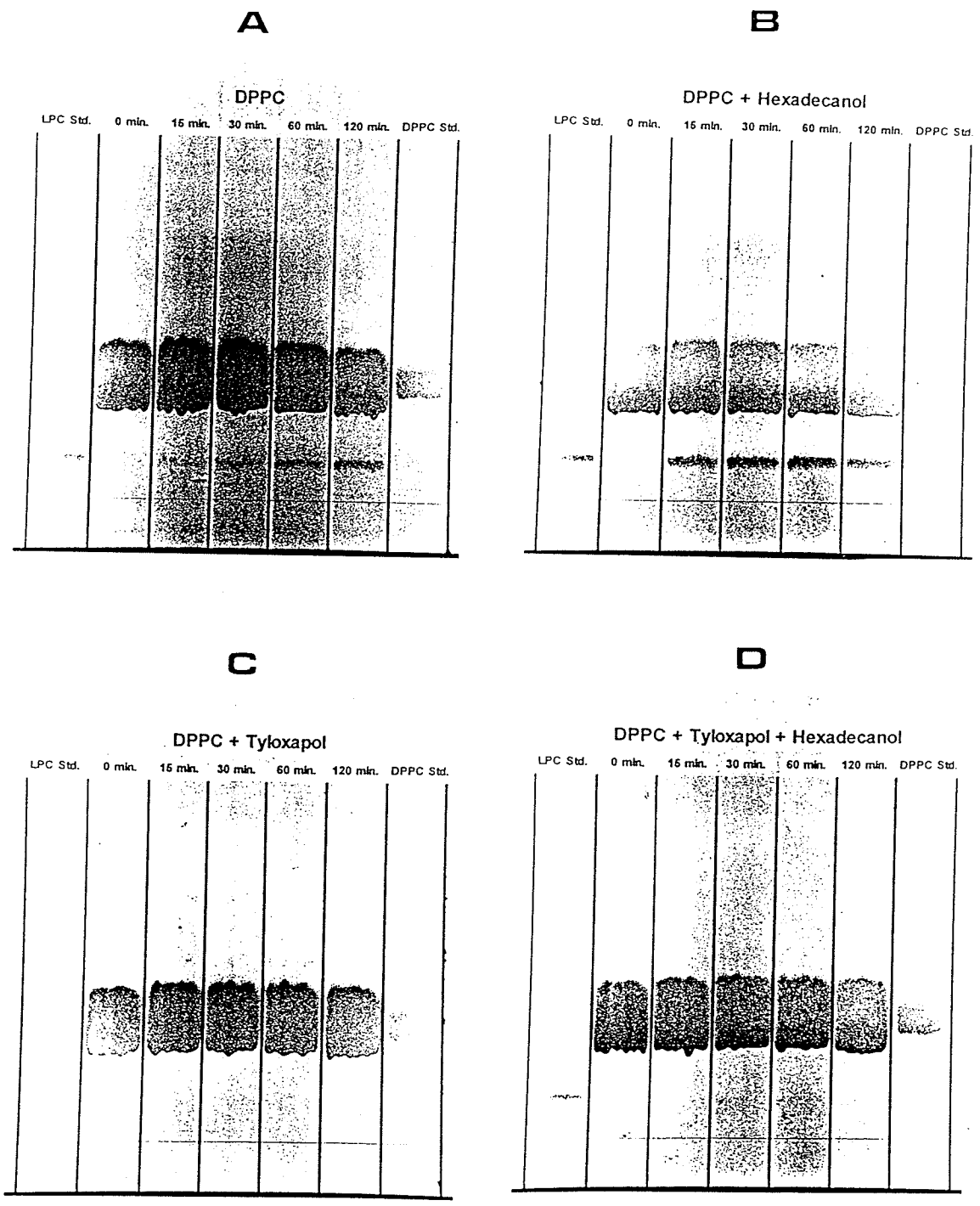

Figure 17. Thin Layer Chromatograms of Phospholipase A_2 (PLA_2) Degradation of Synthetic Dipalmitoyl Phosphatidylcholine (DPPC) in the Presence of Tyloxapol and/or Hexadecanol. Thin layer chromatograms showing the effect of 9% hexadecanol **(B)**, 6% tyloxapol **(C)**, and a combination of 9% hexadecanol and 6% tyloxapol **(D)**, on PLA_2 mediated generation of lysophosphatidylcholine preparations of pure synthetic DPPC **(A)**. Note that tyloxapol completely suppresses PLA_2 mediated DPPC deacylation.

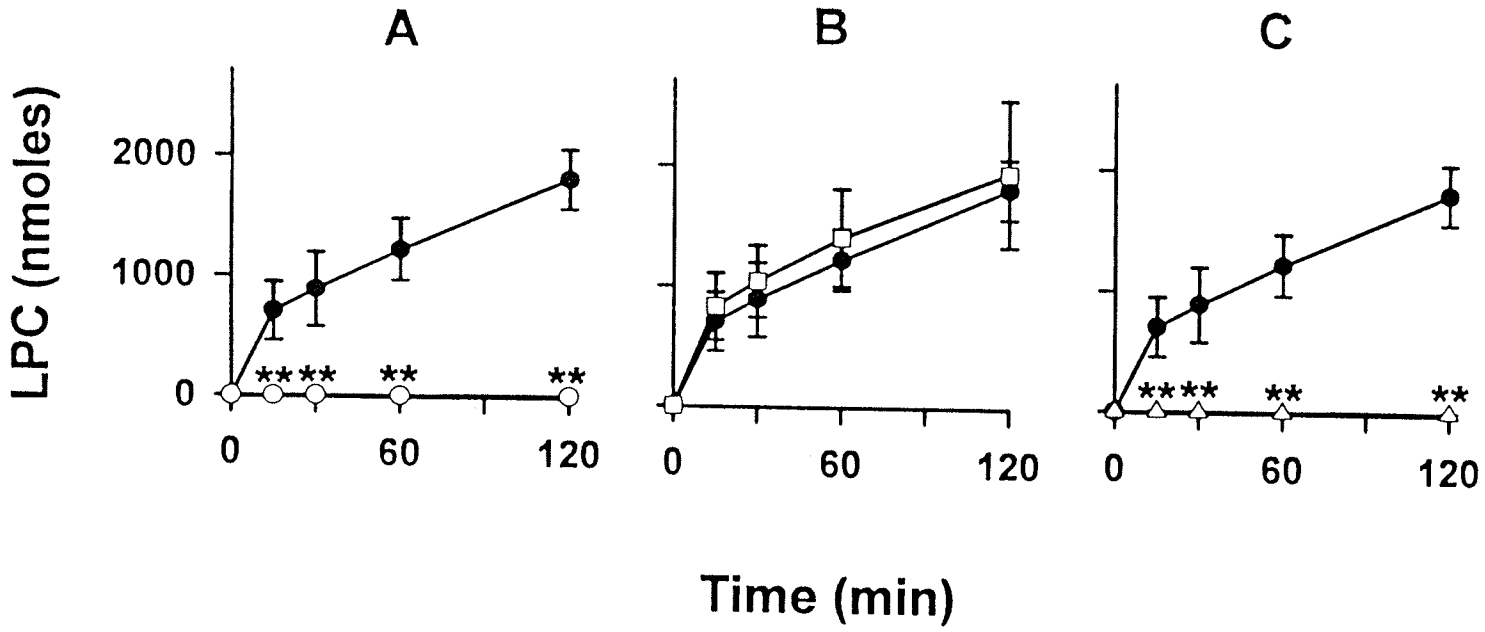

Figure 18. Quantification of Phospholipase A_2 (PLA_2) Degradation of Synthetic Dipalmitoyl Phosphatidylcholine (DPPC) in the Presence of Tyloxapol and/or Hexadecanol. Quantification of the effect of 6% tyloxapol (○), Panel **A**, 9% hexadecanol (□), Panel **B**, and a combination of 6% tyloxapol and 9% hexadecanol (△), Panel **C**, on secretory PLA_2 mediated lysophosphatidylcholine generation in preparations of pure synthetic DPPC (●) (15 mg/ml) (**$p<0.01$).

Addition of tyloxapol inhibited PLA_2 catalyzed deacylation of DPPC (Figure 17, Figure 18), yielding negligible LPC generation by 120 minutes (12 ± 3 nmoles).

Addition of hexadecanol to the synthetic DPPC preparation did not alter the rate or amount of LPC generated over the two hour assay period compared to control (Figure 17, Figure 18). LPC generation was also non-linear, increasing rapidly at 55.3 ± 18.6 nmol/min during the first 15 minutes and declining to a rate of 10.7 ± 3.2 nmol/min for the remainder of the assay. A maximum of 1951 ± 608 nmoles of LPC was generated by 120 minutes incubation.

The presence of both tyloxapol and hexadecanol attenuated LPC generation over the 120 minute assay period (Figure 17, Figure 18), similar to the results obtained for preparations in which tyloxapol alone was added. A maximum of 13 ± 1 nmoles of LPC was generated by 120 minutes incubation.

Preparations of Survanta® were incubated with PLA_2 for up to 120 minutes in the absence or presence of 6% tyloxapol, 9% hexadecanol or both and the generation of LPC determined by TLC (Figure 19). In the presence of PLA_2, LPC generation was non-linear increasing rapidly during the first 15 minutes of the assay at a rate of 18.7 ± 5.1 nmol/min and decreasing to 5.2 ± 0.8 nmol/min for the remainder of the assay period ($p<0.05$), yielding 829 ± 154 nmoles of LPC generated by 120 minutes (Figure 19, Figure 20). In the presence of tyloxapol, LPC generation over the two hour assay was significantly attenuated compared to control values. LPC generation during the first 15 minutes was 2.8 ± 1.2 nmol/min and was reduced to 1.0 ± 0.1 nmol/min for the remainder of the assay ($p<0.05$). After 120 minutes duration, 143 ± 13 nmoles of LPC were generated in Survanta® preparations incubated with PLA_2 in the presence of tyloxapol as compared with

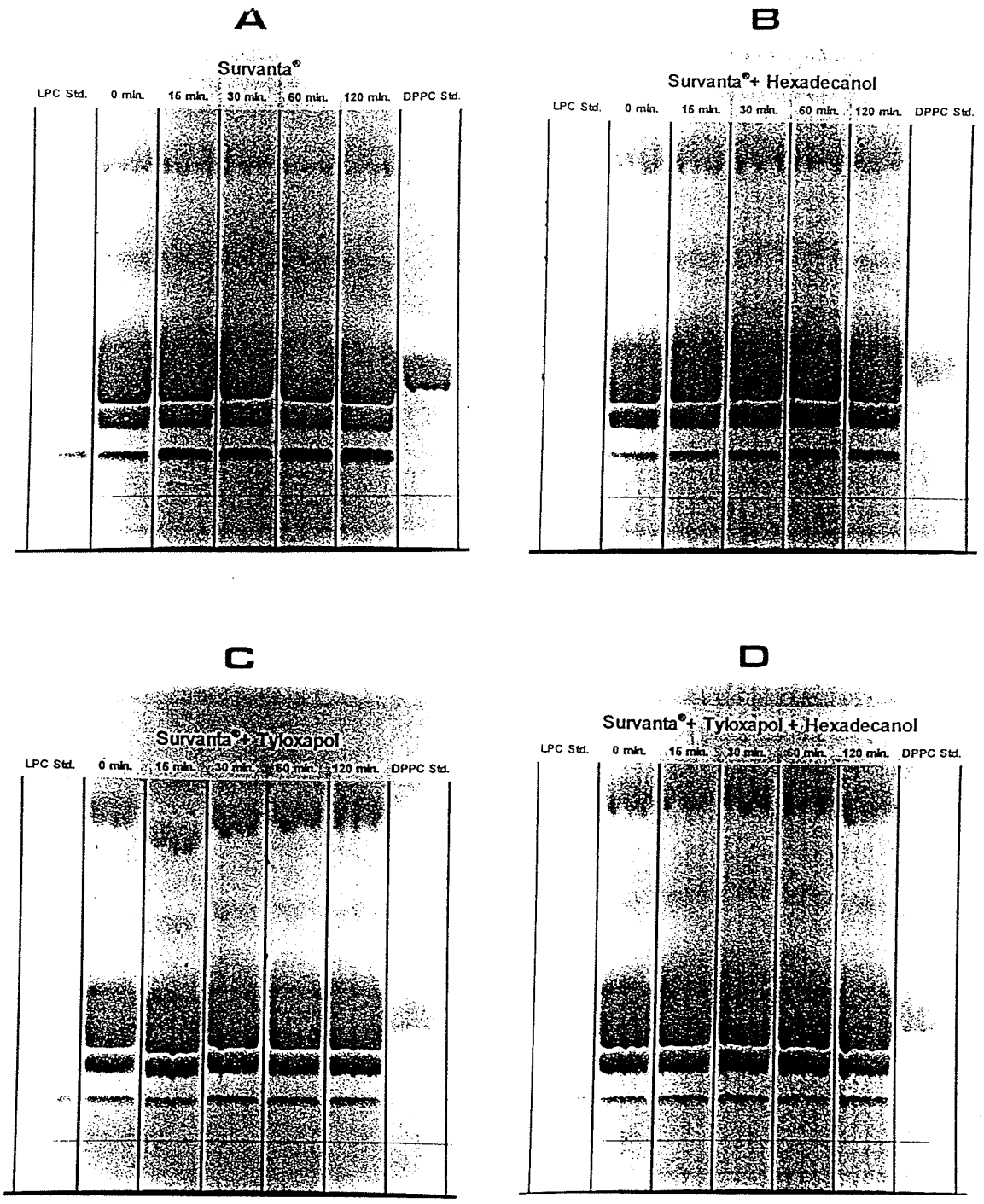

Figure 19. Thin Layer Chromatograms of Phospholipase A_2 (PLA_2) Degradation of Survanta® Phosphatidylcholine (PC) in the Presence of Tyloxapol and/or Hexadecanol. Thin layer chromatograms showing the effect of 6% hexadecanol **(B)**, 9% tyloxapol **(C)**, and a combination of 6% tyloxapol and 9% hexadecanol **(D)**, on PLA_2 mediated generation of lysophosphatidylcholine (LPC) in preparations of Survanta® **(A)**. Note that Survanta® naturally contains LPC as part of its composition, but this LPC content is not increased by PLA_2 deacylation in the presence of 6% tyloxapol.

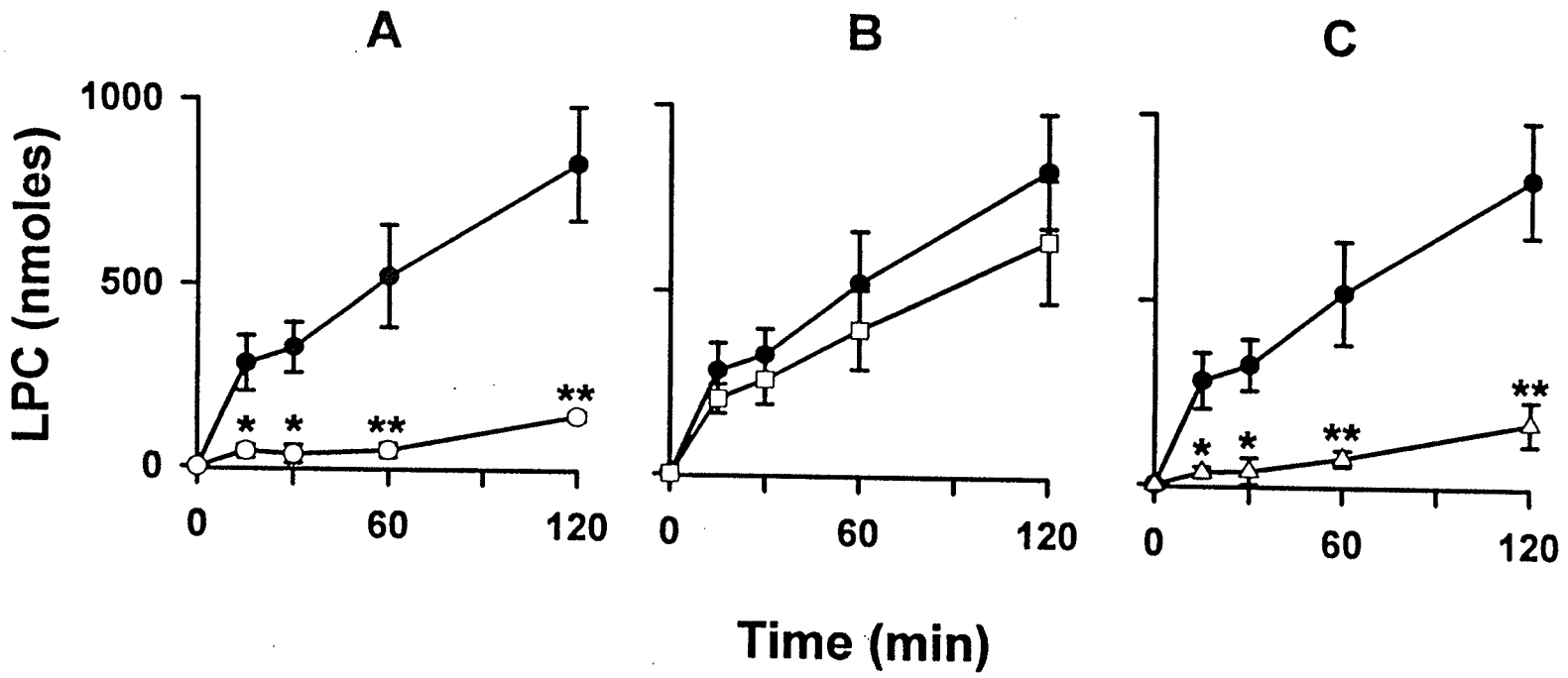

Figure 20. Quantification of Phospholipase A_2 (PLA_2) Degradation of Survanta® Phosphatidylcholine (PC) in the Presence of Tyloxapol and/or Hexadecanol. Quantification of the effect of 6% tyloxapol (○), Panel **A**, 9% hexadecanol (□), Panel **B**, and a combination of 6% tyloxapol and 9% hexadecanol (△), Panel **C**, on secretory PLA_2 mediated generation of lysophosphatidylcholine (LPC) in preparations of Survanta® (●) (15 mg/ml) (*$p<0.05$, **$p<0.01$).

12 ± 3 nmoles LPC in synthetic DPPC preparations incubated with tyloxapol.

In the presence of hexadecanol, PLA_2 mediated LPC generation in the Survanta® preparation over the two hour assay period was not significantly different from control values (Figure 19, Figure 20). LPC generation increased at a rate of 15.7 ± 3.8 nmol/min during the first 15 minutes and decreased to a rate of 4.1 ± 1.3 nmol/min for the duration of the assay. A total of 639 ± 288 nmoles of LPC was generated by 120 minutes incubation.

In the presence of both tyloxapol and hexadecanol, PLA_2 mediated LPC generation in Survanta® preparations was significantly attenuated compared to control values (Figure 19, Figure 20). The rate of LPC generation observed during the first 15 minutes was 2.3 ± 0.9 nmol/min, decreasing to a rate of 1.3 ± 0.4 nmol/min for the remainder of the assay, yielding 172 ± 59 nmoles of LPC by 120 minutes. The rate and amount of LPC generated in Survanta® incubations containing tyloxapol and hexadecanol were not significantly different from trials in which tyloxapol was the only additive.

Effect of Tyloxapol and Hexadecanol on Lysophosphatidylcholine (LPC) Separation by Thin Layer Chromatography (TLC)

Survanta® is a natural preparation of bovine surfactant which contains LPC as part of it's composition. In order to exclude the possibility that the presence of tyloxapol and/or hexadecanol influenced the separation and quantification of LPC, the amount of extractable LPC was determined in Survanta® preparations mixed with tyloxapol, hexadecanol or both. Survanta® preparations contained 564 ± 18 nmoles of LPC. The presence of tyloxapol, hexadecanol or both did not affect LPC separation by TLC or quantification by the sensitive lipid phosphorus assay.

Discussion

This study evaluated the susceptibility of preparations of Exosurf® and Survanta® to secretory type PLA_2 deacylation *in vitro*. We demonstrated that the PC component of the Survanta® preparation was readily susceptible to secretory PLA_2 deacylation whereas in the Exosurf® preparation there was negligible deacylation of the DPPC component, suggesting that one or more of its components protects its DPPC moiety from hydrolysis by PLA_2. Consequently, we investigated the effect of tyloxapol, hexadecanol and a combination of both on LPC generation in preparations of Survanta® and pure synthetic DPPC. We demonstrated that the addition of 6% tyloxapol (Figure 21) by weight to both DPPC and Survanta® preparations inhibited PLA_2 deacylation of synthetic DPPC and significantly attenuated deacylation of Survanta® PC. Addition of 9% hexadecanol did not effect the rate of deacylation by PLA_2 in either synthetic DPPC or Survanta®, but a combination of both tyloxapol and hexadecanol corresponded with results observed by the addition of tyloxapol alone.

Suppression of soluble PLA_2 activity by non-ionic detergents has been previously reported. Bennet and coworkers (1990) demonstrated that other non-ionic detergents such as Triton X-100, n-octylglucoside, and 3-((3-cholamidopropyl)-di-methyl-ammonio)-1-propanesulfonate (CHAPS) inhibited the activity of a secretory PLA_2, purified from guinea pig lung and immunologically related to pancreatic PLA_2. Tyloxapol is a non-ionic surfactant and the observed suppression of LPC generation when added to Survanta® and pure synthetic DPPC indicates that it too has an inhibitory effect upon secretory PLA_2 activity. Interestingly, addition of tyloxapol to the Survanta® preparation at similar concentrations as present in Exosurf® did not completely suppress PLA_2 mediated LPC generation. This evidence suggests that the PLA_2 inhibitory effect of this compound might

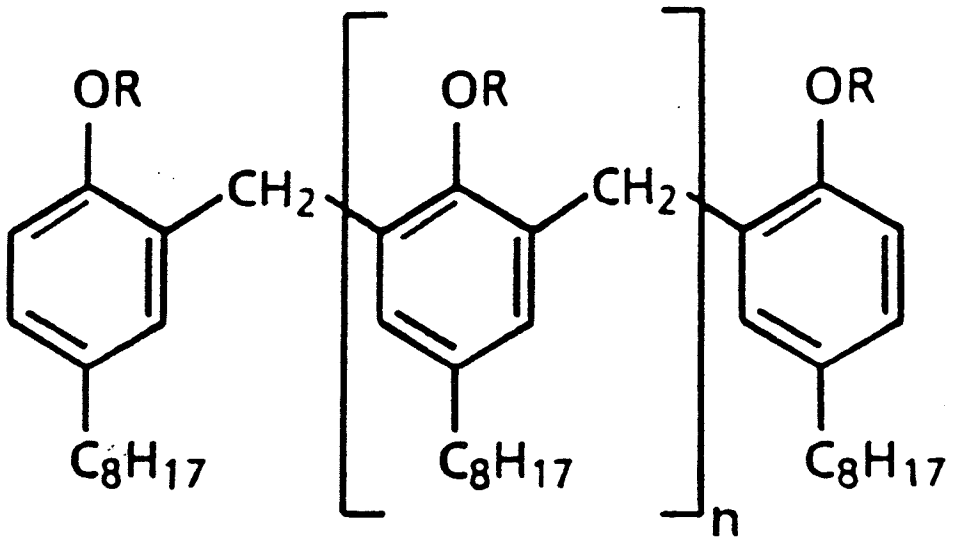

Figure 21. Chemical Name and Structural Formula of Tyloxapol. Tyloxapol is a nonionic, liquid, polymeric detergent used as an adjuvant in the synthetic lung surfactant Exosurf®. R=$CH_2CH_20(CH_2CH_2O)mCH_2CH_2O$; m=6 to 8; n=not more than 5 (from Burroughs Wellcome Co, Research Triangle Park, NC 27709).

be modulated by other substances present in Survanta® such as the apoproteins and free fatty acids. Finally, the present data clearly indicate that hexadecanol, the other component of Exosurf® has no effect upon the rate of LPC generation from PC following addition of PLA_2.

The susceptibility of DPPC, the main component of exogenous and natural surfactants, to PLA_2 activity may have significant clinical implications. A 42% decrease in DPPC, associated with a 5-fold increase in PLA_2 activity, was observed in lung lavage samples from dogs with induced pancreatitis (Das *et al.*, 1987). It is also known that in adults PLA_2 activity is present in broncho-alveolar lavage (BAL) material and is significantly increased in patients with respiratory conditions (Gregory *et al.*, 1991). It is apparent that the enzyme is active *in vivo* as demonstrated by the 4-fold greater LPC content, and corresponding decrease in DPPC content, in BAL material of patients with respiratory failure (Hallman *et al.*, 1982). In this study, the reported value of PLA_2 activity in BAL from adult patients with various lung disease was in the order of 0.1 nmoles/mg protein/min (Hallman *et al.*, 1982). Limited data regarding the lung content and activity of PLA_2 in newborns with RDS are available. Preliminary data from our laboratory (Belik *et al.*, 1994) indicated that PLA_2 was present in TBS material from infants with RDS in the order of 2-3 pmoles/mg protein/min. PLA_2 activity appears to be markedly higher in adults than in newborns, but direct comparisons between these two studies is difficult since the phospholipid substrate used in each was different. In the adult study, [^{3}H]DPPC was used as a substrate for PLA_2 activity whereas in the newborn study [^{14}C]dipalmitoyl phosphatidylglycerol ([^{14}C]DPPG) was used. It is well documented that secretory-type PLA_2 found in mammalian tissue exhibits a substrate preference for [^{14}C]DPPG over [^{3}H]DPPC (Kinkaid and Wilton, 1992; Ono *et al.*, 1988; Verger *et al.*, 1982). Differences

in the volume of BAL and TBS material recovered may also play a role in the observed differences.

Increased secretory PLA_2 activity, *in vivo*, could mediate significant DPPC deacylation rendering exogenous or even natural surfactant less physiologically active. An exogenous surfactant preparation resistant to PLA_2 deacylation could possibly result in longer lasting surface tension lowering properties. This may account for the observation that in premature infants with RDS administered exogenous surfactant on a single dose regimen, the reduction in oxygen supplementation with Exosurf® was much longer lasting than with animal-derived products (Kendig et al., 1988; Merrit *et al.*, 1986a; Stevenson *et al.*, 1992). In the present study we have demonstrated that the presence of tyloxapol confers the observed resistance to PLA_2 mediated DPPC hydrolysis in Exosurf®. DeAngelis and Findlay (1993) reported that upon administration, tyloxapol was retained by the lung and released slowly, with a half-life of 5-6 days, into the systemic circulation. This difference in duration of action between Exosurf® and animal-derived products may be a reflection of the presently observed differences in susceptibility Exosurf® and Survanta® to PLA_2 mediated PC deacylation.

The present data indicate that Exosurf® *in vitro* is resistant to PLA_2 deacylation and thus perhaps superior to the Survanta® preparation in this regard, yet the comparative human and animal data on the physiological efficacy of these two preparations are not fully supportive of my speculation. Two observations illustrate this point. Although differences were observed in initial response to treatment, the reduction in morbidity and mortality associated with the administration of these two preparations was of similar magnitude (Horbar *et al.*, 1993; Mondalou *et al.*, 1994). In the premature lamb, Exosurf® administration has been reported to be of no physiological value in comparison to

Survanta® (Cummings *et al.*, 1992).

The mechanism by which tyloxapol inhibits PLA_2 mediated deacylation of DPPC is presently unknown. Clearly, in spite of inhibition of its enzymatic activity by tyloxapol, the sole presence of PLA_2 should alter the biophysical properties of surfactants, as does the presence of non-enzymatic serum proteins (Cockshutt *et al.*, 1991; Fuchimukai *et al.*, 1987; Holm *et al.*, 1988). However, in a study on the inhibition of pulmonary surfactant function by phospholipases, Holm *et al.* (1991) demonstrated that the inactivation of surfactant biophysical activity was predominantly related to the effect of PLA_2 by-products, LPC and fatty acid, on the surfactant monolayer rather than the PLA_2 mediated decrease in DPPC, the principal surface active component. They reported a significant decrease in surfactant biophysical activity at LPC concentrations representing 10% hydrolysis of DPPC. Using pure synthetic DPPC in the present study, we demonstrated that control preparations were hydrolysed 15.5 ± 2.5% in the presence of 0.672 units PLA_2/ml at 120 minutes incubation. According to Holm and coworkers (1991), this percentage of DPPC deacylation was enough to significantly compromise surfactant biophysical activity. Yet, in the presence of 6% tyloxapol, DPPC deacylation was completely inhibited suggesting the potential maintenance of surfactant biophysical activity in the presence of PLA_2. In this regard, the prevention of generation of products of DPPC deacylation by tyloxapol may be a key to maintained surfactant biophysical activity.

Care must be exercised when extrapolating the present *in vitro* findings to the clinical arena. The observed *in vitro* differences in PC deacylation may not occur *in vivo* or be further modulated by other components of the alveolar micro-environment. Further studies addressing the importance of PLA_2-induced DPPC deacylation in newborns are necessary before any clinical decisions relative to the superiority of tyloxapol containing

versus tyloxapol free surfactant products is claimed.

In conclusion, significant differences in the susceptibility of Exosurf® and Survanta® to secretory-type PLA_2 were observed. These differences were due to the presence of tyloxapol, a non-ionic inhibitor of secretory-type PLA_2, in Exosurf® which inhibits PLA_2 mediated DPPC deacylation. This unique feature may explain the difference in duration of action between Exosurf® and animal derived products and may allow for a clinical advantage in using tyloxapol containing surfactant preparations.

Secretory Phospholipase A_2 (PLA_2) Activity in Tracheo-Bronchial Secretion (TBS) Samples

Introduction

The inhibition of surfactant biophysical activity *in vivo* is mediated by many factors including serum proteins, particularly enzymatic proteins such as phospholipases (Notter, 1984). PLA_2 is an important enzyme in the recycling and remodelling pathways of pulmonary surfactant PC, however increased activity of secretory PLA_2 in association with lung inflammation or bacterial infection are quite likely the cause of faster than normal breakdown of surfactant.

BAL samples have been previously analyzed for determination of PLA_2 activity and phospholipid profile in a limited number of primarily adult patients (Hallman *et al.*, 1982). These studies have demonstrated a moderate increase in lysolecithin fraction and PLA_2 activity, expressed on the basis of total BAL phospholipid, in the respiratory failure and lung disease group compared to control groups. This study attempted to determine the levels of secretory-type PLA_2 levels in the lung of preterm infants with and without NRDS by evaluating TBS samples collected during routine chest physiotherapy sessions. Merrit et al. acknowledge that there are presently no means of performing BAL in sick preterm infants, but TBS samples have yielded data which are consistent with BAL data (Merrit *et al.*, 1986b).

Materials and Methods

Study Patient Population

The study group used to evaluate the role of saline in tracheal suctioning consisted of 18 term and pre-term infants (Gest Age (GA) 33.1±1.0 weeks (mean±SE) (range 25-39

weeks), Birth Weight (BW) 2563.2±246.5 grams (range 630-3980 grams), Postnatal Age (PNA) 37.5±9.2 days (range 4-187 days)). Infants enroled included any neonate with an endotracheal tube in place and requiring chest physiotherapy irrespective of the primary lung pathology.

The study group used to determine secretory-PLA_2 activity in tracheal bronchial secretions consisted of 35 term and preterm infants (GA 30.5±0.8 weeks (range 24-41 weeks), BW 1705.2±191.8 grams (range 485-4200 grams). Of the 35 infants enroled, 27 were pre-term (GA 28.5±0.7 weeks (range 24-35 weeks), BW 1218.2±121.9 grams (range 485-2860)) and 8 were term (GA 37.1±0.6 weeks (range 36-41 weeks), BW 3349.0±308.4 grams (range 1500-4200 grams)). 26 infants (GA 29.04±0.89) were diagnosed with RDS and 9 infants with complications other than RDS including PPHN and MAS. A total of 129 TBS samples were collected from all individuals and analyzed for secretory PLA_2 activity. The number of samples collected from each individual was dependent on the duration of stay in the NICU and the period of tracheal intubation; 26 individuals had <5 TBS samples in total collected, 5 individuals had ≥5 but <10 samples collected, and 4 individuals were studied intensively over time having ≥10 samples collected.

Materials

L-α-LPC, L-α-DPPC, potassium chloride and calcium chloride were purchased from Sigma Chemical (St. Louis, MO). EcoLite(+) scintillation cocktail was purchased from ICN Biomedicals (Mississauga, ON). L-3-phosphatidyl[N-methyl-^{3}H]choline,1,2-dipalmitoyl was purchased from Amersham Life Science (Oakville, ON). Chloroform, methanol, acetic acid were purchased from Fisher Scientific (Edmonton, AB). Thin layer chromatography (TLC) plates were purchased from CanLab Division of Baxter Co. (Winnipeg, MB).

Tracheo-Bronchial Secretions (TBS)-Secretory Phospholipase A_2 (PLA_2) Activity Study

Infants studied were intubated (nasally or orally) for ventilatory support. When clinically indicated for pulmonary toilet, tracheal bronchial aspirations were performed by endotracheal instillation of 0.5 to 1.0 ml of 0.9% NaCl and routine manual ventilation. Following manual ventilation, suction catheters (No. 6F and 8F) were introduced not beyond the distal end of the endotracheal tube and secretions suctioned. Aspirates were collected in 40 cc specimen traps (Sherwood medical). Up to 1.5 ml of saline was aspirated through the catheter into the collection trap to rinse the aspirates from the side wall of the catheter. The volume of sample recovered was recorded to allow quantification of PLA_2 activity on a per ml TBS recovered. Samples were refrigerated (4°C) immediately following collection for a period not exceeding 6 hours.

Tracheo-Bronchial Secretions (TBS)-Saline Instillation Study

Two tracheal bronchial secretion samples were collected, four hours apart, from each infant during routine chest physiotherapy. The first sample was obtained as described above and the second obtained at a subsequent session was also collected as described above without saline instillation. The collection order was reversed when samples were obtained from infants previously enroled. Samples were refrigerated (4°C) immediately following collection for a period not exceeding 6 hours.

Determination of Protein Content

Tracheal bronchial secretion samples were transferred from aspirate specimen traps and placed into 4ml flint glass vials. The aspirate traps were rinsed with 1 ml 0.9%

NaCl solution also collected in the glass vials. The samples were frozen and maintained at -80°C for no longer than 2 weeks prior to analysis. This storage period was previously demonstrated not to significantly alter the PLA_2 activity (Hallman *et al.*, 1982). Preparations were lyophilized and resuspended in 0.3 ml of ddH_2O. A 20 µl aliquot was removed and the protein concentration determined using the Bio-Rad microassay procedure based on the method of Bradford (1976). Protein was determined using bovine serum albumin as a standard.

Determination of Phospholipase A_2 (PLA_2) Activity

Tracheal bronchial secretions (TBS) were analyzed for phospholipase A_2 activity using a modified protocol of Tijburg and coworkers (1991). The reaction mixture contained, in a total volume of 1.0 ml, 50 mM TRIS-HCl (pH 8.0) 3mM $CaCl_2$, 100-200 µg of TBS protein, and 100 nmoles of dipalmitoyl phosphatidyl[N-methyl-^{3}H]choline substrate (0.5 µCi/assay). The assay was incubated at 37°C in an oscillating bath for 60 minutes, and was terminated by the addition of 2 ml of chloroform:methanol (2:1 v/v). A 1 ml aliquot of 1% KCl was added to the incubation mixture in chloroform methanol to effect phase separation. Preparations were vortex-mixed and centrifuged at 2000 x g for 5 minutes. The upper aqueous phase was removed by aspiration and the organic phase washed twice with another 1 ml of 1% KCl. The remaining isolated organic phase was dried under nitrogen gas.

Isolation of Lysophosphatidylcholine (LPC)

The dried organic phase for all samples was resuspended in 100 µl chloroform/methanol (2:1 v/v). A 25 µl aliquot of the organic phase of each sample was

spotted on a 2.5 cm x 20 cm lane of a silica gel TLC plate, which had been cooled to room temperature following heat activation over a 3 hour incubation period (145°C) to remove moisture from the silica gel. A lane containing LPC standard was added to each plate for identification. The plates were immediately developed in a solvent system containing chloroform:methanol:acetic acid:water (50:30:8:3 v/v/v/v) for separation of LPC. LPC was completely resolved from PC and migrated with an R*f* value ≈ 0.09-0.10. After complete drying, the plates were stained in an iodine chamber for lipid visualization. An equal area of silica gel corresponding to LPC for each lane was removed and placed into scintillation vials with 5 ml counting scintillant (Ecolite®) and radioactivity measured in a scintillation counter 24 hours later.

PLA_2 activity is expressed in units of picomoles (pmole) LPC generated/min incubation/mg protein present in sample (pmole/min/mg). Standardized PLA_2 activity is expressed in units of pmoles LPC generated/min incubation/mg protein present in sample/volume of sample (pmole/min/mg/ml).

Statistical Analysis

Results are reported as mean±SEM. Data were analyzed using Student's t-test for paired measures, Student's t-test for unpaired measures, and multi-way analysis of variance (MANOVA) and multiple comparison by Newman Keuls test.

Results

Tracheo-Bronchial Secretions (TBS)-Saline Instillation Study

The TBS samples obtained following normal saline instillation prior to routine tracheal suctioning of intubated neonates had a significantly greater protein content

(1.18±0.11 μg/μl) compared to those TBS samples obtained without saline instillation (0.98±0.11 μg/μl) ($p<0.05$) (Figure 22 A). There was no significant difference observed between protein content removal of TBS samples using saline instillation prior to suctioning in term (1.27±0.12 μg/μl) and preterm (1.09±0.18 μg/μl) infants (Figure 22 B).

Tracheo-Bronchial Secretions (TBS)-Secretory Phospholipase A_2 (PLA_2) Activity Study

The overall average of all TBS samples obtained following normal saline instillation prior to routine tracheal suctioning demonstrated no significant difference in protein content removal between intubated Non-RDS (1.07±0.13 μg/μl) and RDS (0.85±0.06 μg/μl) neonates (Figure 23 A). However, intubated RDS infants did demonstrate a significantly increased average PLA_2 activity (2.79±0.31 pmole/min/mg) compared with intubated Non-RDS (1.33±0.20 pmole/min/mg) individuals ($p<0.05$) (Figure 23 B). As well, a significant increase in standardized PLA_2 activity was also demonstrated between RDS (7.68±0.93 pmole/min/mg/ml) and Non-RDS (3.10±0.71 pmole/min/mg/ml) individuals ($p<0.05$) (Figure 23 C).

PLA_2 activity was also determined to be an early marker for respiratory distress in severely premature individuals as demonstrated by multi-way comparison of average PLA_2 activity of three groups of individuals; 1) RDS - 24-29 weeks GA, 2) RDS - >30 weeks GA, and 3) Non-RDS >30 weeks GA over three sampling time periods; 1) <24 hours, 2) 24-72 hours, and 3) 73-200 hours post parturition (Table 2).

Discussion

The instillation of normal saline during routine collection of TBS samples yielded

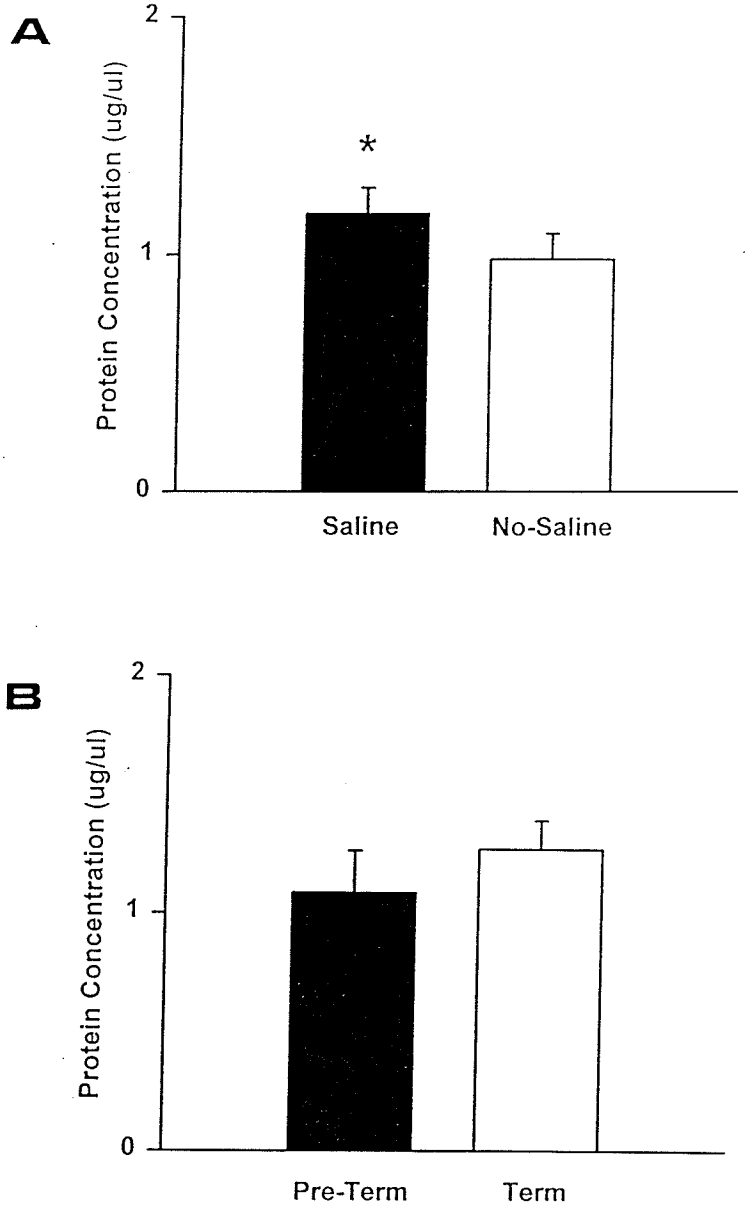

Figure 22. Effect of Saline Instillation on Tracheal Secretion Removal During Tracheo-Bronchial Suctioning in Infants. A) The use of saline prior to tracheal suctioning results in a significantly increased removal of tracheo-bronchial secretions (TBS) as quantified by protein concentration (*$p<0.05$). **B)** The use of saline in term and pre-term infants was equally beneficial in removing TBS samples as quantified by protein concentration.

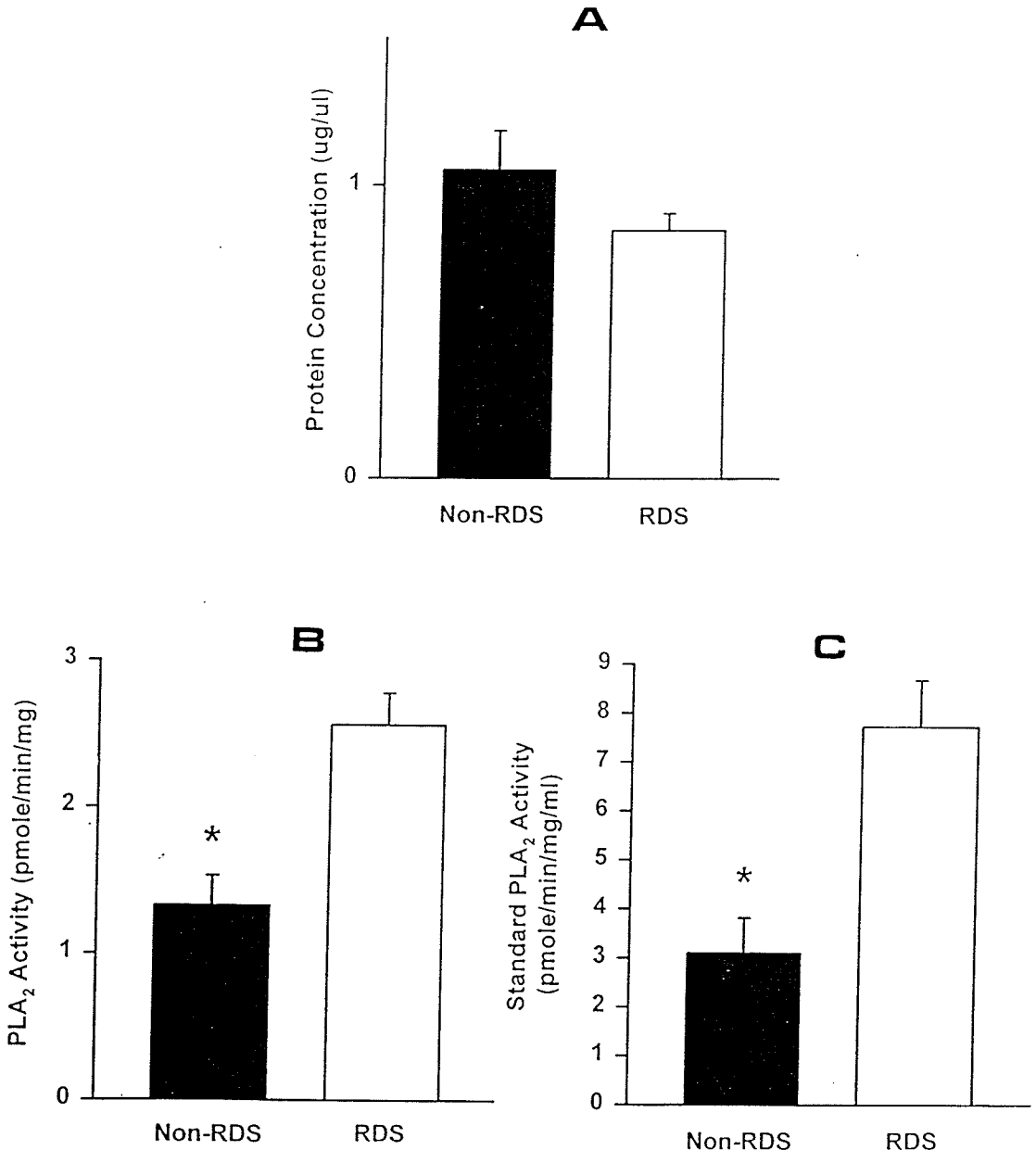

Figure 23. Phospholipase A_2 (PLA_2) Activity in Tracheo-Bronchial Secretion (TBS) Samples. Although Non-RDS infants and RDS infants had an equal protein content in the TBS samples collected with saline instillation **(A)**, measurable PLA_2 activity was much greater in RDS infants **(B)** even after standardizing to volume of TBS sample collected **(C)**.

Table 2. Tracheal Bronchial Secretion Phospholipase A_2 Activity in Premature Neonates. Phospholipase A_2 activity is given as pmole/min/mg/ml

	Sample Time		
	<24 hours	**24-72 hours**	**72-200 hours**
Gestational Age Group			
* **NRDS 24-29 weeks**	8.84±2.47	11.57±2.21	5.24±1.43
** **NRDS >30 weeks**	3.52±1.87	5.65±3.49	3.11±2.47
Non-NRDS >30 weeks	6.69±2.85	2.59±4.94	2.73±2.21

*** p<0.02, **p<0.03**

a greater amount of TBS, as quantified by protein concentration, compared to those collected without saline ($p<0.05$). A greater recovery of TBS yield during tracheal suctioning increases the efficacy of the procedure and possibly reduces the incidence of lung atelectasis as a consequence of protein mediated inhibition of lung surfactant biophysical activity. An equal benefit regarding the use of saline during tracheal suctioning was observed in both term and pre-term infants.

Although analysis of TBS samples revealed no significant difference in protein content removed between intubated Non-RDS and RDS individuals, RDS individuals did demonstrate a significantly increased average PLA_2 activity ($p<0.05$) as well as an increased standardized PLA_2 activity ($p<0.05$). TBS PLA_2 activity was found to be significantly increased, in the first 8⅓ days (200 hours) of life, in severely premature RDS infants born at <29 weeks GA compare to more mildly premature RDS infants (>30 weeks GA) ($p<0.02$) and control Non-RDS infants (>30 weeks GA) ($p<0.03$).

The observation of increased PLA_2 activity in the lungs of RDS infants could be the result of increased lung permeability associated with RDS allowing the introduction of plasma proteins into the airspace or the result of the ventilation of surfactant deficient lungs yielding desquamation of lung epithelium culminating in an inflammatory response mediated by PLA_2 (Vadas and Pruzanski, 1989). The latter explanation seems most likely as there was no significant difference between measurable protein content in TBS samples from both RDS and Non-RDS infants. The PLA_2 which initiates the propagation of an inflammatory response is a non-pancreatic, secretory PLA_2 (Vadas and Pruzanski, 1989).

A natural, powerful inhibitor of secretory PLA_2 activity is uteroglobin (Levin *et al.*, 1986) , originally described as blastokinin, discovered in the rabbit uterus (Miele *et al.*,

1987). It is thought that its presence in the uterus confers protection to the fetal lung from maternal inflammatory responses during gestation (Mukherjee *et al.*, 1980). High levels of uteroglobin have also been observed in neonatal TBS samples (Samuelsson et al., 1987) and it is postulated that it functions in maintaining a balance between antigen, prostaglandin and leukotriene activity in the tracheobronchial tree (Samuelsson *et al.*, 1987). It is also hypothesised that uteroglobulin in the lung may be involved in the prevention of NRDS by inhibiting PLA_2 activity which degrades pulmonary surfactant (Kaiser *et al.*, 1990). It must, therefore, also be considered that the observed increase in PLA_2 activity in RDS TBS samples may be a reflection of an altered or decreased production of uteroglobulin in the tracheobronchial tree. Since the content of neither PLA_2 nor uteroglobin were measured in the TBS samples, it is unknown whether the increased PLA_2 activity is a reflection of the PLA_2 content or specific activity.

Lung Ventilation-Perfusion Studies

Introduction

Lung ventilation-perfusion studies were conducted, according to a modification of the protocol of Kraft et al. (1995), to determine the vasoactive potential of LPC administered both intra-vascularly and intra-tracheally. The intravascular administration of LPC was an *in vivo* confirmation of the results that were previously observed *in vitro* with pulmonary arterial rings from the main branch of the pulmonary artery of adult and newborn guinea pigs. The intratracheal administration of LPC was performed to determine the potential of LPC to cross the epithelial-endothelial barrier to mediate pulmonary vasodilation. Based on the observed endothelial-dependence of LPC to mediate relaxation of vascular smooth muscle, it is hypothesized that LPC must cross both the epithelial barrier within the lung and the endothelium which lines the vasculature to mediate vasorelaxation. It is unclear if LPC may act on the basal aspect of the endothelial cell to mediate vasorelaxation.

Material and Methods

Materials

L-α-LPC palmitoyl (synthetic), L-phenylephrine hydrochloride, albumin (bovine, fraction V), potassium chloride, sodium bicarbonate, sodium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium phosphate monobasic, magnesium sulfate, calcium chloride, and D-glucose anhydrous were purchased from Mallinckrodt division of Anachemia Science (Winnipeg, MB). Heparin sodium injection was obtained from Leo Laboratories (Ajax ON). Exosurf® neonatal (colfosceril palmitate) was obtained from

Burroughs Wellcome Inc. (Kirkland, PQ). Ketalean® (ketamine hydrochloride) was purchased from MTC Pharmaceuticals (Cambridge, ON) and Rompun® (xylazine) obtained from Bayvet Division, Chemagro Limited (Etobicoke, ON). Pentobarbitone sodium was purchased from BDH Inc. (Toronto, ON).

Animal Preparation

Lung perfusion studies, modified from the protocol of Kraft et al. (1), were performed on adult female guinea pigs (800-1000 grams) under a protocol approved by the University of Manitoba Ethics Committee on animal experimentation. Animals were sedated with an intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine (2.2 mg/kg) prior to an administration of lidocaine hydrochloride (3 mg) in the upper thoracic region. Following tracheostomy, an endotracheal tube (2 mm ID) was introduced and the animal was mechanically ventilated (Harvard Apparatus Respirator) with room air at 24 breaths/min (15 ml/breath). The chest cavity was immediately opened and, following the removal of the pericardium, a canula was introduced into the main trunk of the pulmonary artery at the junction of the pulmonary artery and the conus arteriosus through an incision in the right ventricle. A second cannula was introduced into the left atrium through an incision in the left ventricle. Both cannula were secured by ligature through the right and left ventricles respectively.

Perfusion System

Two perfusion mediums, equilibrated with a gas mixture of 95% O_2-5% CO_2 (pH 7.4), were incorporated in this study. The first, which consisted of Krebs-Henseleit solution with heparin (5 Units/ml) was utilized to prepare the perfusion system prior to

experimentation and to flush the pulmonary vasculature of blood and blood borne products. The second, recirculated perfusion medium consisted of Krebs Henseleit solution containing 3% bovine serum albumin (BSA) pre-filtered with a 0.20 µm cellulose acetate filter. The initial perfusion medium was delivered at a low flow rate (1-1.5 ml/min) until the exiting perfusate became blood free. The second perfusion medium was initially delivered at 2 ml/min, but during experimentation was delivered at a flow rate of 5 ml/min. The perfusion system consisted of a Masterflex® peristaltic pump delivery system; polyethylene tubing (Intramedic® - ID 0.062", OD 0.082"), 2 in line bubble traps, an in line filter system and an in line pressure transducer (Gould P23ID). The perfusion medium was maintained at 37°C via a servo controller (Fisher and Paykel) and the animal preparation was also maintained at 37°C for the duration of the experiment with an electric blanket. The chest and tracheal region was covered with plastic wrap to conserve humidity.

Changes in pulmonary vascular resistance were measured with an in line pressure transducer and recorded at a frequency of 5 Hz by means of a customized data acquisition program (AT Lever Data Capture Program - Cunningham Engineering).

Intra-Vascular Perfusion of Lysophosphatidylcholine (LPC)

Following the perfusion of the Krebs + 3% BSA medium through the preparation, 2 cumulative doses of PE ($3x10^{-6}$ M) were added to the perfusate medium to effect an increase in PVR. When the achievement and maintenance of an steady state increase in pulmonary arterial pressure was observed, an intravascular dose of LPC (10^{-4} M) was administered directly into the perfusate line as a 10 ml bolus. Care was taken to ensure that the 10 ml LPC bolus was administered carefully so as not to alter the pulmonary

arterial pressure. The perfusate medium exiting the preparation was collected for a 2 minute period following introduction of LPC and disposed to ensure that the recycled perfusion medium remained free of LPC.

Intra-Tracheal Administration of Lysophosphatidylcholine (LPC)

LPC (50 mM) was administered intra-tracheally using 300 µl Exosurf®, a synthetic surfactant preparation which is LPC free, as a vehicle. The preparation was sonicated and gently vortex-mixed to completely dissolve LPC into solution. The Exosurf®-LPC preparation was administered intermittently over 30 seconds, immediately following exhalations to allow the proceeding inhalation of the respirator to "push" the Exosurf®-LPC mixture into the lungs and facilitate an even distribution into each lung lobe. The administration of 300 µl of Exosurf® alone acted as control trials.

Results

Intra-Vascular Perfusion of Lysophosphatidylcholine (LPC)

Baseline pulmonary arterial pressure was approximately 12 mmHg. The intravascular adminstration of LPC in the ventilation perfusion model was performed twice yielding a complete reversal of the increased PVR achieved with $6x10^{-6}$ M PE (40 mmHg) during both trials (Figure 24A). The vasorelaxation and concomitant decrease in pulmonary arterial pressure was short lived as the 10 ml bolus briefly passed through the pulmonary vasculature. Following complete return of pulmonary arterial pressure to baseline values and passage of the LPC through the pulmonary vasculature, the perfusion of medium containing PE reached the vasculature and once again returned pulmonary arterial pressure to increased PVR levels.

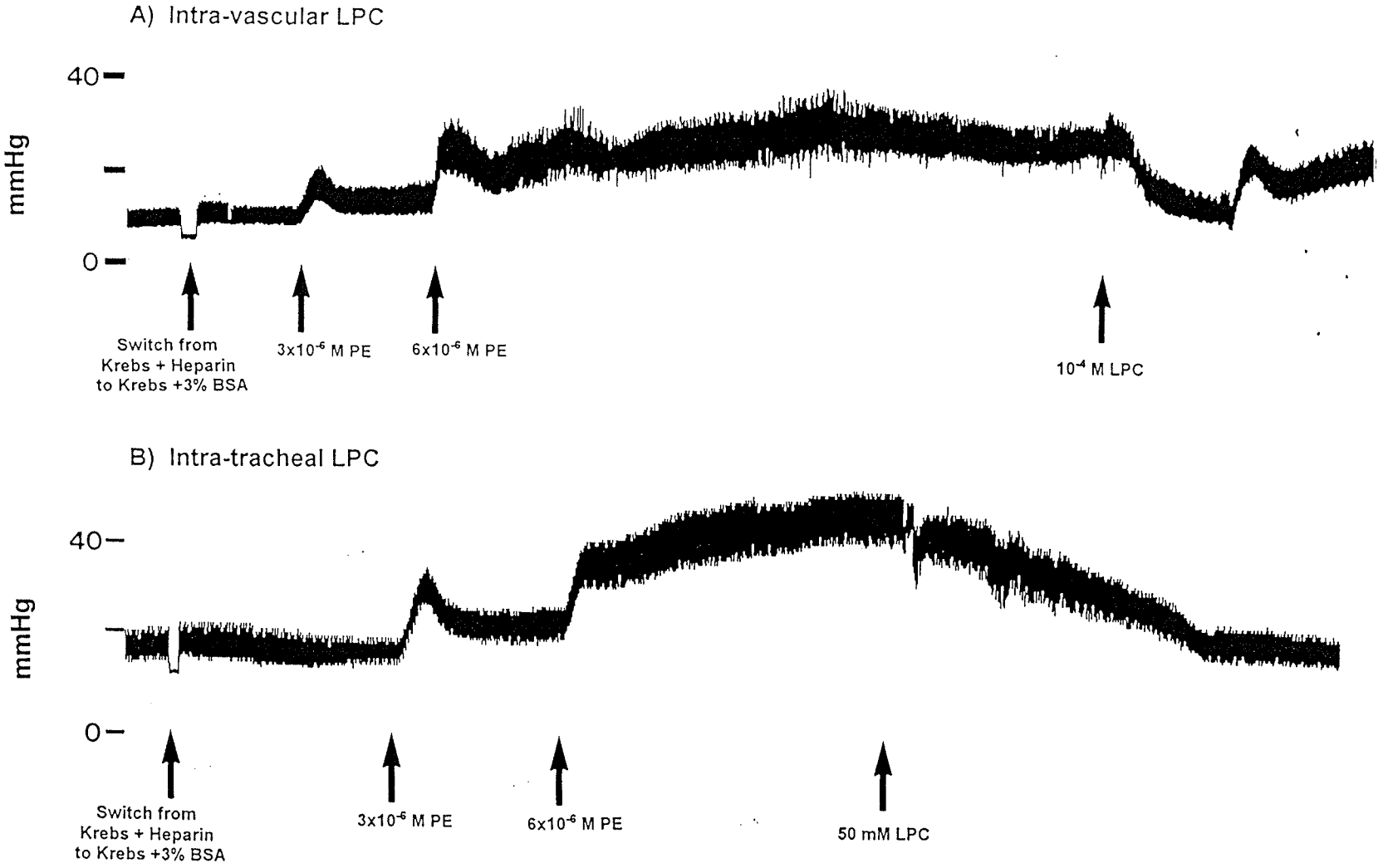

Figure 24. Lung Perfusion Studies - Effect of Intra-vascular and Intra-tracheal Administration of Lysophosphatidylcholine (LPC) on Pulmonary Vascular Resistance. The administration of a 10 ml bolus of LPC (10^{-4} M) intravascularly, following an increase in pulmonary arterial pressure with $6x10^{-6}$ M PE, resulted in a brief return to baseline pulmonary arterial pressure **(A)**. Intra-tracheal adminstration of LPC (50 mM) intra-tracheally with Exosurf® yielded a slower return to baseline pulmonary arterial pressure **(B)**. A decrease in pulmonary arterial pressure following intra-tracheal administration of LPC was observed once in four trials.

Intra-Tracheal Administration of Lysophosphatidylcholine (LPC)

The intra-tracheal adminstration of Exosurf® alone did not alter pulmonary hemodynamics, but did result in increased compliance of the lungs as determined by the slight increase in the size of the lungs during inspiration. Four trials of intra-tracheally administered LPC were conducted and an observed decrease in PVR was observed in one of these (Figure 24B) whereas no significant alteration in PVR was observed in the remaining three.

Discussion

Vasodilation of the precontracted pulmonary vascular bed *in vivo* was observed following the intravascular administration of LPC, confirming our results obtained *in vitro* and previous *in situ* studies (Bing et al., 1988). This vasodilation was observed as a complete reversal in the increased pulmonary arterial pressure achieved with $6x10^{-6}$ M PE. A decrease in pulmonary arterial pressure following the intra-tracheal administration of LPC fortified doses of Exosurf® was observed only once in four trials. Although this contradicts the hypothesis that LPC can cross the epithelial-endothelial barrier to mediate relaxation of the pulmonary vasculature, it does not discredit the hypothesis. The intra-tracheal adminstration of LPC was tested on an adult, non surfactant deficient guinea pig animal model. The clinically reported decrease in pulmonary vascular resistance following surfactant administration was observed in surfactant deficient pre-term infants suffering NRDS (Kääpä *et al.*, 1993) which were ventilated prior to the administration of exogenous surfactant. The pre-term lung is highly permeable, compared to the adult lung, and tends to leak large amounts of intravascular proteins into the airway after birth (Jobe *et al.*, 1983), a phenomenon which decreases with advancing gestational age (Jobe *et al.*, 1985).

This would suggest the potential for diffusion of LPC across the epithelial-endothelial barrier into the lung vasculature of the pre-term newborn as opposed to the adult. As well, in the clinical studies infants were intubated and ventilated prior to the administration of exogenous surfactant. The ventilation of the premature surfactant deficient lung prior to the administration of surfactant is associated with irregular alveolar expansion and necrosis and desquamation of airway epithelium (Grossman *et al.*, 1986; Robertson 1988). Breakdown of the epithelial barrier causes an increased permeability of the epithelial barrier of the lung (Robertson, 1988) which would facilitate the diffusion of LPC into the pulmonary vasculature. In our adult model, this could have possibly been achieved by broncho-alveolar lavage of the lungs prior to ventilation or by the ventilation of the lungs with ozone to facilitate epithelial damage.

Trace amounts of LPC administered with exogenous surfactant are rapidly removed from the airspace of adult rabbits, partially by epithelial type-II cells and remodelled, with 60-80% efficiency, to PC (Seidner *et al.*, 1988). In the preterm lamb, LPC is also removed from the airspace and remodelled to PC, in a much less efficient manner than observed in the adult rats (Ikegami et al., 1989). This may indicate an increased loss of LPC from the lung into the vasculature following administration into the preterm animal. Although the fate of LPC in the pre-term lung following exogenous surfactant replacement therapy has not been completely clarified, it is possible that some may cross the epithelial-endothelial barrier and reach the pulmonary vasculature.

Although the aforementioned explanations for the lack of an observed decrease in pulmonary arterial pressure following intra-tracheal administration of LPC seem reasonable, it must also be considered that LPC may not cross the epithelial/endothelial barrier to the pulmonary vasculature at all or in sufficient concentration to yield vasodilation of the pulmonary vasculature.

Summary

It was the purpose of this research thesis to address the vasoactive potential of LPC, a component of administered exogenous surfactant and a potential product of surfactant degradation in the lung, following exogenous surfactant therapy to neonates with NRDS (Figure 25).

Exogenous surfactant preparations contain DPPC as their main, active ingredient. We have demonstrated, *in vitro*, that the naturally derived exogenous surfactant preparation Survanta® is readily deacylated by secretory PLA_2 whereas the synthetic exogenous surfactant preparation Exosurf® is resistant to secretory PLA_2 activity. We have also demonstrated that TBS samples from infants with RDS contain a significantly increased PLA_2 activity compared with Non-RDS infants.

It has been demonstrated that LPC is a potent vasodilator of the pulmonary vasculature both *in vitro* and *in vivo*. Although it has not been demonstrated that LPC is capable of crossing the epithelial-endothelial barrier to mediate pulmonary vasorelaxation, this is a reflection of the adult animal model used which is not a true reflection of the ventilated, surfactant deficient preterm lung in which a decrease in PVR has been observed following exogenous surfactant administration.

Limitations of the Study

Although this study has seemingly demonstrated the potential for LPC to be generated in the NRDS lung and mediating a decrease in PVR by vasodilation of the pulmonary vasculature, there are several inherent limitations to this study.

Due to the large calibre and relative ease with which preparations could be

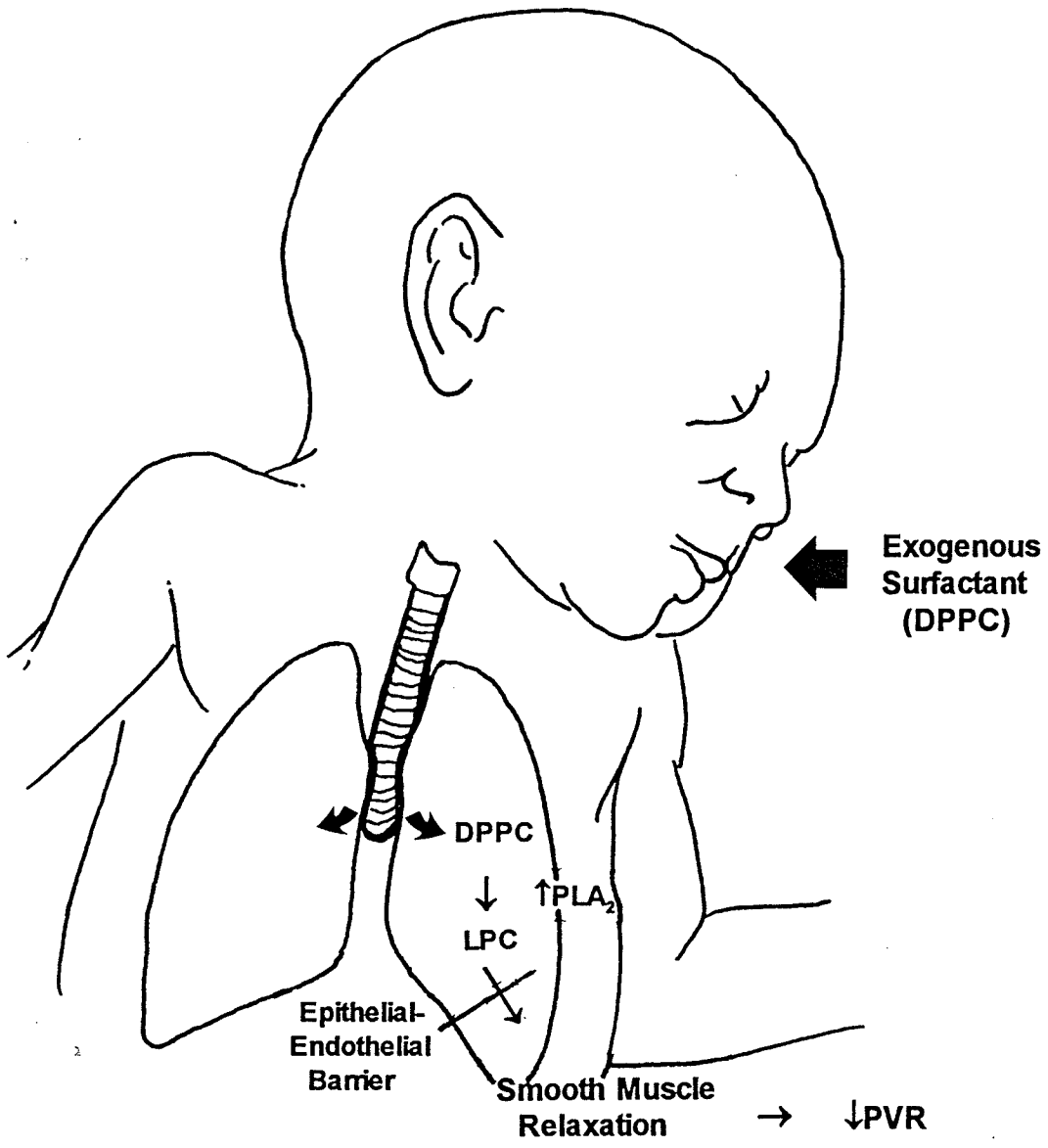

Figure 25. **Pulmonary Vasoactive Potential of Lysophosphatidylcholine (LPC).** Adminstration of surfactant, which contains large quantities of dipalmitoyl phosphatidylcholine (DPPC), may be degraded in the lung by phospholipase A_2 (PLA_2) yielding LPC which may transverse the epithelial-endothelial barrier and mediate pulmonary arterial vasodilation. Significantly increased levels of PLA_2 in tracheal bronchial secretions (TBS) of infants with neonatal respiratory distress syndrome (NRDS) has been demonstrated. The vasodilatory properties of LPC on the pulmonary vasculature has been demonstrated *in vitro* and *in vivo*. The ability of LPC to transverse the epithelial-endothelial barrier was not confirmed.

obtained and handled *in vitro*, smooth muscle mechanics studies to determine the vasoactive potential of LPC were conducted on arterial rings from the pulmonary trunk of guinea pigs. The control of pulmonary vascular resistance, however, is mediated by the pulmonary arterioles which are not only much smaller in scale but are also embedded deep in the parenchyma of the lungs making them difficult to obtain and handle *in vitro*. Preparations from the pulmonary trunk were therefore used with the assumption that their vasoactive behaviour in the presence of PE, ACh and LPC were much the same as the smaller pulmonary arterioles. The effect of the administration of PE and LPC intravascularly on pulmonary arterial pressure in the lung ventilation perfusion model supports this contention.

Analysis of the susceptibility of the exogenous surfactant preparations Exosurf® and Survanta® to secretory PLA_2 activity *in vitro* is based on the assumptions that the increased lung PLA_2 activity associated with RDS is of a pancreatic isoform and that the conditions in which the assays are conducted are similar to those present *in vivo*. The increased lung PLA_2 activity, if a result of lung epithelial inflammation due to ventilation as speculated, would primarily be of a secretory non-pancreatic isoform (Vadas and Pruzanski, 1990). It is assumed that the susceptibility of both Exosurf® and Survanta® to pancreatic and non-pancreatic isoforms would be equivalent. The inference that the results of this study would be similar to what is expected *in vivo* cannot be accepted with a great deal of certainty. Surfactant exists as many forms in the lung, such as LB's, TM, and relatively pure DPPC in the monolayer. PLA_2 will hydrolyse DPPC in the surfactant monolayer itself (Enhorning *et al.*, 1992; Holm *et al.*, 1991) but its potential to hydrolyse other DPPC fractions of the surfactant remain unclear.

At present there are no means of performing broncho alveolar lavage (BAL) in

intubated pre-term neonates. TBS samples were alternatively obtained for quantification of PLA_2 activity as an indicator of PLA_2 activity in the alveolar microenvironment itself. Although TBS samples may differ in their composition from BAL samples, Merritt et al. (1986b) contend that TBS samples yield data comparable to that obtained with BAL samples, used in the monitoring and cytopathology of lung disease. Although the use of TBS is limited and there is no certainty that the increased PLA_2 measured is a true reflection of what is occurring in the lung, it is assumed that TBS samples are a reasonable indicator of the alveolar microenvironment. Regardless, exogenous surfactant must pass through the tracheobronchial tree during its administration into the lungs.

Lung ventilation perfusion studies were performed on adult, surfactant efficient, guinea pig preparations. It was assumed that the adult preparations would be a representative model of the newborn, surfactant deficient pre-term infants in which clinical observations regarding decreased PVR following surfactant administration have been made. Desquamation and inflammation of airway epithelium following ventilation of surfactant-deficient lungs (Robertson, 1988) and the permeability of the premature lung (Jobe *et al.*, 1983) suggest that the adult model incorporated in this study was not reflective of the state of the lung of the premature, surfactant deficient pre-term and probably a poor model to use in this regard.

Conclusions

1a. This study demonstrated that lysophosphatidylcholine (LPC) is an effective, endothelium dependent vasodilator of pulmonary arterial smooth muscle preparations *in vitro* from both adult and newborn guinea pigs.

1b. LPC induced vasorelaxation is mediated by both guanylate cyclase (GC) and nitric oxide synthase (NOS) activity, however, in the newborn guinea pig the response is mediated to a greater extent by the NOS pathway compared to the adult which is more dependent on the GC pathway.

2a. The phosphatidylcholine (PC) component of the exogenous surfactant preparation Survanta® is readily susceptible to secretory-phospholipase A_2 (PLA_2) deacylation *in vitro* whereas the dipalmitoyl phosphatidylcholine (DPPC) component of the exogenous surfactant preparation Exosurf® is resistant,

2b. The resistance of Exosurf® DPPC to secretory-PLA_2 deacylation is a reflection of the presence of tyloxapol, a non-ionic surfactant component of Exosurf® which facilitates rapid adsorption of DPPC in the lung following administration.

3a. Saline instillation prior to tracheal suctioning of intubated neonates yields a greater removal of tracheal bronchial secretions (TBS) in both term and pre-term infants than tracheal suctioning without saline instillation, as quantified by TBS protein concentration.

3b. Infants with respiratory distress syndrome (NRDS) have an increased PLA_2 activity in their TBS samples compared to Non-NRDS infants.

4a. LPC administered into the pulmonary vasculature mediates a decrease in PVR.

4b. Preliminary investigations regarding intra-tracheal administration of LPC into the lung of a ventilated, non-surfactant deficient adult guinea pig are too few to allow rigorous analysis and significant conclusions to be drawn regarding the potential of LPC to cross the epithelial-endothelial barrier to mediate a decrease in pulmonary vascular resistance.

References

Auten RL, Notter RH, Kendig JW, Davis JM, Shapiro DL 1991 Surfactant treatment of full-term newborns with respiratory failure. Pediatrics 87:101-107

Avery MD, Mead J 1959 Surface properties in relation to atelectasis and hyaline membrane disease. Am J Dis Child 97:517-523

Ballard PL 1989 Hormonal Regulation of pulmonary surfactant. Endocrine Rev 10:165-181

Bar T 1978 Morphometric evaluation of capillaries in different laminae of rat cerebral cortex by automatic image analysis:changes during development and aging. Adv Neurol 20:1-9

Batenburg JJ 1992 Surfactant phospholipids:synthesis and storage. Am J Physiol 262(Lung Cell Mol Physiol 6):L367-L385

Batenburg JJ, Van Golde LMG 1979 Formation of Pulmonary Surfactant in Whole Lung and Isolated Type-II Alveolar Cells. IN: Reviews in Perinatal Medicine (vol. 3). Scarpelli EM, Cosmi EV (eds). Raven Press, New York, New York. p73

Bates SR, Ibach PB, Fisher AB 1989 Phospholipids coisolated with rat surfactant protein C account for the apparent protein-enhanced uptake of liposomes into lung granular pneumocytes. Exp Lung Res 15:695-708

Belik J, Duncan JE, Hatch GM 1994 Exogenous surfactant administration and broncho-alveolar fluid (BAF) phospholipase A (PLA) activity in neonates Pediatr Res 35:325A

Bennet CF, McCarte A, Crooke ST 1990 Purification and characterization of a soluble phospholipase A_2 from guinea pig lung. Biochim Biophys Acta 1047:271-283

Bing RJ, Saeed M 1987 The role of lysolecithin in the relaxation of vascular smooth muscle. Bioscience Reports 7:783-789

Bing RJ, Saito T, Wolf A, Menon NK 1988 The effect of phospholipid (lysophosphatidylcholine) on arterial relaxation *in vitro* and *in situ*. Trans Assoc Am Physicians 101:70-78

Bing RJ, Termin A, Conforto A, Dudek R, Hoffman MJ 1993 Membrane function and vascular reactivity. Bioscience Reports 13:61-67

Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA 1994 Nitric oxide activates calcium-dependent potassium channels in vascular smooth muscle. Nature 368(6474):850-853

Bradford MM 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Chander A, Fisher AB 1990 Regulation of lung surfactant secretion. Am J Physiol (Lung Cell Mol Physiol 2):L241-L253

Chander A, Fisher AB, Strauss JF 1982 Role of an acidic compartment in synthesis of disaturated phosphatidylcholine by rat granular pneumocytes. Biochem J 208:651-658

Chander A, Claypool Jr WD, Straus III F, Fisher AB 1983 Uptake of lysosomal phosphatidylcholine by granular pneumocytes in primary culture. Am J Physiol 245(Cell Physiol 14):C397-C404

Chander A, Johnson Jr JG, Reicherter J, Fisher AB 1986 Lung lamellar bodies maintain an acidic internal pH. J Biol Chem 261:6126-6131

Charpie JR, Schreur KD, Papadopoulos SM, Webb RC 1994 Endothelium dependency of contractile activity differs in infant and adult vertebral arteries. J Clin Invest 93:1339-1343

Chevalier G, Collet AJ 1972 *In vivo* incorporation of choline-^{3}H, leucine ^{3}H and galactose-^{3}H in alveolar type II pneumocytes in relation to surfactant synthesis. A quantitative radioautographic study in mouse by electron microscopy. Anat Rec 174:289-310

Clements JA 1977 Functions of the alveolar lining of the lung. Am Rev Respir Dis 115(Suppl):67-71

Clyman RI, Jobe AH, Heymann M, Ikegami M, Roman C, Payne B, Mauray F 1982 Increased shunt through the patent ductus arteriosus after surfactant replacement therapy. Pediatrics 100:101-107

Cockshutt AM, Possmayer F 1991 Lysophophatidylcholine sensitizes lipid extracts of pulmonary surfactant to inhibition by serum proteins. Biochim Biophys Acta 1086:63-71

Corbet A, Long W 1992 Symposium on synthetic surfactant: Introduction. J Pediatr 120:S1-S2

Corbet AJ, Long WA, Murphy DJ, Garcia-Prats JA, Lombardy LR, Wold DE 1991a Reduced mortality in small premature infants treated at birth with a single dose of synthetic surfactant. J Paediatr Child Health 27:245-249

Corbet A, Bucciarelli R, Goldman S, Mammel M, Wold D, Long W, and the American Exosurf Pediatric Study Group 1 1991b Decreased mortality rate among small premature infants treated at birth with a single dose of synthetci surfactant: A

multicenter controlled trial. J Pediatr 118:277-284

Corr PB, Cain ME, Witkowski FX, Price DA, Sobel BE 1979 Potential arrhythmogenic electrophysiological derangements in canine purkinje fibers induced by lysophosphoglycerides. Circ Res 44:822-832

Corr PB, Gross RW, Sobel BE 1984 Amphipathic metabolites and membrane dysfunction in ischemic myocardium. Circ Res 55:135-154

Cosmi EV, DiRenzo GC 1988 Respiratory Distress Syndrome: Requirements of Perinatal Diagnosis, Prevention and Treatment. IN: Surfactant Replacement Therapy in Neonatal and Adult Respiratory Distress Syndrome. Lachmann B (ed). Springer-Verlag, Heidelberg, Germany. pp49-57

Crapo JD, Young SL, Fram EK, Pinkerton KE, Barry BE, Crapo RD 1983 Morphometric characteristics of cells in the alveolar region of mammalian lungs. Am Rev Respir Dis 128(suppl. 2):S42-S46

Cummings JJ, Holm BA, Hudak ML, Hudak BB, Ferguson WH, Egan EA 1992 A controlled clinical comparison of four different surfactant preparations in surfactant-deficient preterm lambs. Am Rev Respir Dis 145:999-1004

Curstedt T, Johansson J, Barros-Soderling J, Robertson B, Nilsson G, Westberg M, Jornvall H 1988 Low molecular-mass surfactant protein type I. The primary structure of a hydrophobic 8 kDa polypeptide with 8 half-cysteine residues. Eur J Biochem 172:521-525

Das SK, Scott MT, McCuiston S 1987 Effects of experimental acute pancreatitis in dogs on metabolism of lung surfactant phosphatidylcholine. Biochem Biophys Res Commun 145:612-618

DeAngelis RL, Findlay JWA 1993 Metabolism of synthetic surfactants. In. Long W (eds). Clinics in Perinatology: Surfactant Replacement Therapy. WB Saunders Co., Philadelphia, pp 697-710

Den Breejen JN, Batenburg JJ, Van Golde LMG 1989 The species of acyl-CoA in sub-cellular fractions of type II cells isolated from adult rat lung and their incorporation into phosphatidic acid. Biochim Biophys Acta 1002:277-282

Dobbs LG 1990 Isolation and culture of alveolar type II cells. Am J Physiol 258(Lung Cell Mol Physiol 2):L134-L147

Dobbs LG, Wright JR, Hawgood S, Gonzalez R, Venstrom K, Nellenbogen J 1987 Pulmonary surfactant and its components inhibit secretion of phosphatidylcholine from cultured type-II cells. Proc Natl Acad Sci USA 84:1010-1014

Dudek R, Conforto A, Bing RJ 1993 Lysophosphatidylcholine-induced vascular relaxation and production of cGMP are mediated by endothelium-derived relaxing factor. Proc

Soc Exp Biol Med 203:474-479

Duncan JE, Hatch GM, Belik J 1995 Inhibition of secretory-type phospholipase A_2 (PLA_2) deacylation of surfactant phosphatidylcholine (PC) by the non-ionic surfactant tyloxapol. Pediatr Res (Submitted)

Dunn MS 1994 Surfactant replacment therapy: new developments. Canadian Neonatal/Perinatal Rev 1(2):3-5

Dunn MS, Shennan AT, Possmayer F 1990 Single- versus multiple-dose surfactant replacement therapy in neonates of 30 to 36 weeks' gestation with respiratory distress syndrome. Pediatrics 86:564-571

Enhorning G, Hill D, Sherwood G, Cutz E, Robertson B, Bryan C 1978 Improved ventilation of prematurely delivered primates following tracheal deposition of surfactant. Am J Obstet Gynecol 132:529-536

Enhorning G, Shumel B, Keicher L, Sokolowski J, Holm BA 1992 Phospholipases introduced into the hypophase affect the surfactant film outlining a bubble. J Appl Physiol 73:941-945

Filgueiras OMO, Possmayer F 1990 Purification and characterization of a phospholipase A_2 associated with rabbit lung microsomes: some evidence for its mitochondrial origin. Biochim Biophys Acta 1046:258-266

Floros J, Phelps D, Taeusch HW 1985 Biosynthesis and *in vitro* translation of the major surfactant-associated protein from human lung. J Biol Chem 260:495

Förstermann U, Goppelt-Strübe M, Frölich JC, Busse R 1986 Inhibitors of acyl coenzyme A:lysolecithin acyltransferase activate the production of endothelium-derived vascular relaxaing factor. J Pharm Exp Ther 238:352-359

Fuchimukai T, Fujiwara T, Takahashi A, Enhorning G 1987 Artificial surfactant inhibited by proteins. J Appl Physiol 62:429-437

Fujiwara T, Chida S, Watabe Y, Maeta H, Morita T, Abe T 1980 Artificial surfactant therapy in hyaline-membrane disease. Lancet 1980:55-59

Furchgott RF, Zawadzki JV 1980 The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373-376

Gil J, Reiss OK 1973 Isolation and characterization of lamellar bodies and tubular myelin from rat lung homogenates. J Cell Biol 58:159-171

Gil J 1985 Histological preservation and ultrastructure of alveolar surfactant. Annu Rev Physiol 47:753-763

Gluck L, Kulovich MV, Borer RC, Brenner PH, Anderson GG, Spellacy WM 1971

Diagnosis of the respiratory distress syndrome by amniocentesis. Am J Obstet Gynecol 109:440-445

Gregory TJ, Longmore WJ, Moxley MA, Whitsett JA, Reed CA, Fowler AA, Hudson LD, Maunder RJ, Crim C, Hyers TM 1991 Surfactant chemical composition and biophysical activity in acute respiratory distress syndrome. J Clin Invest 88:1976-1981

Grossman G, Nilsson R, Robertson B 1986 Scanning electron microscopy of epithelial lesions induced by artificial ventilation of the immature neonatal lung; the prophylactic effect of surfactant. Eur J Pediatr 145:361-367

Hallman M 1984 Antenatal Diagnosis of Lung Maturity. IN: Pulmonary Surfactant. Robertson B, Van Golde LMG, Batenburg BB (eds). Elsevier, Amsterdam. pp 419-439

Hallman M, Epstein BL, Gluck L 1981 Analysis of labelling and clearance of lung surfactant phospholipids in rabbit. Evidence of bidirectional surfactant flux between lamellar bodies and alveolar lavage. J Clin Invest 68:742-751

Hallman M, Spragg R, Harrell JH, Moser KM, Gluck L 1982 Evidence of lung surfactant abnormality in respiratory failure: A study of bronchoalveolar lavage phospholipids, surface activity, phospholipase activity, and plasma myoinositol. J Clin Invest 70:673-683

Harwood JL, Desai R, Hext P, Tetley T, Richards R 1975 Characterization of pulmonary surfactant from ox, rabbit, rat and sheep. Biochem J 151:707-714

Hawgood S 1989 Pulmonary surfactant apoproteins: a review of protein and genomic structure. Am J Physiol 257:L13-L22

Hawgood S, Benson BJ, Hamilton RL 1985 Effects of surfactant-associated proteins and calcium ions on the structure and surface activity of lung surfactant lipids. Biochemistry 24:184-190

Hawgood S, Benson BJ, Schilling J, Damm D, Clements JA, White RT 1987 Nucleotide and amino acid sequences of pulmonary surfactant SP18 and evidence for cooperation between SP18 and SP23-36 in surfactant lipid adsorption. Proc Natl Acad Sci USA 84:66-70

Hawkins DF, DiRenzo GC 1986 Preterm Premature Rupture of Membranes. IN: Perinatal Medicine: Problems and Controversies. DiRenzo GC, Hawkins DF (eds). Raven, New York, New York. pp 157-162

Heath MF, Jacobson W 1976 Phospholipase A_1 and A_2 in lamellar inclusion bodies of the alveolar epithelium of rabbit lung. Biochim Biophys Acta 441:443-452

Hill RW, Wyse GA 1989 Animal Physiology (2nd ed). Harper and Row Publishers, Inc.

New York. 656 pp.

Hoekstra RE, Jackson JC, Myers TF, Frantz III ID, Stern ME, Powers WF, Maurer M, Raye JR, Carrier ST, Gunkel JH, Gold AJ 1991 Improved neonatal survival following multiple doses of bovine surfactant in very premature neonates at risk for respiratory distress syndrome. Pediatrics 88:10-18

Holm BA, Waring AJ 1993 Designer Surfactants : The Next Generation in Surfactant Replacement. In: Long W (ed) Clinics in Perinatology: Surfactant Replacement Therapy. WB Saunders Co., Philadelphia, Pennsylvania. pp 813-829

Holm BA, Enhorning G, Notter RH 1988 A biophysical mechanism by which plasma proteins inhibit lung surfactant activity. Chem Phys Lipids 49:49-55

Holm BA, Keicher L, Liu M, Sokolowski J, Enhorning G 1991 Inhibition of pulmonary surfactant function by phospholipases. J Appl Physiol 71:317-321

Hongo K, Nakagomi T, Kassell NF, Sasaki T, Lehman M, Vollmer DG, Tsukahara T, Ogawa H, Torner J 1988 Effects of aging and hypertension on endothelium dependent vascular relaxation in rat carotid artery. Stroke 19:892-897

Hook GER, Gilmore LB 1982 Hydrolases of pulmonary lysosomes and lamellar bodies. J Biol Chem 257:9211-9220

Horbar JD, Soll RF, Sutherland JM, Kotagal U, Philip AGS, Kessler DL, Little GA, Edwards WH, Vidyasagar D, Raju TNK, Jobe AH, Ikegami M, Mullett MD, Myerberg DZ, McAuliffe TL, Lucey JF 1989 A multicenter randomized, placebo-controlled trial of surfactant therapy for respiratory distress syndrome. N Engl J Med 320:959-965

Horbar JD, Soll RF, Schachinger H, Kewitz G, Versmold HT, Linder W, Duc G, Mieth D, Linderkamp O, Zilow EP, Lemburg P, Von Loewenich V, Brand M, Minoli I, Moro G, Riegel KP, Roos R, Weiss L, Lucey JF 1990 A European multicenter randomized controlled trial of single dose surfactant therapy for idiopathic respiratory distress syndrome. Eur J Pediatr 149:416-423

Horbar JD, Wright LL, Soll RF. Wright EC, Fanaroff AA, Korones SB, Shankaran S, Oh W, Fletcher BD, Bauer CR, Tyson JE, Lemons JA, Donovan EF, Stoll BJ, Stevenson DD, Papile L, Philips III J, for the National Institute of Child Health and Human Development Neonatal Research Network 1993 A multicenter randomized trial comparing two surfactants for the treatment of neonatal respiratory distress syndrome. National Institute of Child Health and Human Development Neonatal Research Network. J Pediatr 123:757-766

Humason GL 1972 Animal Tissue Techniques. W.H. Freeman and Company, San Francisco, California. 641 pp

Ikegami M, Jobe A, Jacobs H, Lam R 1984 A protein from airways of premature lambs

that inhibits surfactant function. J Aply Physiol 57:1134-1142

Ikegami M, Jobe A, Yamada T, Priestly A, Ruffini L, Rider E, Seidner S 1989 Surfactant metabolism in surfactant preterm ventilated lambs. J Appl Physiol 67:429-437

Ikegami M, Ueda T, Absolom D, Baxter C, Rider E, Jobe AH 1993 Changes in exogenous surfactant in ventilated preterm lamb lungs. Am Rev Respir Dis 148:837-844

Jacobs KA, Phelps DS, Steinbrink R, Fisch J, Kriz R, Mitsock L, Dougherty JP, Taeusch HW, Floros J 1987 Isolation of a c-DNA clone encoding a high molecular weight precursor to 6 kDa pulmonary surfactant-associated protein. J Biol Chem 262:9808-9811

Jobe AH 1994 Surfactant Function and Metabolism. IN: New Therapies for Neonatal Respiratory Failure: A Physiological Approach. Boynton BR, Carlo WA, Jobe AH (eds). Cambridge University Press, New York, New York. pp16-35

Jobe A, Ikegami M 1987 Surfactant for the treatment of respiratory distress syndrome. Am Rev Respir Dis 136:1256-1275

Jobe AH, Ikegami M, Jones S, Conaway D 1983 Permeability of premature lamb lungs to protein and the effect of surfactant on that permeability. J Appl Physiol 55:169-176

Jobe AH, Jacobs H, Ikegami M, Berry D 1985 Lung protein leaks in ventilated lambs: effects of gestational age. J Appl Physiol 58:1246

Jobe A, Ikegami M, Glatz T, Yoshida Y, Diakomanolis E, Padbury J 1988 Duration and characteristics of treatment of premature lambs with natural surfactant. J Clin Invest 67:370-375

Kääpä P, Kero P, Saraste M 1992 Synthetic surfactant replacement therapy decreases estimated pulmonary artery pressure in RDS. Am J Dis Child 146:961-964

Kääpä P, Seppänen M, Kero P, Saraste M 1993 Pulmonary hemodynamics after synthetic surfactant replacement in neonatal respiratory distress syndrome. J Pediatr 123:115-119

Kaiser E, Chiba P, Zaky K 1990 Phospholipases in biology and medicine. Clin Biochem 23:349-370

Katyal SL, Singh G 1984 Analysis of pulmonary surfactant apoproteins by isoelectric focusing. Biochim Biophys Acta 794:411-418

Kendig JW, Notter RH, Cox C, Aschner JL, Benn S, Bernstein RM, Hendricks-Munoz K,

Maniscalo WM, Metlay LA, Phelps DL, Sinkin RA, Wood BP, Shapiro DL 1988 Surfactant replacement therapy at birth: Final analysis of a clinical trial and comparisons with similar trials. Pediatrics 82:756-762

Kendig JW, Notter RM, Maniscalco WM et al. 1989 Clinical Experience With Calf Lung Surfactant. IN: Surfactant Replacement Therapy. Shapiro DL, Notter RH (eds). Alan Liss, Inc., New York. pp 257-271

King RJ 1974 The surfactant system of the lung. Fed Proc 33:2238-2247

King RJ 1982 Pulmonary Surfactant. J Appl Physiol 53:1-8

King RJ 1984 Isolation and Chemical Composition of Pulmonary Surfactant. IN: Pulmonary Surfactant. Robertson B, Van Golde LMG, Batenburg JJ (eds). Elsevier Science Publishers, Amsterdam. pp1-15

King RJ, Clements JA 1972 Surface active materials from dog lung. II. Composition and physiological correlations. Am J Physiol 223:715-726

King RJ, Klass DJ, Gikas EG, Clements JA 1973 Isolation of apoproteins from canine surface active material. Am J Physiol 224:788-795

Kinkaid AR, Wilton DC 1992 A study of the 14-kDa phospholipase A_2 from rat liver using a continuous fluorescent displacement assay for the enzyme. Biochem Soc Trans 20:287S

Kinnaird AAA, Choy PC, Man RYK 1988 Lysophosphatidylcholine accumulation in the ischemic canine heart. Lipids 23:32-35

Kirschbaum BB, Bosmann HB 1973 Lysolecithin enhancement of glycoprotein:glycosyl transferase activity. FEBS Lett 34:129-132

Kirschbaum BB, Bosmann HB 1974 Glomerular glycoprotien:galactosyl transferase activity in rat kidney. J Lab Clin Med 83:271-280

Klaus MH, Clements JA, Havel RJ 1961 Composition of surface-active material isolated from beef lungs. Proc Natl Acad Sci USA 47:1858-1859

Kraft SA, Fujishima S, McGuire GP, Thompson JS, Raffin TA, Pearl RG 1995 Effect of blood and albumin on pulmonary hypertension and edema in perfused rabbit lungs. J Appl Physiol 78:499-504

Kramer RM, Johansen B, Hession C, Pepinsky RB 1990 Structure and Properties of a Secretable PLA_2 From Human Platelets. IN: Phosholipase A_2:Role and Function in Inflammation. Wong PYK, Dennis EA (eds). Plenum Press Inc., New York, New York. pp35-53

Kuroki Y, Mason RJ, Voelker DR 1988 Pulmonary surfactant apoprotein A structure and

modulation of surfactant secretion by rat alveolar type II cells. J Biol Chem 263:3388-3394

Kwong MS, Edmund EA, Notter RH, Shapiro DL 1985 Double-blind clinical trial of calf lung surfactant extract for the prevention of hyaline membrane disease in extremely premature infants. Pediatrics 76:585-592

Leff AR, Schumacker PT 1993 Respiratory Physiology:Basics and Applications. W.B. Saunders Company, Philadelphia, Pennsylvania. 198pp.

Levin SW, Butler JD, Schumacher UK, Wightman PD, Mukherjee AB 1986 Uteroglobin inhibits phospholipase A_2 activity. Life Sci 38:1813-1819

Liechty EA, Donovan E, Purohit D, Gilhooly J, Feldman B, Noguchi A, Denson SE, Sehgal SS, Gross I, Stevens D, Ikegami M, Zachman RD, Carrier ST, Gunkel JH, Gold AJ 1991 Reduction of neonatal mortality after multiple doses of bovine surfactant in low birth weight neonates with respiratory distress syndrome. Pediatrics 88:19-28

Lincoln TM, Cornwell TL 1993 Intracellular cyclic GMP receptor proteins. FASEB J 7:328-338

Lincoln TM, Komalavilas P, Cornwell TL 1991 Pleiotropic regulation of vascular smooth muscle tone by cyclic GMP-dependent protein kinase. Hypertension 23(2):1141-1147

Long W 1993a The Newborn - Update 1993 Focus on Surfactant. A Medi-View Report From A Department of Pediatrics, McGill University Continuing Medical Education Program. Express Highlights. Medical Education Network Canada Inc, 3535 Blvd St. Charles #303, Kirkland QC H9H 3C4

Long W 1993b Preface:Surfactant Replacement Therapy. Clinics in Perinatology 20:4 (Dec): ix-x

Long W, Thompson T, Sundel H, Schumacher R, Volberg F, Guthrie R, and the American Exosurf Neonatal Study Group 1 1991 Effects of two rescue doses of a synthetic surfactant on mortality rate and survival without bronchopulmonary dysplasia in 700- to 1350-gram infants with respiratory distress syndrome. J Pediatr 118:595-605

Menon NK, Bing RJ 1991 Nitroarginine does not inhibit lysophosphatidylcholine (LPC)-induced vascular relaxation and accumulation of cyclic GMP. Proc Soc Exp Biol Med 196:461-463

Menon NK, Saito T, Wolf A, Bing RJ 1989 Correlation of lysophosphatidylcholine-induced vs spontaneous relaxation to cyclic GMP levels in rabbit thoracic aorta. Life Sci 44:611-618

Merritt TA, Hallman M, Bloom BT, Berry C, Benirschke K, Sahn D, Key T, Edwards D, Jarvenpaa AL, Pohjavuori M, Kankaanpaa K, Kunnas M, Paatero H, Rapola J, Jaaskelainen J 1986a Prophylactic treatment of very premature infants with human surfactant. N Engl J Med 315:785-790

Merritt TA, Hallman M, Holcomb K, Strayer D, Bloom B, Revak S, Cochrane CG 1986b Human surfactant treatment of severe respiratory distress syndrome: pulmonary effluent indicators of lung inflammation. J Pediatr 108:741-748

Merritt TA, Amirkhanian JD, Helbock H, Halliwell B, Cross CE 1993 Reduction of the surface-tension-lowering ability of surfactant after exposure to hypochlorous acid. Biochem J 295:19-22

Miele L, Cordella-Miele E, Mukherjee AB 1987 Uteroglobin: structure, molecular biology and new perspectives on its function as a phospholipase A_2 inhibitor. Endocrin Rev 8:474-484

Mines AH 1993 Respiratory Physiology (3rd ed). Raven Press Ltd., New York, New York. 182 pp.

Mondalou H, Beharry K, Norris K, Bottoli I, Aranda JV 1994 Comparative effects of Survanta® (SURV) and Exosurf® (EXO) on the outcome of respiratory distress syndrome (RDS). Pediatr Res 35:242A(1436)

Mookerjea S, Yung JWM 1974a Stimulation of galactosyltransferase in liver microsomes by lysolecithin. Biochem Biophys Res Comm 57:815-822

Mookerjea S, Yung JWM 1974b A study on the effect of lysolecithin and phospholipase A on membrane-bound galactosyltransferase. Can J Biochem 52:1053-1066

Morgan AP, Jenny ME, Haessler H 1968 Phospholipids, acute pancreatitis, and the lungs: Effect of lecithinase infusion on pulmonary surface activity in dogs. Ann of Surg 167:329-335

Moritoki H, Hosoki E, Ishida Y 1986 Age related decrease in endothelium-dependent dilator response to histamine in rat mesenteric artery. Eur J Pharmacol 126:61-67

Murad F 1986 Cyclic guanosine monophosphate as a mediator of vasodilation. J Clin Invest 78:1-5

Mukherjee AB, Laki K, Agrawal AK 1980 Possible mechanism of success of an allotransplantation in nature: mammalian pregnancy. Med Hypotheses 6:1043-1055

Nogee LM, DeMello DE, Dehner LP, Colten HR 1993 Deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. N Engl J Med 328:406-410

Notter RH 1984 Surface Chemistry of Pulmonary Surfactant: The Role of Individual

Components. IN: Pulmonary Surfactant. Robertson B, Van Golde LMG, Batenburg JJ (Eds). Elsevier Science Publishers. New York, NY. pp 17-65

Offenstadt G, Pinta P, Masliah J, Alcindor LG, Hericord P, Amstutz P 1981 Phospholipasic and prophospholipasic activities in bronchoalveolar lavage fluid in severe acute pulmonary disease with or without ARDS. Intensive Care Med 7:285-290

Ohashi Y, Katayama M, Hirata K, Suematsu M, Kawashima S, Yokoyama M 1993 Activation of nitric oxide synthase from cultured aortic endothelial cells by phospholipids. Biochem Biophys Res Comm 195:1314-1320

Oishi K, Raynor RL, Charp PA, Kuo JF 1988 Regulation of protein kinase C by lysophospholipids. J Biol Chem 263:6865-6871

Olafson RW, Rink U, Kielland S, Yu SH, Chung J, Harding PG, Possmayer F 1987 Protein sequence analysis studies on the low molecular weight hydrophobic proteins associated with bovine pulmonary surfactant. Biochem Biophys Res Comm 148:1406-1411

Ono T, Hiromasa T, Kuramitsu S, Kagamiyama H, Okamoto M 1988 Purification and characterization of a membrane-associated phospholipase A_2 from rat spleen. J Biol Chem 263:5732-5738

OReilly MA, Nogee L, Whitsett JA 1988 Requirement of the collagenous domain for carbohydrate processing and secretion of a surfactant protein, SP-A. Biochim Biophys Acta 969:176-184

Oyarzún MJ, Clements JA, Baritussio A 1980 Ventilation enhances pulmonary alveolar clearance of radioactive dipalmitoyl phosphatidylcholine in liposomes. Am Rev Respir Dis 121:709-721

Parthasarathy S, Bauman WJ 1979 Lysolecithin as regulator of *de novo* lecithin synthesis in rat liver microsomes. Biochem Biophys Res Commun 91:637-642

Pastrana-Rios B, Flach CR, Brauner JW, Mautone AJ, Mendelsohn R 1994 A direct test of the "squeeze-out" hypothesis of lung surfactant function. External reflection FT-IR at the air/water interface. Biochemistry 17:5121-5127

Pattle RE 1958 Properties, function and origin of the alveolar lining layer. Proc R Soc Lond (Biol) 148:217-240

Persson A, Chang D, Rust K, Moxley M, Longmore W, Crouch E 1988 CP4: a pneumocyte-derived collagenous surfactant associated protein. Evidence for heterogeneity of collagenous surfactant proteins. Biochemistry 27:8576-8584

Persson A, Chang D, Rust K, Moxley M, Longmore W, Crouch E 1989 Purification and biochemical characterization of CP4 (SP-D), a collagenous surfactant associated

protein. Biochemistry 28:6361-6367

Petty TL, Reiss OK, Paul GW, Silvers GW, Elkins ND 1977 Charactersitics of pulmonary surfactant in adult respiratory distress syndrome associated with trauma and shock. Am Rev Respir Dis 115:531-536

Pfeilschifter J Regulation of Synthesis and Secretion of Group II Phospholipase A_2. IN: Phospholipase A_2 in Clinical Inflammation: Endogenous Regulation and Pathological Actions. Glaser KB, Vadas P (eds). Boca Raton, FL: CRC, 1995. In Press

Phibbs RH, Ballard RA, Clements JA, Heilbron DC, Phibbs CS, Schleuter MA, Sniderman SH, Tooley WH, Wakeley A 1991 Initial clinical trial of Exosurf, a protein-free synthetic surfactant, for the prophylaxis and early treatment of hyaline membrane disease. Pediatrics 88:1-9

Plückthun A, Dennis EA 1985 Activation, aggregation, and product inhibition of cobra venom phospholipase A_2 and comparison with other phospholipases. J Biol Chem 269:11099-11106

Possmayer F 1984 Biochemistry of Pulmonary Surfactant During Fetal Development and in the Perinatal Period. In: Pulmonary Surfactant. Robertson B, Van Golde LMG, Batenburg JJ (eds). Elsevier Science Publishers, Amsterdam. pp295-355

Possmayer F 1988 A proposed nomenclature for pulmonary surfactant-associated-proteins. Am Rev Respir Dis 138:990-998

Possmayer F 1989 Metabolism of Phosphatidylcholine in Lung. IN: Phosphatidylcholine Metabolism. Vance DE (ed). CRC Press, Inc., Boca Raton, pp. 205-223

Post M, Schuurmans EAJM, Batenburg JJ, Van Golde LMG 1983 Mechanisms involved in the synthesis of disaturated phosphatidylcholine by alveolar type II cells isolated form rat lung. Biochim Biophys Acta 750:68-77

Pryhuber GS, Hull WM, Fink I, McMahon MJ, Whitsett JA 1991 Ontogeny of surfactant proteins A and B in human amniotic fluid as indices of fetal lung maturity. Pediatr Res 30:597-605

Rannels SR, Gallaher KJ, Wallin R, Rannels DE 1987 Vitamin K-dependent carboxylation of pulmonary surfactant-associated proteins. Proc Natl Acad Sci USA 84:5952-5956

Rice WR, Sarin VK, Fox JL, Baatz J, Wert S, Whitsett JA 1989 Surfactant peptides stimulate uptake of phosphatidylcholine by isolated cells. Biochim Biophys Acta 1006:237-245

Roberston BA 1988 Surfactant and Lung Injury. IN: Surfactant Treatment of Lung Diseases: Report of the Ninety-Sixth Ross Conference on Pediatric Research.

Jobe AH, Taeusch HW (eds). Columbus, Ohio, Ross Laboratories. pp 62-69

Romaschin AD, DeMajo WC, Winton T, D'Costa M, Chang G, Rubin B, Gamliel Z, Walker PM 1992 Systemic phospholipase A_2 and cachetin levels in adult respiratory distress syndrome and multiple organ failure. Clin Biochem 25:55-60

Rooney SA, Canavan PM, Motoyama EK 1974 Identification of phosphatidylglycerol in the rat, rabbit, monkey and human lung. Biochim Biophys Acta 360:56

Rouser G, Fleischer S, Yamamoto A 1969 Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5:494-496

Rudolph AM 1974 Congenital Diseases of the Heart. Year Book Medical Publishers Inc., Chicago, Illinois. p 47

Saito T, Wolf A, Menon NK, Saeed M, Bing RJ 1988 Lysolecithins as endothelium-dependent vascular smooth muscle relaxants that differ from endothelium-derived relaxing factor (nitric oxide). Proc Natl Acad Sci USA 85:8246-8250

Sakuma I, Stuehr DJ, Gross SS, Nathan C, Levi R 1988 Identification of arginine as a precursor of endothelium-derived relaxing factor. Proc Natl Acad Sci USA 85:8664-8667

Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN 1987 Leukotrienes and lipoxins: structures, biosynthesis and biological effects. Science 237:1171-1176

Sandermann Jr H 1978 Regulation of membrane enzymes by lipids. Biochim et Biophys Acta 515:209-237

Sanders RL, Hasset RJ, Vatter AE 1980 Isolation of lung lamellar bodies and their conversion to tubular myelin figure *in vitro*. Anat Rec 198:485-501

Scarpelli EM 1990 Pulmonary Physiology: Fetus, Newborn, Child, Adolescent (2nd ed). Lea and Febiger, Philadelphia, Pennsylvania. pp500

Seidner S, Jobe AH, Ikegami M, Pettenazzo A, Priestly A, Ruffini L 1988 Lysophosphatidylcholine uptake and metabolism in the adult rabbit lung. Biochim Biophys Acta 961:328-336

Shaul PW, Farrar MA, Magness RR 1993 Pulmonary endothelial nitric oxide production is developmentally regulated in the fetus and the newborn. Am J Physiol 265:H1056-H1063

Shier WT, Trotter JT 1976 Stimulation of liver microsomal sialyltransferase activity by lysolecithin. FEBS Lett 62:165-168

Shier WT, Baldwin JH, Nilsen-Hamilton M, Hamilton RT, Thanassi NM 1976 Regulation

of guanylate cyclase activities by lysolecithin. Proc Natl Acad Sci USA 73:1586-1590

Soll R, Hoekstra RE, Fangman JJ, Corbet AJ, Adams JM, James LS, Schulze K, Oh W, Roberts Jr JD, Dorst JP, Kramer SS, Gold AJ, Zola EM, Horbar JD, McAuliffe TL, Lucey JF, and the Ross Colaborative Surfactant Prevention Study Group 1990 Multicenter trial of a single-dose modified bovine surfactant extract (Survanta) for prevention of respiratory distress syndrome. Pediatrics 85:1092-1102

Soltis EE 1988 Effect of age on blood pressure and membrane-dependent vascular responses in the rat. Circ Res 62:889-897

Speer CP, Robertson B, Curstedt T, Halliday HL, Compagnone D, Gefeller O, Harms K, Herting E, McClure G, Reid M, Tubman R, Herin P, Noack G, Kok J, Koppe J, van Sonderen L, Laufkötter E, Köhler W, Boenisch H, Albrecht K, Hanssler L, Maim M, Oetomo SB, Okken A, Altfeld PC, Groneck P, Kachel W, Relier JP, Walti H 1992 Randomized European multicenter trial of surfactant replacement therapy for severe neonatal respiratory distress syndrome: single versus multiple doses of Curosurf. Pediatrics 89:13-20

Stevens PA, Wright JR, Clements JA 1989 Surfactant secretion and clearance in the newborn. J Appl Physiol 67(4):1597-1605

Stevenson D, Walther D, Long W, Sell M, Pauly T, Gong A, Easa D, Pramanik A, LeBlanc M, Anday E, Dhanireddy R, Burchfield D, Corbet A, and the American Exosurf Neonatal Study Group 1992 Controlled trial of a single dose of synthetic surfactant at birth in premature infants weighing 500 to 699 grams. J Pediatr 120:S3-S12

Stewart PA, Magliocco M, Hayakawa K, Farrell CL, Del Maestro RF, Girvin J, Kaufmann JCE, Vinters HV, Gilbert J 1987 A quantitative analysis of blood-brain barrier ultrastructure in the aging human. Microvasc Res 33:270-282

Survanta (Beractant) intratracheal suspension package insert. Columbus, Ohio: Ross Laboratories, 1993

Ten Centre Study Group 1987 Ten centre trial of artificial surfactant (artificial lung expanding compound) in very premature infants. Br Med J 294:991-996

Tijburg LBM, Nishimaki-Mogami T, Vance DE 1991 Evidence that the rate of phosphatidylcholine catabolism is regulated in cultured rat hepatocytes. Biochim Biophys Acta 1085:167-177

Tooley WH, Clements JA, Muramatsu K, Brown CL, Schluetter MA 1987 Lung function in prematurely delivered rabbits treated with a synthetic surfactant. Am Rev Respir Dis 136:651-656

Vadas P 1984 Elevated plasma phospholipase A_2 levels: Correlation with the

hemodynamic and pulmonary changes in gram-negative septic shock. J Lab Clin Med 104:873-881

Vadas P, Pruzanski W 1986 Role of secretory phospholipases A_2 in the pathobiology of disease. Lab Invest 55:391-404

Vadas P, Pruzanski W 1990 Phospholipase A_2 is the Pivotal Step in the Effector Pathway of Inflammation. IN: Phospholipase A_2: Role and Function in Inflammation. Wong PYK, Dennis EA (eds). Adv Exp Med Biol 275:83-101

Van De Graaff KM, Fox SI 1989 Concepts of Human Anatomy (2nd ed). Wm C Brown Publishers, Inc. Dubuque, Iowa. 1096 pp.

Van Golde LMG 1976 Metabolism of phospholipids in the lung. Am Rev Respir Dis 114:977-1000

Van Golde LMG, Batenburg JJ, Robertson B 1994 The pulmonary surfactant system. News in Physiological Science 9:13-20

Van Heusden GPH, Vianen GM, Van Den Bosch H 1980 Differentiation between acyl coenzyme A:lysophosphatidylcholine acyltransferase and lysophosphatidylcholine: lysophosphatidylcholine transacylase in the synthesis of DPPC in the rat lung. J Biol Chem 255:9312-9318

Van Iwaarden FB, Welmers B, Verhoef J, Haagsman HP, Van Golde LMG 1990 Pulmonary surfactant protein A enhances the host-defense mechanism of rat alveolar macrophages. Am J Respir Cell Mol Biol 2:91-98

Verger R, Ferrato F, Mansbach CM, Pieroni G 1982 Novel intestinal phospholipase A_2: Purification and some molecular characteristics. Biochem 21:6883-6889

Vermont Oxford Trials Network 1994 A multicenter randomized trial comparing synthetic surfactant to modified bovine surfactant extract in the treatment of neonatal respiratory distress syndrome: Pediatr Res 35:259A(1542)

Vidyasagar D, Maeta H, Raju TNK, John E, Bhat R, Go M, Dahiya U, Roberson Y, Yamin A, Narula A, Evans M 1985 Bovine surfactant (surfactant TA) therapy in immature baboons with hyaline membrane disease. Pediatrics 75:1132-1142

Von Wichert P, Temmesfeld M, Meyer W 1981 Influence of septic shock upon phosphatidylcholine remodeling mechanism in rat lung. Biochim Biophys Acta 664:487-497

Voorhout WF, Veenendaal T, Haagsman HP, Weaver TE, Whitsett JA, Van Golde LMG, Geuze HJ 1992 Intracellular processing of pulmonary surfactant protein B in an endosomal/lysosomal compartment. Am J Physiol 263:L479-L486

Walker SR, Williams MC, Benson B 1986 Immunocytochemical localization of the major

surfactant apoproteins in type II cells, Clara cells and alveolar macrophages of rat lung. J Histochem Cytochem 34:1137-1148

Weaver TE 1988 Pulmonary surfactant-associated proteins. Gen Pharmac 19:361-368

Weaver TE, Kropp KL, Whitsett JA 1982 *In vitro* sulfation of pulmonary surfactant-associated protein-35. Biochim Biophys Acta 869:330-336

Weaver TE, Whitsett JA 1989 Processing of hydrophobic pulmonary surfactant protein B in rat type II cells. Am J Physiol 257 (Lung Cell Mol Physiol):L100-L108

Weaver TE, Whitsett JA 1991 Function and regulation of expression of pulmonary surfactant-associated proteins. Biochem J 273:249-264

Weltzien HU 1979 Cytolytic and membrane-perturbing properties of lysophosphatidylcholine. Biochim Biophys Acta 559:259-287

White AA, Lad PJ 1975 Activation of the soluble guanylate cyclase from rat lung by preincubation. Fed Proc 34:232

Whittsett JA 1994 Composition and Structure of Pulmonary Surfactant. IN: New Therapies for Neonatal Respiratory Failure: A Physiological Approach. Boynton BR, Carlo AA, Jobe AH (eds). Cambridge University Press, New York, NY. pp3-15

Whitsett JA 1988 Structure and Function of Pulmonary Surfactant Protein SP-A. IN: Surfactant Treatment of Lung Diseases, Report of the Ninety-Sixth Ross Conference on Pediatric Research. Jobe AH, Taeusch HW (eds). Columbus, Ohio, Ross Laboratories. pp 9-19

Whitsett JA, Weaver T, Hull W, Ross G, Dion C 1985 Synthesis of surfactant-associated glycoprotein A by rat type-II epithelial cells: primary translation products and post-transcriptional modification. Biochim Biophys Acta 828:162-171

Williams MC 1977 Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. J Cell Biol 72:260-277

Wolf A, Saito T, Dudek R, Bing RJ 1991 The effect of lysophosphatidylcholine on coronary and renal circulation in the rabbit. Lipids 26:223-226

Wright JR 1990 Clearance and recycling of pulmonary surfactant. Am J Physiol 259 (Lung Cell Mol Physiol):L1-L12

Wright JR, Clements JA 1987 Metabolism and turnover of lung surfactant. Am Rev Respir Dis 136:1256-1275

Wright JR, Hawgood S 1989 Pulmonary surfactant metabolism. Clin Chest Med 10:83-93

Wright JR, Dobbs LG 1991 Regulation of pulmonary surfactant secretion and clearance. Annu Rev Physiol 53:395-414

Wright JR, Wager RE, Hawgood S, Dobbs L, Clements JA 1987 Surfactant apoprotein M_r=26-36000 enhances uptake of liposomes by type II cells. J Biol Chem 262:2888-2894

Zwiller JJ, Ciesielski-Treska J, Mandel P 1976 Effect of lysolecithin on guanylate and adenylate cyclase activities in neuroblastoma cells in culture. FEBS Lett 69:286-290