Surfactant Phosphatidylcholine Metabolism in Preterm Infants Studied with Stable Isotopes

CIP-data Koninklijke Bibliotheek, Den Haag
ISBN: 90-73235-39-1

Surfactant Phosphatidylcholine Metabolism in Preterm Infants Studied with Stable Isotopes
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Printed by Optima Grafische Communicatie, Rotterdam

Surfactant Phosphatidylcholine Metabolism in Preterm Infants Studied with Stable Isotopes

Het metabolisme van surfactant fosfatidylcholine bij te vroeg geboren kinderen, bestudeerd met stabiele isotopen

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.Dr. P.W.C. Akkermans, M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
vrijdag 16 juni 2000 om 11:00 uur

door

Jan Erik Hendrik Bunt

geboren te Utrecht

Promotiecommissie

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to my parents
to Daphne

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Chapter 1

Introduction

INTRODUCTION

Respiratory distress syndrome (RDS) is an important cause of morbidity and mortality in the preterm infant despite the introduction of prenatal glucocorticosteroids, postnatal surfactant treatment, and improved perinatal care. The important factor causing the development of RDS is primary surfactant deficiency. Pulmonary surfactant is necessary to maintain alveolar patency by reducing the surface tension at the air-liquid interface in the alveoli.

Surfactant is a complex mixture of lipids and surfactant specific proteins. Of the surfactant lipids, phosphatidylcholine is the most abundant and main surface tension lowering component. Surfactant is synthesized in the alveolar type II pneumocyte and secreted to the alveolar space to form a surface active monolayer. Surfactant components can be recycled by the type II cell or catabolized and cleared from the lung. Knowledge about surfactant production and catabolism is important to understand the pathophysiology of RDS and to improve treatment.

Administration of large amounts of exogenous surfactant to the preterm infant with RDS is now standard treatment. Some *in vitro* and animal studies suggest the presence of a feedback mechanism to regulate endogenous surfactant synthesis. It is unknown whether exogenous surfactant interferes with endogenous surfactant synthesis in preterm infants.

In vitro studies show that corticosteroids stimulate surfactant synthesis but studies in animals give conflicting results. Preterm infants that received corticosteroids prenatally have a decreased incidence and severity of RDS. Whether this is due to increased surfactant synthesis is unknown.

In animals surfactant metabolism is studied with radioactive isotopes, however, this approach is not acceptable in human subjects, especially not in human preterm infants. The ionizing radiation may cause injury to the molecular structure of the cell, leading to chromosome aberrations or cell death. Surfactant metabolism has been studied in many experiments in different animals, at different developmental stages, under different conditions, with hormonal and surfactant treatments. Surfactant analyses of sequential tracheal aspirates of human infants have been performed and provide data on the quality and concentration of surfactant in the epithelial lining fluid. However, the data are difficult to interpret as the total volume of the epithelial lining fluid is unknown and the surfactant tissue pools have not been measured, and therefore, do not give direct information on the processes of synthesis and catabolism. In addition, the study of endogenous surfactant metabolism with the current methods is complicated by the administration of large doses of exogenous surfactant as therapy for severe RDS.

To study metabolic pathways and turnover in humans, stable isotopes have been applied for the last 60 years. Stable isotopes have the obvious advantage of being nonradioactive and thus can be used safely in preterm infants. The use of stable isotopes is a potential method to study surfactant metabolism in humans. There are different stable isotopes, like ^{2}H (deuterium), ^{15}N, and ^{13}C. In the studies presented in this thesis, we used the tracers [U-^{13}C]glucose, [U-^{13}C]palmitic acid, and [U-^{13}C]linoleic acid, all containing the stable isotope ^{13}C. The stable isotope ^{13}C has a natural occurrence (enrichment) of ~1.11 % of the total carbon atoms in the human body. Small differences are present between individuals, mainly due to differences in diet. To account for this natural enrichment, baseline samples are taken prior to isotope administration. In the samples, the ratio of $^{13}C/^{12}C$ are measured directly, so sampling techniques and sample amount are of little influence. The stable isotopes are administered to the infants and are incorporated into the fatty acids in surfactant phosphatidylcholine by endogenous synthesis of phosphatidylcholine. The rate of incorporation is a measure for synthesis of surfactant phosphatidylcholine. The disappearance of the stable isotope from phosphatidylcholine is a reflection clearance of surfactant phosphatidylcholine.

AIM OF THE STUDIES

1. To develop and use a novel method to study surfactant metabolism in preterm and older infants.
(chapters 3 and 4).
2. To study endogenous surfactant synthesis in relation to prenatal glucocorticosteroids.
(chapters 5 and 6).
3. To study the influence of surfactant therapy on endogenous surfactant metabolism.
(chapters 7 and 8).
4. To study surfactant composition and concentration after surfactant therapy.
(chapter 8).

Chapter 2

Surfactant Metabolism in the Preterm Infant

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Summary

Pulmonary surfactant phosphatidylcholine (PC) is synthesized and stored as lamellar bodies in the alveolar type II pneumocyte. In respiratory distress syndrome (RDS), surfactant alveolar pool sizes are low (5 to 10 mg PC/kg) and the composition is immature with less disaturated PC. The process of synthesis and secretion to the alveolus is a slow process *in vivo*, as shown by studies with labeled precursors in animals and human infants. The slow incorporation of labeled precursors into surfactant PC over days corresponds to slow increases in PC concentrations in alveolar fluid and total lung pool sizes after preterm birth. The synthesis of endogenous surfactant is stimulated by administration of prenatal corticosteroids in preterm animals and human preterm infants. Because the synthesis of surfactant PC is low, the accumulation of sufficient amounts in the alveolus for adequate ventilation requires several days.

Surfactant is cleared from the alveoli mainly by uptake by the type II pneumocyte and is recycled to a large extent (~90% in the premature animal). The apparent half-life of exogenously administered surfactant PC ranges from 30 to 110 h in preterm infants with RDS. Therefore, the concentration of surfactant in the alveoli remains elevated for several days after exogenous surfactant therapy for RDS. There are data that show that exogenous surfactant does not suppress endogenous surfactant synthesis in preterm infants.

Submitted

INTRODUCTION

After premature delivery, neonatal respiratory distress syndrome (RDS) is an important cause of morbidity and mortality.[1] Avery and Mead showed in 1959 that pulmonary surfactant deficiency is a major factor in the pathophysiology of RDS.[2] In 1980, for the first time, exogenous surfactant was administered endotracheally to preterm infants to treat RDS successfully by Fujiwara *et al.*[3] Later, multicenter trials demonstrated decreased death rates and complications of RDS after surfactant administration.[4-6] Although most infants respond favorably to surfactant treatment, some infants have no or little response and some complications of RDS like bronchopulmonary dysplasia, intraventricular and pulmonary hemorrhage have not decreased significantly by the introduction of surfactant therapy. Surfactant deficiency in RDS is characterized by small surfactant pools, immature surfactant composition and functional inhibition by plasma proteins. Because the intracellular and alveolar surfactant reserves are small, they are tightly regulated by synthesis, secretion, and recycling.[7] In the present review, surfactant phospholipid metabolism in the human preterm infant will be discussed and *in vitro* and animal studies will be used to put the data from human studies into perspective or when little information from humans is available. Other reviews have focused mainly on surfactant metabolism *in vitro* and in animals.[8-14]

Liggins *et al.* showed in 1972 that one course of prenatal corticosteroids decreased the incidence and severity of RDS in preterm infants.[15] Multiple courses can decrease birth weight and increase mortality.[16] Enhancing lung maturation by administration of prenatal corticosteroids seems more effective than surfactant therapy in reducing RDS and its complications.[17,18] The combined use of prenatal corticosteroids and postnatal surfactant is more beneficial than either treatment alone.[19] The effects of prenatal corticosteroid therapy and postnatal surfactant administration on surfactant PC metabolism will be discussed. A better understanding of surfactant metabolism could help to fine-tune the treatment of the preterm infant with surfactant deficiency.

SURFACTANT FUNCTION, COMPOSITION, AND POOL SIZE IN THE PRETERM NEONATE

Adequate amounts of pulmonary surfactant decrease the surface tension at the air-liquid interface in the alveoli and distal bronchioli which promotes lung expansion during inspiration and prevents alveolar collapse at expiration. Insufficient amounts of surfactant lead to decreased pulmonary compliance, decreased functional residual capacity, atelectasis, and enlargement of the functional right-to-left shunt, decreased gas exchange, respiratory acidosis, and pulmonary edema with further inactivation of surfactant by plasma constituents.

Surfactant is a complex mixture of lipids (~90%) and proteins (~10%) and its composition is similar across species including the human.[20-22] Surfactant is synthesized and stored in the alveolar type II pneumocyte and secreted to the alveolus. Of the surfactant lipids, 80 to 90% are phospholipids, and the other lipids, in decreasing order, are cholesterol, triacylglycerol, and free fatty acids.[23] Phosphatidylcholine (PC) is the major phospholipid (70 to 80% of the phospholipids) and is the main surface tension lowering component of surfactant. The PC molecule comprises a glycerol backbone, two fatty acids, phosphate, and a choline moiety. Approximately 50 to 60% of the PC is disaturated (Sat PC, DSPC) of which ~75% is the dipalmitoyl (16:0/16:0) species (DPPC).[20,24,25] Other surfactant phospholipids are phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, lysophosphatidylcholine, and sphingomyelin.[22]

The surfactant proteins are either hydrophilic (SP-A and SP-D) or hydrophobic (SP-B and SP-C).[26] The surfactant proteins are either exclusively lung-associated or predominantly found in the lung. SP-A reduces the secretion of PC and stimulates the formation of tubular myelin

from secreted lamellar bodies.[27] SP-B and possibly SP-C are required for the formation of tubular myelin and both proteins are necessary for rapid spreading of surfactant onto the air-liquid interface.[28-32] Surfactant protein A and D play a role in the first line defense against inhaled pathogens[33] The present review will not deal extensively with the surfactant proteins, and the reader is referred to other reviews.[22,26,34,35]

The composition and concentration of surfactant phospholipids in amniotic fluid reflect lung maturation and the risk of developing RDS. Several measures to estimate lung maturity or risk of developing RDS have been described such as the lecithin:sphingomyelin ratio (L/S ratio),[36,37] Sat PC concentration in amniotic fluid,[38] the palmitic acid:stearic acid (P/S) ratio of PC,[39,40] and the presence or absence of phosphatidylglycerol.[36,41] Such measurements, however, are rarely made in clinical practice and should be interpreted with caution as reviewed by Gluck *et al.*[42,43] After birth, lung maturation continues as reflected by alveolar surfactant measurements.[44-46] Hunt *et al.* found in human fetal lung tissue that PC became more disaturated during gestation with predominantly PC (16:0/16:0) and PC (14:0/16:0).[20] Surfactant PC from preterm infants at birth with RDS contains a lower percentage palmitic acid than infants that do not develop RDS, and the percentage palmitic acid increases during the first weeks of life.[47,48]

Jackson *et al.* measured an alveolar pool size of Sat PC of ~5 mg/kg by lavage in preterm monkeys with RDS at birth.[49] Preterm sheep and young pigs have alveolar pool sizes of PC of ~10 mg/kg.[50-52] The pool size estimates are remarkably constant across animal species and humans. The first pool size studies of phospholipids in humans were performed by Adams *et al.* using autopsy material from premature infants.[44] The total lung and alveolar phospholipid and PC pool sizes remained constant up to 24 weeks and increased afterwards.[44,45] Hallman *et al.* administered exogenous surfactant containing PG and measured the dilution by endogenous alveolar surfactant, as endogenous surfactant in infants with RDS does not contain PG[53] Hallman found an alveolar pool size of 8 to 10 mg PC/kg.[46] Using the same method with either sphingomyelin or PG as markers, Griese *et al.* found an alveolar pool size of ~20 mg phospholipid/kg.[54] Torresin *et al.* administered exogenous surfactant labeled with [U-^{13}C]DPPC, to preterm infants with RDS and calculated the alveolar pool size to be ~8 mg PC/kg.[55] These methods using dilution of an exogenous marker to calculate pool size have to be considered with some caution. After surfactant administration approximately 50% of the introduced surfactant becomes directly lung tissue associated and cannot be retrieved by lavage, which would lead to an overestimation of the dilutional effect by endogenous alveolar surfactant, resulting theoretically in a 50% overestimation of the alveolar endogenous pool size.[12] Furthermore, the exogenous label probably equilibrates with surfactant in the type II cell and lung tissue phospholipids. It is not clear to which extent the extra-alveolar surfactant phospholipid pool is included in these calculations. However, the remarkable similarity with the autopsy and animal data is reassuring.

Surfactant synthesis and secretion

Surfactant PC is synthesized in the endoplasmatic reticulum (proteins) and/or Golgi apparatus (lipids) (fig. 1).[56] Precursors (glucose, ketone bodies, acetate) and components (fatty acids, choline, phosphorus, glycerol) are used for surfactant PC synthesis.[57-60]

The CDP-choline pathway is the principle pathway involved in the *de novo* synthesis of surfactant PC.[14,24,25,61,62] The formation of glycerol-3-phosphate is the principle starting point for the synthesis of diacylglycerolipids. The glycerol-3-phosphate is mainly formed by reduction of dihydroxyacethone phosphate. Cholinephosphotransferase catalyses the formation of PC from diacylglycerols and CDP-choline. The required CDP-choline is made by CTP-phosphocholine cytidylyltransferase from phosphocholine, which in turn is formed from

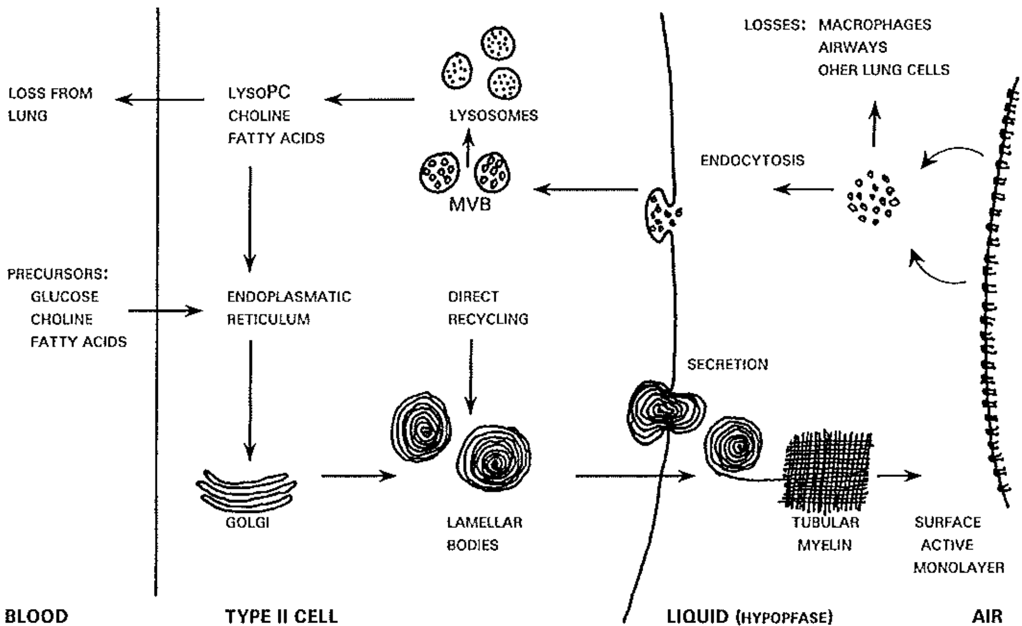

Figure 1. Illustration of surfactant phosphatidylcholine metabolism. The precursors for surfactant PC are taken up by the type II pneumocyte from blood. The secreted surfactant forms a surface tension lowering monolayer. The surfactant is degraded after some cycles of compression and expansion and mainly recycled. TB = tubular myelin, MVB = multi vesicular body, LysoPC =lysophosphatidylcholine.

choline by choline kinase. There is ample evidence that the enzyme CTP-phosphocholine cytidylyltransferase plays an important regulatory role in the *de novo* synthesis of PC.[63-66] In the type II cell of the fetal lung, intracellular glycogen stores appear to be a major source of the gly-cerol backbone of PC,[67] and in the adult lung glucose from the blood stream is a major substrate for glycerol. The choline is mainly derived from the diet.[68] The fatty acids required for the surfactant phospholipids are derived from blood supply and from *de novo* fatty acid synthesis. There is evidence that the lipogenesis of fatty acids from glucose and lactate among other substrates, is of particular importance to supply fatty acids for phospholipid synthesis in the prenatal lung.[69-71] In more mature lungs, the fatty acids of surfactant PC are derived mainly from uptake of fatty acids from plasma.[52] The composition of the newly formed PC is modified to achieve high levels of DPPC, by acyl remodeling, mainly involving sequential actions of phospholipase and acyltransferase enzymes.[72-74]

Intracellular surfactant is stored as lamellar bodies which are condensed, highly structured lipoprotein packages (fig. 1). Lamellar bodies can be detected in human type II cells from approximately 20 weeks gestation[75] and are secreted thereafter, as reflected by increasing PC concentrations in amniotic fluid.[38] Secretion of lamellar bodies is stimulated by mechanical stretch of the alveolus during inspiration[76-81] Various agents, including agonists for β-adrenergic-, purino-, and vasopressin-receptors increase cytosolic Ca^+, cellular c-AMP, or activate protein kinases which can stimulate secretion of lamellar bodies.[9,76,80,82-85] At secretion, the lamellar bodies lose their limiting membrane[86] and enter the hypophase (epithelial lining fluid, ELF). The lamellar bodies unravel to form loose membranous arrays and lattice-like structures, called tubular myelin (fig. 1).[87-89] In cultured human fetal type II cells at 16 to 21 week of gestation SP-A is already being synthesized and mainly secreted apart from the

lamellar bodies.[90] SP-A deficient mice do not have tubular myelin in the alveolar surfactant fraction. This lack has no effects on overall metabolism of Sat PC or SP-B in mice.[91,92] At alveolar surface expansion during inspiration, surfactant components insert from the hypophase into the monolayer. At expiration the alveolar surface reduces and the monolayer is compressed, thereby squeezing out some surfactant proteins, unsaturated PC, and other lipids. By this mechanism, the monolayer comprises mainly the most surface tension lowering component DPPC during compression.[93,94]

The time required for *de novo* PC synthesis, secretion, and significant alveolar accumulation has been studied in animals using radioactive labeled substrates. In the newborn rabbit and sheep, following the intravascular injection of radiolabeled palmitic acid, recovery of labeled alveolar PC reaches its peak at 35 to 45 h, as shown in figure 2, which suggests a slow *de novo* surfactant PC synthesis in these animals.[8,95-97] In preterm ventilated lambs, by 24 h, only 0.5% of the endogenously synthesized PC had been secreted to the alveolus, indicating slow movement of the PC from the synthetic sites to the alveolus.[50]

Preterm infants (<34 wk) that do not develop RDS have higher surfactant PC concentrations[48,98,99] and percentages of DPPC in tracheal aspirates and alveolar lavages[100] implying a more developed surfactant production compared to infants that develop RDS. In preterm infants with RDS, surfactant concentration increased slowly on day one and two of life, and then remained constant at values similar to infants that were treated with surfactant for RDS or that did not have RDS.[48,98] Others report in preterm infants ventilated for RDS that alveolar concentrations of Sat PC and SP-A are very low at birth, with concentrations still significantly lower three days after birth than in preterm ventilated infants without RDS.[99] These slow increases in PC and SP-A concentration could imply a slow endogenous surfactant synthesis and corresponds to the usual clinical improvement several days after birth in infants with RDS. In preterm infants with RDS that received the stable isotope [U-^{13}C]glucose, the palmitic acid in surfactant PC became labeled after approximately 18 h and was maximally labeled after about 70 h (fig. 3).[101] In ventilated critically ill infants surfactant PC became labeled after about 10 h after infusion of [U-^{13}C]palmitic acid or [U-^{13}C]linoleic acid and was labeling maximal after ~65 h.[102]

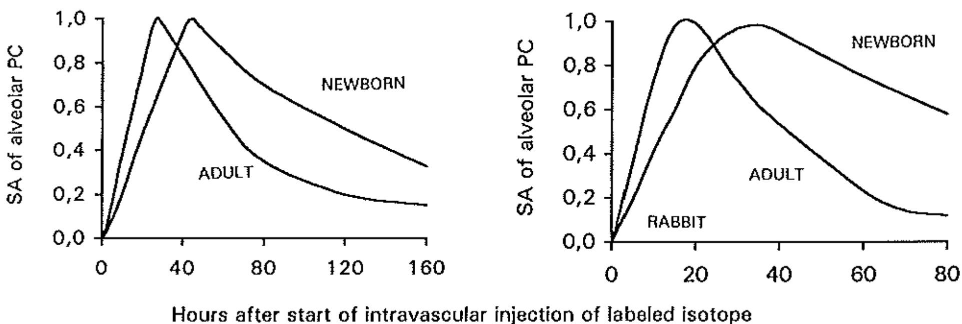

Figure 2. Kinetics of secretion of de novo synthesized phosphatidylcholine (PC). The curves show the accumulation of labeled PC in the airspaces following the intravascular injection of the radiolabeled precursor palmitic acid. This precursor is incorporated into lung PC within minutes of intravascular injection. The curves were normalized such that the maximal specific activities for each curve expressed as CPM/ mol PC were given a value of 1.0. The curves show slower accumulation in the newborn than in the adult. Data are derived from several sources as reviewed by Jobe.[8]

In preterm infants with RDS, the first incorporation of linoleic acid from intravenous feeding into surfactant PC had a delay of ~3 d.[48] The patterns of ^{13}C-incorporation in infants are in line with results in preterm ventilated lambs and baboons (fig. 2)[8,95,96,103] and the slow increase in surfactant PC concentrations after birth measured by Hallman *et al.*[98] Cogo *et al.* used plasma [U-^{13}C]palmitic acid and plasma [U-^{13}C]linoleic acid as tracers for surfactant PC metabolism in critically ill ventilated infants (with a mean age of 50 d).[102] It was estimated that 34 and 50% of the total surfactant PC pool was synthesized per day, when calculated with palmitic and linoleic acid, respectively.[102] Absolute synthesis rates have been estimated *in vivo* in RDS and range from minimally 5 mg/kg/d in preterm infants to ~12 mg/kg/d in preterm monkeys.[49,101]

In summary, the endogenous synthesis and secretion of surfactant PC in the preterm infant is a slow process. The lung of the preterm infant seems not to be able to adapt rapidly to extra-uterine life which requires a significant alveolar surfactant pool and therefore exogenous surfactant administration is necessary to rapidly augment surfactant pools in RDS.

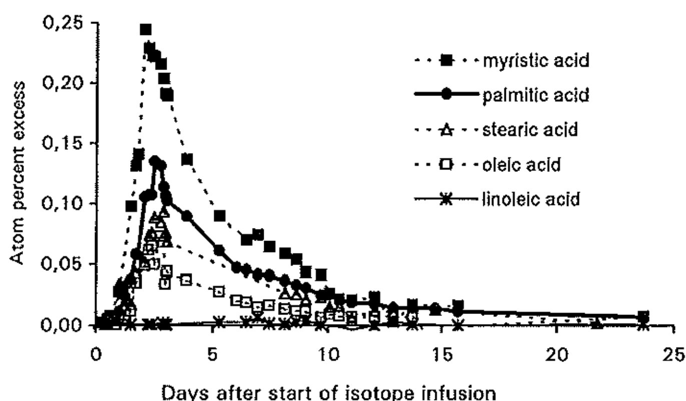

Figure 1. ^{13}C-enrichment of selected fatty acids of surfactant phosphatidycholine in a patient with RDS. At birth a 24-h [U-^{13}C]glucose infusion was administered. Tracheal aspirates were obtained. The ^{13}C-enrichment in surfactant phosphatidylcholine (PC) was measured and expressed in atom percent excess.[101]

SURFACTANT CLEARANCE

Due to the cyclic surface changes during respiration the surfactant monolayer is degraded to small aggregates representing "used surfactant". By differential centrifugation of alveolar fluid, these "small aggregates" (= inactive surfactant) can be separated from the lamellar bodies and tubular myelin, the "large aggregates" (= active surfactant).[104-106]

The major route for clearance of alveolar surfactant is through uptake of the small aggregates by the type II cell,[107] which is stimulated by SP-A *in vitro*.[108] The PC molecule is not degraded by phospholipase prior to reuptake.[109] The PC molecule is probably reutilized mainly intact directly[110,111] via lamellar bodies or becomes degraded in lysosomes to degradation products which can be used for surfactant synthesis or lost from the type II cell.[112] In the newborn, only small amounts of alveolar surfactant (~10%) are lost via uptake by macrophages and other lung cells, via the airways, and lost to the blood. After instillation of radiolabeled surfactant, radioactivity can be measured in liver, brain, kidney, and serum indicating loss of surfactant components from the lung.[113] In the term newborn rabbit and in preterm lambs, the turnover time (the time to fill the pool if it were empty) for alveolar Sat PC has been estimated to be about 10 and 13 h, respectively.[111,114] This is a short period relative to the slow *de novo* surfactant PC synthesis due to a high degree of alveolar recycling. The surfactant PC recycling was calculated to be >90% in the newborn rabbit and 50% in adult rabbits[115] and 90% in young pigs.[52]

The half-life of a surfactant component is often described as "apparent" because surfactant components are lost from the total lung much slower than from the alveolus due to recycling. Alveolar and total lung half-life are difficult to separate in *in vivo* studies. Measurement of half-life of alveolar surfactant is complicated and findings are different between studies because of many difficulties with the models. When a labeled precursor is injected intravenously, the alveolar pool of surfactant PC is not pulse labeled.[97,116] This factor along with reutilization tends to prolong the apparent half-life. After an intravenous tracer, the half-life probably represents the disappearance of label from the total lung surfactant pool. When labeled preparations of exogenous surfactant are administered endotracheally, the alveolar pool PC becomes pulse labeled and therefore only the reutilization would be involved in prolonging the apparent half-life. Labeled components of surfactant PC can be used for lung tissue synthesis. Degradation of lung tissue can then again release labeled substrates for surfactant PC synthesis, which prolongs the measured surfactant PC half-life.[117] The continued entrance of labeled PC precursors from other body pools (e. g. liver or adipose tissue) long after the initial label administration could in theory also prolong a measured half-life.

It is not clear how surfactant pools change after birth and after surfactant administration in the human preterm infant. In 3-day-old rabbits, total lung surfactant pool sizes remained constant for 3 d after increasing the pool three- to five-fold by exogenous surfactant.[118] Half-lives calculated in animal studies differ depending on gestational and postnatal age, the labeled substrate used and the surfactant pool studied. The difference between species is small when similar methods are applied.[119] The half-life of alveolar surfactant PC in newborn rabbits after intravenous palmitic acid ranged from 97 to 136 h, and after intravenous choline the half-life of PC was >200 h.[120] In lung parenchyma, the half-lives of PC-palmitate ranged from 50 to 94 h and for PC-choline it was >100 h.[120] In term newborn lambs, [^{3}H]palmitate labeled PC in alveolar samples had a half-life of approximately 50 h, after intravenous administration of [^{3}H]palmitate.[95] These values indicate a very slow catabolism and/or considerable reutilization of endogenous surfactant PC in the newborn. After endotracheal injection of [^{3}H]PC in the newborn lamb, the half-life of alveolar PC were 12 and 6 d for alveolar and lung tissue PC, respectively.[117] These values are different from half-lives of alveolar [^{14}C]DPPC in the very premature baboon of ~30 h after intratracheal administration,[121] possibly due to more recycling in premature animals, and species difference. Although ~80% of the labeled surfactant had disappeared from the total baboon lung on day six, the lung tissue contained increased Sat PC pools with low amounts in the alveolus, indicating extensive synthesis of PC but little secretion.[121]

Studies on surfactant catabolism have been performed mainly in premature infants treated with surfactant for RDS. In preterm infants who received human amniotic fluid-derived surfactant containing phosphatidylglycerol (PG), the half-life of PG calculated from tracheal aspirates was ~30 h.[46] This value is similar to the half-life of PG ~35 h, calculated in preterm lambs with the same method.[50] In preterm infants treated with Survanta®, alveolar PG and sphingomyelin had half-lives of ~105 and ~97 h, respectively, but in infants treated with Alveofact®, alveolar PG had a half-life of ~43 h.[54] Differences in the lipid composition or the higher content of SP-B in Alveofact® could play a role in the different half-lives of PG between the preparations. The similar half-lives of PG and sphingomyelin in the Survanta® treated infants indicate that these two phospholipids may be metabolized similarly. Hallman *et al.* treated three preterm infants with human surfactant with [^{2}H]DPPC and unlabeled PG.[122] In these neonates, the PG concentration had a shorter half-life than the ^{2}H-enrichment of DPPC (~33 *versus* ~50 h), which could reflect a difference in the metabolism between these phospholipids.[122] The apparent half-life of PG in natural human surfactant containing SP-A was significantly shorter compared to the half-life of PG in Alveofact® without SP-A. This could be related to the presence of SP-A which enhanced lipid uptake by the type II cell.[123-125] However, SP-A did not promote lipid uptake in SP-A knock-out mice.[126]

Indirect evidence for a slow clearance of surfactant is reflected in several studies about alveolar surfactant concentration and composition after surfactant administration. In preterm infants the concentration of PC did not decrease significantly for many days - even up to four weeks - after therapy.[98,99] Ashton et al showed that the percentage DPPC of total lipids in tracheal aspirates increased due to surfactant (containing a high percentage of DPPC) and returned to baseline by d 6 after administration.[100] Bunt *et al.* showed that the administration of Survanta® containing 85% palmitic acid in PC, increased the PC palmitate percentage in tracheal aspirates directly, with a subsequent half-life of ~34 h after one dose and ~53 h after two doses of surfactant.[48]

Torresin *et al.* treated preterm infants with RDS with Curosurf® with [U-^{13}C]palmitic acid-labeled DPPC and calculated the half-life to be ~36 h[55] Using labeled DPPC has the advantage that it is the major component while PG and sphingomyelin are minor components of surfactant.In preterm infants the half-life of ^{13}C-labeled PC-palmitate measured in tracheal aspirates was ~99 h (71 to 144) after an intravenous infusion of [U-^{13}C]glucose.[101,127] Half-lives were similar after one, two, or three doses of surfactant, but tended to be lower (~70 h) when the infants had received no exogenous surfactant.[127]

In infants with acute respiratory distress syndrome that did not receive exogenous surfactant, the half-life of ^{13}C-labeled PC-palmitate ranged from 17 to 178 h and the half-life of ^{13}C-labeled PC-linolate ranged from 24 to 144 h after a [U-^{13}C]palmitic acid and [U-^{13}C]linoleic acid infusion.[102] This variability between individual patients could be related to differences in lung diseases and could possibly affect the clinical course of the respiratory failure.[102]

In infants with severe RDS, the first dose of surfactant (100 mg/kg) is sufficient to increase the surfactant pool size immediately, and when more doses are necessary, this is probably required to overcome inhibition,[128] as the lungs of neonates with RDS are abnormally permeable to small solutes and proteins.[129]

In term rabbits, the clearance of labeled exogenous surfactant from the total lung was a fixed (16%) percentage of the dose per day and showed that the lung can adapt to large amounts of exogenous surfactant and that clearance mechanisms were not saturated.[130] In the human preterm infant, some studies reported that half-lives were unrelated to the number of doses.[46,48] Others showed longer half-lives in relation to more doses exogenous surfactant.[100,127] However, the number of patients were small in each study, with large inter individual variability, and therefore the effect of exogenous surfactant on surfactant clearance in preterm infants should be studied further.

In summary, compared to animal studies, there exist only few studies on surfactant metabolism in the human infants and there are no data on recycling of surfactant. The preterm and newborn lung is characterized by a slow turnover of PC with very efficient recycling, and little clearance.

EFFECTS OF SURFACTANT THERAPY ON SURFACTANT SYNTHESIS

Although the administration of exogenous surfactant has become routine treatment of RDS in the preterm infant, there is very little information regarding effects of exogenous surfactant on endogenous surfactant metabolism. In healthy adult rabbits *in vivo*, administration of surfactant to the left lung only, resulted in increased incorporation of palmitic acid from plasma into surfactant PC in the left lung but not in the right lung[131] suggesting stimulation of endogenous synthesis. In preterm ventilated lambs, surfactant treatment stimulated [^{3}H]palmitic acid incorporation into surfactant PC after correction for the increased surfactant pool.[50] In 3-day-old rabbits, the administration of surfactant did, however, not influence the incorporation of labeled precursor in the total lung surfactant pool.[118]

In preterm infants with RDS, the half-life of phosphatidylglycerol was independent of the dose of exogenous surfactant (60 *versus* 120 mg/kg).[46] This indicates that the absolute turnover had doubled after a two doses of surfactant compared to one dose. In preterm infants, the concentration of SP-A increased after exogenous surfactant while this preparation did not contain any SP-A, which suggests that endogenous SP-A secretion was stimulated and not suppressed.[132,133] In preterm infants the incorporation of ^{13}C from intravenous [U-^{13}C]glucose into alveolar surfactant PC palmitate increased after exogenous surfactant treatment.[127]

In summary, there is little information from animal and human studies about effects of exogenous surfactant on surfactant metabolism. Exogenous surfactant treatment in the premature with RDS, seems not to suppress endogenous surfactant synthesis.

PRENATAL CORTICOSTEROIDS

The role of corticosteroids on surfactant PC synthesis and structural lung development has been described mainly in *in vitro* and animal studies.[134,135] In many *in vitro* studies corticosteroids increase the activity of the enzymes involved in surfactant PC synthesis[64,136-139] and increase surfactant protein synthesis.[137,140-142] The rate limiting enzyme CTP:phosphocholine cytidylyltransferase (CT) in the surfactant PC synthetic pathway, is enhanced by corticosteroids probably in the following way: corticosteroids stimulate the synthesis of the fibroblast pneumocyte factor (FPF) in the lung fibroblast. The FPF stimulates the formation of CT directly in the type II cell, and FPF induces via fatty acid synthetase the synthesis of fatty acids that can stimulate the activity of CT[139,143,144] and stimulates surfactant phospholipid synthesis by the type II cell.[145] Corticosteroids can probably also stimulate PC synthesis in the absence of fibroblasts.[64] Corticosteroids stimulate glycogen storage in the type II cell during early gestation[146,147] and stimulate glycogenolysis in the type II cell to supply acetate for fatty acids and the glycerol for PC synthesis later in gestation during the period of surfactant synthesis.[148-150]

In *in vitro* experiments with lung slices and isolated type II cells, corticosteroids increase the incorporation of radiolabeled precursors into surfactant PC, reflecting increased PC synthesis.[136,139,144,151-156] Corticosteroids at low concentrations *in vitro* seem to increase SP-A and SP-B synthesis and at high concentration inhibit their synthesis.[157-159] The *in vivo* prenatal corticosteroid treatment of preterm lambs increases surfactant proteins mRNA's rapidly and surfactant protein amounts in lung tissue.[160] In a study by Kessler *et al.* in premature baboons, a 72-h treatment with prenatal dexamethasone did not increase radioactive palmitate incorporation in lung lipids, but it increased total lung phospholipid and alveolar lavage DPPC in lung lavage fluid at birth.[161] In preterm baboons, the synthesis rate of surfactant PC from plasma glucose was doubled after a 48 h treatment with prenatal corticosteroids.[162]

Data on pool size measurements as an indication of surfactant synthesis *in vivo* are conflicting. In some studies with newborn rabbits, preterm monkeys, and lambs chronically catheterized *in utero*, corticosteroid treatment 2 to 3 d before delivery increased surfactant PC pool sizes in alveolar lavage and lung tissue significantly after birth.[136,161,163,164] In contrast, other studies in large animals show no prenatal steroid induced increase of the alveolar[165-169] or total lung Sat PC pool size after birth.[165-168] Ballard *et al.* and Ikegami *et al.* did not find an increase in total lung Sat PC pool size at birth in preterm lambs when corticosteroids were administered 2 to 4 d before preterm delivery.[166,170] However, when corticosteroids were administered one week before delivery, the total lung Sat PC pool sizes at birth increased significantly.[166,170]

Obladen *et al.* found increased concentrations of both PC and PG in tracheal and pharyngeal aspirates in preterm infants after stressed pregnancies.[171] In preterm infants that had received prenatal corticosteroids, the alveolar surfactant showed improved function *in vitro*

during the first days of life although PC concentrations in tracheal aspirates were not elevated, which would imply qualitative rather than quantitative changes.[172] Arias *et al.* found increased L/S ratios in amniotic fluid from mothers who had been treated with dexamethasone.[173] We recently found in preterm infants that two doses of prenatal corticosteroids doubled the endogenous surfactant synthesis from the precursor plasma glucose.[174]

In summary, prenatal corticosteroids enhance surfactant PC synthesis *in vivo*, but the *de novo* synthesis rates remain low, and alveolar pool sizes are not increased within 48 h. In preterm animals with RDS, prenatal corticosteroids improve pulmonary compliance within 15 h[168] accompanied by stimulated structural development.[175-180] Therefore, it could well be that the improved outcome of preterm infants after a relative short period of exposure to prenatal corticosteroids is more the result of non-surfactant phospholipid mechanisms by which pulmonary function is improved.

CONCLUSIONS

Pulmonary surfactant deficiency in the preterm infant is a major factor in the pathophysiology of RDS. The incidence and severity of RDS has decreased by the use of prenatal corticosteroid therapy and surfactant therapy. A clear understanding of surfactant metabolism will help to gain more insight in the pathophysiology and treatment of RDS and other neonatal lung diseases. The information about surfactant phosphatidylcholine metabolism in the newborn animal is extensive, but there are few studies in human infants. Studies in newborn animals suggest a slow *de novo* synthesis and clearance of surfactant and a rapid recycling in order to "reactivate" used surfactant. Surfactant PC concentrations are low in RDS and increase slowly in several days. Several studies in the human preterm infant indicate a slow synthesis and clearance from the lung. Surfactant treatments do not seem to interfere with endogenous metabolic pathways for surfactant synthesis and secretion. Data suggest that prenatal corticosteroids increase surfactant synthesis in the preterm infant. To which extent this stimulation contributes to improved neonatal outcome after a relative short period of exposure to prenatal corticosteroids remains unclear.

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Chapter 3

Endogenous Surfactant Turnover in Preterm Infants Measured with Stable Isotopes

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Summary

We studied surfactant synthesis and turnover *in vivo* in preterm infants using the stable isotope [U-^{13}C]glucose, as a precursor for the synthesis of palmitic acid in surfactant phosphatidylcholine (PC). Six preterm infants (birth weight, 916±244 g; gestational age, 27.7±1.7 wk) received a 24-h [U-^{13}C]glucose infusion on the first day of life. The ^{13}C-enrichment of palmitic acid in surfactant PC, obtained from tracheal aspirates, was measured by gas chromatography-combustion interface-isotope ratio mass spectrometry.

We observed a significant incorporation of carbon-13 from glucose into surfactant PC palmitate. PC palmitate became enriched after 19.4±2.3 (16.5 to 22.3) h and reached maximum enrichment at 70±18 (48 to 96) h after the start of the label infusion. The fractional synthesis rate (FSR) of surfactant PC palmitate from glucose was 2.7±1.3%/d. We calculated the absolute production rate of surfactant PC to be 4.2 mg/kg/d, and the half-life to be 113±25 (87 to144) h.

Data on endogenous surfactant production and turnover were obtained for the first time in human infants with the use of stable isotopes. This novel and safe method could be applied to address many important issues concerning surfactant metabolism in preterm infants, children, and adults.

Am. J. Respir. Crit. Care Med. 1998; 157: 810-814

INTRODUCTION

pulmonary surfactant deficiency is the main cause of respiratory distress syndrome (RDS) in the preterm infant.[1] Prenatal administration of corticosteroids and postnatal treatment with exogenous surfactant have greatly reduced morbidity and mortality caused by RDS.[2,3] However, some infants respond poorly or only temporarily to surfactant therapy. Different clinical responses to exogenous surfactant could reflect differences in surfactant metabolism between individuals.

To date, surfactant analysis of sequential tracheal aspirates is the only available method for the evaluation of surfactant production in humans.[4,5] Although this method provides data on the quality and concentration of surfactant, it does not give information on the endogenous production and catabolism. In animals surfactant synthesis and catabolism are measured with radioactive tracers.[6-9] This approach is not acceptable in humans, and especially not in infants. We studied endogenous surfactant production and turnover in preterm infants with RDS, using a novel and safe method. We used the stable isotope [U-^{13}C]glucose as a precursor for the fatty acid synthesis of lung phosphatidylcholine (PC). We measured carbon-13 incorporation into the fatty acids of surfactant PC obtained from tracheal aspirates of preterm infants with RDS.

METHODS

Subjects and study design

Six premature infants requiring mechanical ventilation were studied (birth weight, 916±244 g; gestational age, 27.7±1.7 wk) (table 1), starting directly after birth. Inclusion criteria were: gestational age <31 wk, prenatal corticosteroid therapy, severe RDS on chest radiograph (Giedeon grade III-IV), and written parental informed consent. Exclusion criteria were: congenital infection, maternal diabetes, and chromosomal abnormality. Patients received a constant intravenous infusion of [U-^{13}C]glucose (Campro Scientific, Veenendaal, The Netherlands) for 24 h at 0.170 mg/kg/min. The start of the study (t=0) was defined by the start of the infusion of the labeled glucose. This was 4.2±1.1 h after birth. The labeled glucose was infused by a high precision pump (M22, Harvard Apparatus Co. Inc., South Natick, MA). The total glucose intake was 5.7 mg/kg/min, including nonlabeled glucose. Before and during the glucose infusion, 1 ml arterial blood was drawn every 6 h for determination of glucose enrichment. Exogenous surfactant (Survanta®; Abbott Laboratories, North Chicago, IL) was administered endotracheally at t=0, at a dose of 100 mg/kg phospholipids, if the mean airway pressure exceeded 7.5 cm of water, or if the inspiratory oxygen fraction (F_{IO_2}) was higher than 0.40. Infants received a second dose 6 h later if the criteria were still met. Tracheal aspirates were obtained every 6 h during the time that the infant was intubated. The tracheal suctioning procedure was performed during routine patient care and did not deviate from the normal clinical protocol. Twenty seconds after 0.5 ml normal saline was injected into the tracheal tube, suction was done beyond the tip of the endotracheal tube. The suction catheter was flushed with normal saline. Tracheal aspirates were immediately placed at -20°C, until further processing. No tracheal suction was done within 6 h after surfactant administration. Total parenteral nutrition including lipids was started at t=48 h. The study was approved by the local medical ethics committee.

Analytical procedure

Tracheal aspirates. After thawing, normal saline was added to the aspirate. This was vortexed and centrifuged at 450 x g for 10 min at 4°C,[10] lipids were extracted from the supernatant.[11] Surfactant PC was isolated from the lipid extract by thin layer chromatography

(TLC)[12] using Kieselgel 60 TLC plates (Merck, Darmstadt, Germany) and a Camag Linomat IV (Merck, Darmstadt, Germany). The PC was derivatized,[13] and the fatty acid methyl esters were extracted with hexane and stored at -20°C. Tracheal aspirates containing visible blood were not analyzed.

Blood samples. Blood was collected from the patient in lithium-heparin-containing vacutainers and directly centrifuged to separate plasma and cells. The plasma was stored at -70°C until further processing. Plasma was delipidated with chloroform and methanol.[14] The water fraction was passed over a column with anion-exchange resin (AG-1X8 [acetate], 100-200 mesh; BioRad Laboratories, Richmond, CA) and cation-exchange resin (AG-50W X8 [Hydrogen], 200-400 mesh; BioRad). The elute containing the glucose was evaporated to dryness at 80°C and derivatized to an aldonitril pentacetate derivative.[15]

Determination of enrichment

The enrichment of selected fatty acids, components of surfactant PC, and of plasma glucose was measured by gas chromatography-combustion interface-isotope ratio mass spectrometry (GC-CI-IRMS) (VG Isotech, Middlewich, Cheshire, UK) as described.[16] For fatty acid analysis, 1μl was injected on column (30 m Omegawax column; Supelco, Zwijndrecht, The Netherlands). The injection and oven temperature was 45°C for 2 min and raised at 15°C/min to 175°C, held for 15 min at 175°C, and subsequently ramped with 2°C/min to 240°C.

For glucose, 1μl was injected on a 25 m x 0.25 mm, 0.11 μm HT-5 capillary column (Scientific Glass Engineering, Victoria, Australia), with a split ratio of 30:1. The oven temperature was isothermal at 220°C. The enrichment was expressed in atom per cent excess (APE), which represents the increase in the percentage of carbon-13 atoms in total carbon dioxide from the combusted compounds above baseline enrichment (before infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms added during derivatization.

Calculations

Tissue bound and alveolar surfactant were regarded as one pool, since studies in newborn rabbits showed that recycling is ~16 times faster than *de novo* synthesis and clearance.[7-9] As palmitic acid is by far the most abundant fatty acid in surfactant PC, calculations were performed for palmitic acid only.

Secretion time was defined as the time lag between the start of the [U-^{13}C]glucose infusion and the appearance of enriched fatty acids in surfactant PC. The time of appearance of the enrichment in PC palmitate was calculated by plotting the regression line for the linear-increasing part of the enrichment *versus* time curve, and extrapolating it to baseline enrichment.[17]

Fractional synthesis rate (FSR) of palmitic acid is expressed as the percentage of the total surfactant PC palmitate pool synthesized from glucose per day. It is calculated by dividing the slope of the linear increase of enrichment of PC palmitate by the steady state enrichment of plasma glucose.[18]

Half-life of PC palmitate was calculated by exponential curve-fitting at the final, monoexponential part of the downslope of the enrichment *versus* time curve.
Data are presented as mean$\pm$standard deviation (range).

RESULTS

The patients' characteristics are shown in table 1. Patients received two doses of exogenous surfactant, except for patient 2 who received one dose and was extubated on the second day of life. Exogenous surfactant therapy decreased of the $F_{I_{O2}}$ from 0.53$\pm$0.18 to 0.25$\pm$0.06.

Table 1. Patients' characteristics

Patient number	1	2	3	4	5	6	Mean	SD
Gestational age (wk)	27.1	27.7	29.1	26.1	30.3	25.6	**27.7**	**1.7**
Birth weight (g)	1140	840	770	900	1225	590	**916**	**244**
RDS-grade	IV	IV	IV	III	IV	III	-	-
Doses of surfactant	2	1	2	2	2	2	-	-
Apgar at 5 min	9	7	8	8	6	6	**7.3**	**1.2**
Umbilical pH	7.30	7.30	7.17	7.30	6.95	-	**7.21**	**0.15**
F_{IO_2} at t=24	0.26	0.21	0.26	0.35	0.40	0.40	**0.31**	**0.08**
F_{IO_2} at t=36	0.25	0.21	0.35	0.34	0.37	0.44	**0.33**	**0.08**
F_{IO_2} at t=48	0.23	0.21	0.30	0.34	0.35	0.35	**0.30**	**0.06**
Days of intubation	15	2	22	12.5	5.5	31	**14.5**	**11**

In patient 6, the F_{IO_2} decreased after the first dose of surfactant, from 0.50 to 0.25, but it increased again to 0.40, and a second dose did not result in a decrease in oxygen need.

The ^{13}C-enrichment of plasma glucose reached a steady state of 2.63±0.45 APE in all patients between t=6 and t=24 h (data not shown). This was concluded because the slope of the enrichment *versus* time curve per patient did not deviate significantly from zero in this period.

The ^{13}C-enrichments of selected fatty acids of PC in patient 3 are shown in figure 1. The enrichment *versus* time curves of the nonessential fatty acids showed the same pattern. As expected, the essential fatty acids were not enriched (only linoleic acid is depicted).

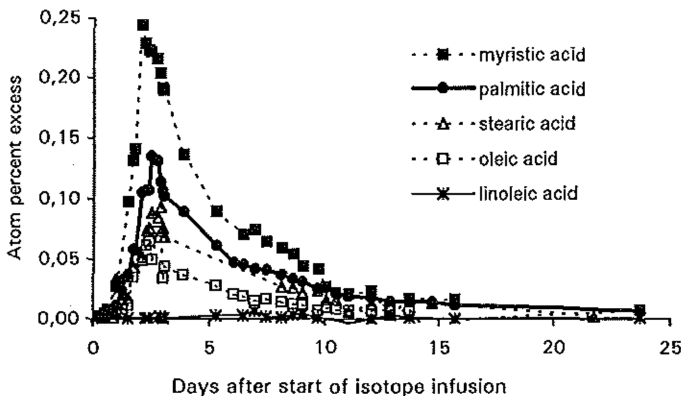

Figure 1. ^{13}C-enrichment of selected fatty acids of surfactant phosphatidylcholine (PC) in patient 3 with RDS. At birth a 24-h [U-^{13}C]glucose infusion was administered. Tracheal aspirates were obtained. The ^{13}C-enrichment of surfactant PC was measured and expressed in atom percent excess.

The increasing enrichment of PC palmitate, for all patients is shown in figure 2. The regression line of the linear part was used for calculating the secretion time, which was 19.4±2.3 (16.5 to 22.3) h (table 2), and for calculating the fractional synthesis rate. The FSR of PC palmitate from glucose was 2.7±1.3 (1.06 to 4.25) % per day. Maximal enrichment was reached at 70±18 (48 to 96) h. Decreasing enrichment of palmitic acid of PC in each individual patient is shown in figure 3. The closed marks represent the monoexponential part used for half-life calculations, in one patient only. The half-life of endogenous synthesized surfactant PC palmitate was 113±25 (87 to 144) h.

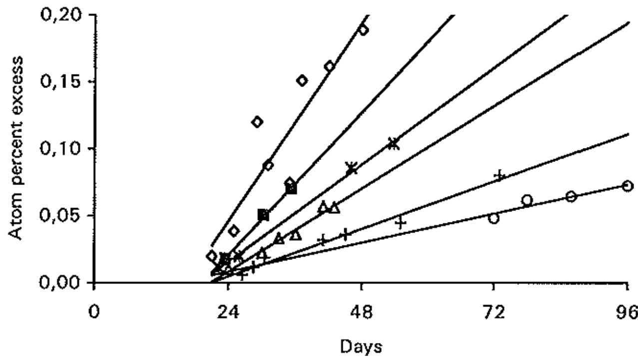

Figure 2. Linear increase in ^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine in each patient. The regression line for each patient is shown. For calculation of secretion time, the regression line was extrapolated to baseline enrichment. The fractional synthesis rate was calculated by dividing the slope of the regression line by steady state enrichment of plasma glucose.

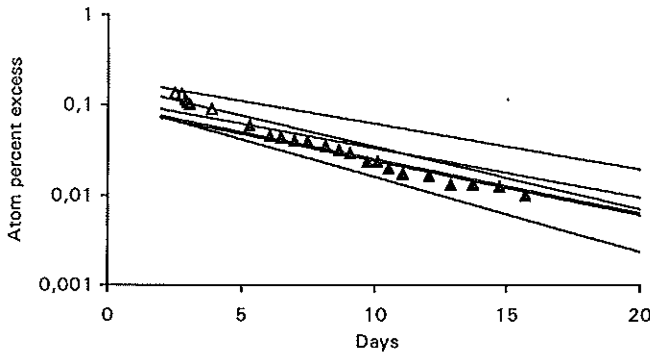

Figure 3. Decrease of ^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine for each patient Patient 2 was extubated on day 2 of life. For clarity, the individual marks are shown for one patient only. The open symbols represent the start of the decreasing enrichment. The closed marks, from the same patient, show the final monoexponential decrease, which was used to calculate half-life.

Discussion

This study measures for the first time endogenous surfactant production and turnover in humans. We infused the stable isotope [U-^{13}C]glucose in preterm infants and measured the ^{13}C-enrichment of selected fatty acids in surfactant PC. The incorporation of the carbon-13 into surfactant PC palmitate began at 19.4±2.3 (16.5 to 22.3) h after the start of the isotope infusion (fig. 2), which likely corresponds to the time required for palmitic acid synthesis from glucose, processing of surfactant PC in the alveolar type II cell, and secretion into the alveolar spaces. In preterm ventilated lambs that received an intravenous injection of [^{3}H]palmitic acid, radioactivity was already detected in surfactant after 5 h.[9] This suggests that incorporation of plasma palmitate into surfactant PC is a faster process than palmitic acid synthesis from glucose with subsequent incorporation into surfactant PC. In our study, the PC palmitate enrichment was maximal 70±18 (48 to 96) h after the start of the isotope infusion. This time to peak enrichment is dependent on the duration of the isotope infusion (24 h) among other factors. The maximal enrichment was found at 46±18 h after the finish of the isotope infusion (fig. 1), which is comparable to the 35 to 60 h found in newborn rabbits and lambs after a single intravenous bolus of radio-labeled palmitic acid.[9,19,20]

Table 2. Kinetics of palmitic acid in surfactant phosphatidylcholine

Patient	Secretion time (h)	FSR (%/d)	Time of maximal enrichment (h)	Half-life (h)
1	16.5	4.25	48	144
2	20.0	4.07	-	-
3	21.6	2.28	60	97.6
4	22.3	1.40	73	86.6
5	18.9	3.14	74	105
6	17.4	1.06	96	133
Mean	**19.4**	**2.70**	**70**	**113**
SD	**2.3**	**1.34**	**18**	**24.5**

Six ventilated preterm infants received a 24-h [U-^{13}C]glucose infusion. During the period the infants were intubated, tracheal aspirates were obtained and surfactant phosphatidylcholine (PC) was isolated. Enrichment of palmitic acid in surfactant PC was measured. Kinetic parameters were calculated using enrichment versus time curves from plasma glucose and surfactant PC palmitate. FSR = fractional synthesis rate.

In this study, surfactant PC half-life was 4.7 ± 1.0 (3.6 to 6.0) d, which indicates that in human preterm infants endogenous surfactant remains in the lung for a long time. To minimize the potential influence of ongoing incorporation of labeled palmitic acid produced via lipogenesis in the liver, we calculated half-life from the final monoexponential part of the enrichment *versus* time curve. To confirm that there is no major influence of ongoing incorporation from labeled palmitic acid during the time frame of half-life calculation, we measured enrichment of palmitic acid in plasma triglycerides and phospholipids in three patients who were clinically comparable to the study patients. The enrichment of palmitic acid in plasma triglycerides and phospholipids was maximal at $t = 25 \pm 0$ h (0.22 ± 0.01APE) and 35 ± 5 h (0.16 ± 0.01 APE), respectively, and decreased afterwards. The enrichment of plasma free palmitic acid was measured in one patient and was very low during the entire study period (maximal at $t = 24$ h, 0.05 APE, and at $t = 70$ h, 0.01 APE). At the time of maximal enrichment of surfactant PC palmitic acid ($t = 70$ h, 0.12 ± 0.047 APE) the enrichment of triglycerides was 0.020 ± 0.01 APE and that of phospholipids was 0.02 ± 0.002 APE. During the period of half-life calculation (starting from approximately 100 h), the enrichment of surfactant PC palmitate was 0.09 ± 0.03 APE. The ^{13}C-enrichment of palmitic acid in triglycerides and phospholipids was much lower (0.01 ± 0.01 and 0.020 ± 0.01 APE, respectively). This indicates that the long half-life for surfactant PC palmitate is not due to the presence of a high enrichment of plasma palmitic acid but a reflection of surfactant PC metabolism in the lungs.

The long half-life of 4.7 d of surfactant PC in our study is compatible with findings in animal studies. In term newborn sheep, after tracheal instillation of PC labeled with [^{3}H]palmitic acid the half-life of PC in the alveolar wash was approximately 11.6 d.[21] In term newborn rabbits, who received a bolus of albumin-bound [^{3}H]palmitic acid intravenously, the specific activity of alveolar PC [^{3}H]palmitate had a half-life ranging from >2 to 5.7 d.[9,22] In one study using surfactant labeled with [^{14}C]DPPC in newborn rabbits, the half-life was approximately 25 h,[23] which is shorter than in most other studies, possibly explained by the short duration of this study (only 16 h). Support of a long half-life of surfactant in human preterm infants is provided by a study by Hallman *et al.* who reported that PC concentrations in tracheal aspirates after surfactant administration in preterm infants with RDS did not decrease during

the first week of life.[5] In preterm infants with RDS treated with surfactant containing phosphatidylglycerol, the half-life of phosphatidylglycerol in tracheal aspirates was calculated by Hallman *et al.* to be 20 to 36 h.[4] This might be different, however, from the half-life of PC, as it is known that not all surfactant components have the same turnover rates.

In the present study all infants received exogenous surfactant, which readily mixes with the endogenous pool. Whether exogenous surfactant influences endogenous surfactant metabolism and whether the half-life of exogenous surfactant is similar remains to be studied.

In our study the rate of PC palmitate synthesis from glucose was low (FSR = 2.7%/d), which could partially explain why preterm infants with RDS who do not receive exogenous surfactant improve only after a few days. Thus the only way to rapidly augment the surfactant pool in newborn infants with RDS is by exogenous surfactant administration. The pool size of endogenous PC in preterm infants with RDS is very low and estimated to be approximately 5 mg/kg.[4,24] Therefore, we assumed the total lung surfactant PC pool size to be the estimated endogenous pool size (5 mg/kg) together with the administered dose of exogenous surfactant, 87 mg/kg PC after one dose or 174 mg/kg PC after two doses. With this assumption, we calculated the production rate of PC palmitate from glucose to be 2.2 ± 1.1 (0.99 to 3.95) mg/kg/d. This corresponds to a PC production rate from glucose of approximately 4.2 mg/kg/d (absolute production rate = FSR x pool size). It is possible, however, that part of the exogenous surfactant is not completely retained within the lungs, which would result in an overestimation of pool size and surfactant PC synthesis. In newborn rabbits and in preterm monkeys surfactant production from palmitic acid and choline was estimated to be 8.4 and 12.5 mg/kg/d, respectively, when measured with labeled choline and palmitic acid.[9,25] The absolute production rate in our study was slightly lower than that in the animal studies mentioned above, possibly because we calculated production from glucose only. The low production rate indicates that only after several days a considerable amount of surfactant is present in the alveolus. This observation is in agreement with the study of Hallman, which showed that after 4 to 5 d the concentration of PC in the alveolus reaches values comparable to preterm infants without RDS.[5]

In the type II pneumocyte, glucose is a primary source for fatty acid synthesis of surfactant PC.[26-28] In the type II cell other substrates are used for surfactant PC palmitate synthesis as well, such as fatty acids derived from lipolysis,[27] lactate, pyruvate, and ketone bodies.[26] We measured endogenous PC palmitate synthesis from glucose only. We have recently demonstrated that lipogenesis from glucose occurs in preterm infants fed solely glucose intravenously on the first day of life.[29] Therefore, with our approach carbon-13 incorporation into surfactant PC occurs via lipogenesis in type II cells directly or via lipogenesis in the liver and subsequent uptake of labeled palmitic acid by type II cells.

In summary, this study shows that in preterm infants with RDS, after prenatal corticosteroid therapy and postnatal surfactant administration, the surfactant PC palmitate production from glucose is a slow process. The long half-life of 4.7 d indicates also a very slow turnover of the total surfactant PC palmitate pool. The novel and safe method described in this report could be applied to address many important issues concerning surfactant metabolism in preterm infants, children, and adults.

Acknowledgments

The authors thank Janine den Ouden and Ingrid H.T. Luijendijk, Erasmus University Rotterdam, Department of Pediatrics, for excellent technical assistance; and the nursing staff, residents, and neonatologists for their help and support with this study.

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Chapter 4

Endogenous Surfactant Metabolism in Critically Ill Infants Measured with Stable Isotope Labeled Fatty Acids

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Summary

Little is known about endogenous surfactant metabolism in infants, because radioactive isotopes used for this purpose in animals cannot be used in humans. We developed a method to measure the endogenous surfactant kinetics *in vivo* in humans by using stable isotope labeled fatty acids.

We infused albumin-bound [U-^{13}C]palmitic acid (PA) and [U-^{13}C]linoleic acid (LLA) for 24 h in eight critically ill infants (mean ± SD; weight; 3.7 ± 1.3 kg, age; 51.3 ± 61.6 d) who required mechanical ventilation. The ^{13}C-enrichment of PA and LLA in surfactant phosphatidylcholine (PC), obtained from tracheal aspirates, was measured by gas chromatography-combustion interface-isotope ratio mass spectrometry.

We measured a significant incorporation of both ^{13}C-PA and ^{13}C-LLA into surfactant PC. PC-PA and PC-LLA became enriched after 8.7 ± 4.9 (3.4 to 17.3) h and 10.0 ± 7.2 (3.0 to 22.4) h, respectively. The times of maximal enrichment were 49.2 ± 8.9 and 45.6 ± 19.3 h, respectively. The fractional synthesis rate (FSR) of PC-PA ranged from 0.4 to 3.4% per h, whereas the FSR of PC-LLA ranged from 0.5 to 3.8% per h. The surfactant PC-PA and PC-LLA half-lives ranged from 16.8 to 177.7 and 23.8 to 144.4 h, respectively.

This method provides new data on surfactant metabolism in infants requiring mechanical ventilation. We found that the synthesis of surfactant from plasma PA and LLA is a slow process and that there were marked differences in PC kinetics among infants. This variability could be related to differences in lung disease and could affect the clinical course of the respiratory failure.

Pediatr. Res. 1999; 45: 242-246

INTRODUCTION

Damage to the surfactant system has been implicated in the pathophysiology of acute lung injury which occur in humans in a wide variety of acute processes such as lung infection, sepsis, trauma, ARDS, or neonatal RDS, or occurs secondary to mechanical ventilation. All these processes lead to a widespread proteinaceous edema within the lungs and to a release of inflammatory mediators that could maintain lung inflammation.[1-6]

Exogenous surfactant has been advocated as therapy in humans to overcome respiratory failure by restoring more uniform mechanical properties of the lungs. This approach has been proven to be beneficial in neonates with RDS,[7] but the results in adults have been less encouraging.[5,8,9] There are no data in humans on endogenous surfactant synthesis and turnover in normal and injured lungs to support the use of exogenous surfactant after the neonatal period.

We have recently published that small preterm infants with PDS have a low fractional synthesis of lung PC palmitate from plasma glucose.[10] Whether this low incorporation is caused by the precursor molecule used or the severity of the lung immaturity of the infants studied remains to be investigated. In the present study, we tested whether the labeling of plasma FFA with stable isotopes is a suitable technique for the measurement of surfactant synthesis in young infants. We developed a method for the measurement of endogenous surfactant production and turnover using a constant intravenous (i.v.) infusion of albumin-bound [U-^{13}C]PA and [U-^{13}C]LLA. We saw a significant incorporation of the tracers into surfactant PC, and new data on endogenous surfactant synthesis and turnover in humans could be obtained.

METHODS

Subjects and study design

Surfactant synthesis was studied in eight infants whose clinical characteristics are reported in table 1. All patients were admitted to the Neonatal or Pediatric Intensive Care Units of the Department of Pediatrics, University of Padua, Italy for respiratory failure. The inclusion criteria were: age <6 months, stable hemodynamic condition during the isotope infusion, and respiratory failure that required endotracheal intubation for at least 48 h. Infants were excluded from the study when they presented with signs of liver and renal failure, seizures, or if they received blood products during the study. All infants had arterial and central venous lines placed for clinical monitoring and no infant received i.v. lipid emulsion during the isotope infusion. Feeding was started gradually, 3 d after the start of infusion. All medications given to the patients during the study were recorded. The study protocol was approved by the ethics committee on Human studies at the Department of Pediatrics of the University of Padua and was conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from the parents.

Study protocol. [U-^{13}C]PA (16:0; purity: 99%) and [U-^{13}C]LLA (18:2n-6; purity: 97%) were purchased from Martek Biosciences (Columbia, MD). Chemical and isotopic purity was confirmed by gas chromatography mass spectrometry. [U-^{13}C]PA and [U-^{13}C]LLA were bound to human albumin (Merieux, Pasteur, Lyon, France) and prepared for i.v. infusion as described previously.[11] The albumin-bound tracers were infused at a constant rate by a high precision calibrated syringe pump (M22, Harvard Apparatus Co, Inc. Natick, MA) over a 24-h period. The rates of infusion were 1.0 ± 0.26 and 1.19 ± 0.23 μmol/kg/h for PA and LLA, respectively. The arterial line was used for blood sampling and the venous line for tracer infusion. Before and during the isotope infusion, 0.6 mL of blood was drawn at 0, 3, 6, 12, 18, and 24 h to determine the isotopic enrichment of PA and LLA enrichment in plasma FFA. The blood

drawn was placed in tubes containing EDTA and immediately centrifuged at 1300 x g. After separation plasma was stored in tubes containing pyrogallol as antioxidant at -20°C until analysis.

Tracheal aspirates were obtained before the start of the isotope infusion and every 6 h thereafter until the patient was extubated. Twenty seconds after 0.5 mL normal saline was injected into the tracheal tube, suction was done beyond the tip of the endotracheal tube.

Analytical procedure

Tracheal aspirates. Normal saline was added to the tracheal aspirate to a total of 5 mL. This was centrifuged at 150 x g for 10 min, and supernatant was stored at -20°C. Lipids were extracted from tracheal aspirates according to Bligh and Dyer.[12] Surfactant PC was separated from other surfactant phospholipids by thin layer chromatography.[13] The PC was derivatized by adding 2 mL of 3 M dry HCl methanol,[14] and the fatty acids were extracted with hexane and stored at -20°C until analysis. No tracheal aspirate contained visible blood.

Plasma lipids were processed as previously described.[11] Plasma was delipidated according to Folch,[15] lipid classes separated by thin layer chromatography, and their fatty acids derivatized as methyl esters. The quantification of plasma FFA and of FA of surfactant PC were performed as previously described.[11]

Determination of enrichment

The enrichment of individual fatty acids of surfactant PC was measured by gas chromatography-combustion interface-isotope ratio mass spectrometry (VG Isotech, Middlewich, Chesire, UK).[10] The enrichment was expressed as APE, which represents the increase in percentage of ^{13}C atoms in total carbon dioxide from the combusted compounds above baseline enrichment (before isotope infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms during derivatization.

Isotopic enrichments of PA and LLA of plasma FFA were carried out on a Fisons MD 800 gas chromatograph quadrupole mass spectrometer (Fisons, Milan, Italy), using the chemical ionization mode with isobutane.[11] Each plasma sample was measured in duplicate.

Calculations

Surfactant in the type II cells and the alveolus were regarded as one pool, because animal studies showed that recycling is much faster than the *de novo* synthesis and clearance.[16,17]

Secretion time is the time of first appearance of the enrichment in surfactant PC and was defined as the time lag between the start of the [U-^{13}C]PA and the [U-^{13}C]LLA infusion and the appearance of the respective labeled fatty acid in surfactant PC. The time of first appearance of the enrichment was calculated by plotting the regression line for the linear increasing part of the enrichment *versus* time curve and extrapolating it to baseline enrichment.[10,18]

Fractional synthesis rate (FSR) of PC-PA and PC-LLA is the percentage of the total surfactant PC pool synthesized from the respective plasma fatty acid per h. It is calculated by dividing the slope of the linear increase of the enrichment of PC by the plasma steady state enrichment of the respective FA of the plasma FFA.[10,18]

Half-life of PC was calculated by exponential curve fitting at the final monoexponential part of the down-slope of the enrichment *versus* time curve.

Peak time is the time of maximal enrichment of surfactant PC.

Table 1. Clinical charateristics of the infants studied

Patient	Sex	Weight (kg) [centile]	Age (d)	GA (wk)	Energy intake Kcal/kg/d	Main Diagnosis	Intubation (d)	MAP cm H_2O	FI_{O2} (%)	NO ppm
1	F	1.9 [50]	11	32	20	Sepsis syndrome	6/4	5	30	
2	M	5.8 [3]	147	39	17	Pneumonia/sepsis	5/2	16	70	
3	F	5 [<3]	149	40	17	Pneumonia/sepsis	30/1	18	50	25
4	M	3.6 [50]	1	38	23	Esophageal atresia, postop	3/1	5	21	
5	F	2.5 [<3]	23	37	14	Streptoccus B meningitis	20/5	8	30	
6	M	4.1 [75]	53	40	21	RSV bronchilolitis	15/2	18	60	
7	M	4.1 [75]	12	34	31	MAS / E. coli spsis	18/12	17	80	6
8	M	3.2 [50]	14	37	29	Pneumothorax	20/12	20	35	
Mean		**3.7**	**51.3**	**37.1**	**21.5**			**13.4**	**47.0**	
SD		**1.3**	**61.6**	**2.9**	**6.0**			**6.3**	**21.4**	

GA = gestational age, MAP = mean airway pressure, NO = nitric oxide, RSV = respiratory syncthial virus, MAS = meconium aspiration syndrome, intubation = number of d intubation/d of intubation before the start of the study, ppm = parts per million.

RESULTS

In all infants, [U-^{13}C]PA and [U-^{13}C]LLA enrichments of plasma FFA reached steady state within 3 h from the start of the i.v. isotope infusion, because the slope of the enrichment curve over time did not deviate significantly between t = 3 h and t = 24 h (fig. 1). Total FFA, PA and LLA plasma concentrations and mol% values of 7 of the 8 infants studied were reported elsewhere.[11]

The percentage values of PA and LLA in tracheal aspirate PC were 64.4 ± 10.6 and 2.0 ± 1.1 mol%, respectively. PC-PA and PC-LLA molar percentages were rather stable in the individual patients with a variability of less than 5% during the study period.

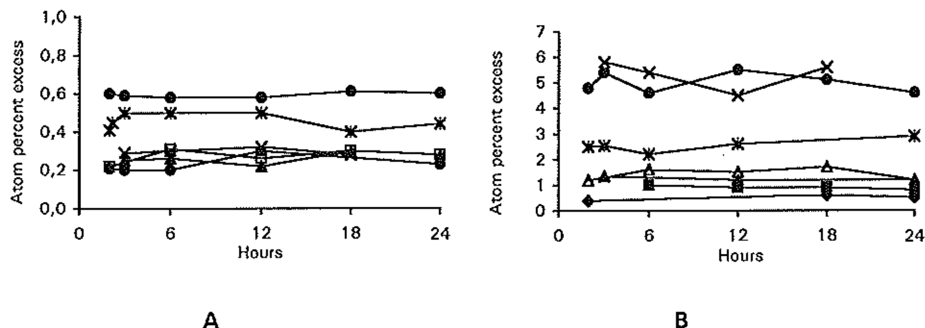

Figure 1. Isotopic enrichment (APE) of plasma free PA (A) and LLA (B) in eight critically ill infants after a 24-h constant infusion of albumin-bound [U-^{13}C]PA and [U-^{13}C]LLA. All patients, identified by different symbols, reached steady state after the third h of the isotope infusion.

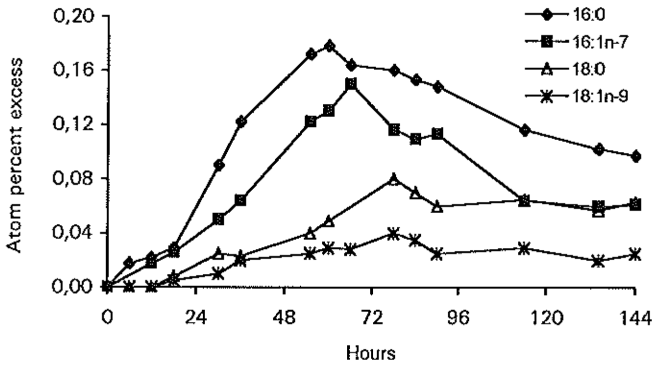

Figure 2. Isotopic enrichments above baseline (APE) of PA and its metabolic derivatives incorporated into surfactant PC after a 24-h i.v. infusion of [U-^{13}C]PA in a patient with respiratory failure.

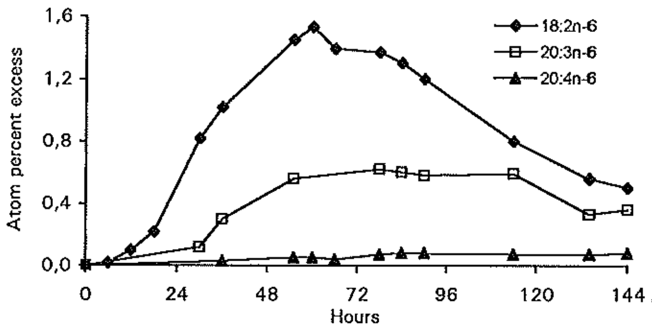

Figure 3. Isotopic enrichments above baseline (APE) of LLA and its metabolic derivatives incorporated into surfactant PC after a 24-h infusion of [U-^{13}C]LLA in a patient with respiratory failure.

The time curves of the ^{13}C-enrichment of individual fatty acids of surfactant PC in patient 7 are shown in figures 2 and 3. Other fatty acids, besides PA and LLA, became enriched in surfactant PC. The nonessential fatty acids derived from the desaturation and/or chain elongation of labeled PA, namely 16:1n-7, 18:0 and 18:1n-9 are shown in figure 2. The ^{13}C-enrichment of LLA and of its metabolic derivatives, namely 20:3n-6 and arachidonic acid (20:4n-6) are shown in figure 3.

Kinetic data calculated for each individual patients are shown in table 2; FSR, secretion time, peak time, and half-life were calculated using both PA and LLA. Surfactant PC-PA and PC-LLA half-lives were calculated in five patients (patients 3, 5-8) because patients 1 and 4 were extubated within 72 h of the beginning of the study and patient 2 died 80 h after the beginning of the study. The FSR for PA and LLA ranged from 0.8 to 3.4 and 0.5 to 3.8% per h, respectively, which correspond to mean values of 34 and 50% per d, respectively. We could not find any correlation between PA and LLA mol% values in tracheal aspirate PC (data not shown). Mean secretion time and time to peak for PA *versus* LLA were similar (8.7$\pm$4.9 *versus* 10.0$\pm$7.2 h and 49.2$\pm$ 8.9 *versus* 45.6$\pm$19.3 h, respectively). Half-lives of PC-PA and PC-LLA showed large differences in the individual patients (range: 16.8 to 177.7 h). In patient 5, half-lives of PC-PA and of PC-LLA were 177.7 *versus* 43.6 h, respectively. In patient 6, we found the reverse , and values were 33.2 *versus* 144.4 h, respectively.

DISCUSSION

In this study we measured the endogenous surfactant production and turnover *in vivo* in human infants by using stable isotope labeled fatty acids. We infused [U-^{13}C]PA and [U-^{13}C]LLA in infants with respiratory failure and measured the ^{13}C-enrichment of selected fatty acids in surfactant PC.

Surfactant synthesis has been studied *in vivo* in animals by using several metabolic precursors of the surfactant PC molecule such as glycerol,[19] glucose,[20] choline,[21] and more frequently, palmitic acid.[17,22] After i.v. administration, these precursor molecules have different volumes of distribution and are subject to a different oxidative and nonoxidative metabolism before taken up by the type II cells in the lungs.

We have recently infused [U-^{13}C]glucose in preterm infants with RDS and measured the isotopic enrichment of PA in surfactant PC.[10] By infusing labeled glucose as a tracer, we measured the PC-PA synthesized *de novo* from glucose via lipogenesis and incorporated into surfactant PC by the type II cells. After i.v. [U-^{13}C]glucose, the site of PA synthesis can be the type II cell with direct incorporation of PA into PC-PA or the liver with the subsequent production of very low density lipoproteins containing labeled PA. Plasma lipids are taken up by the type II cells for PC synthesis.[23] Under these experimental conditions, total surfactant PC synthesis could not be calculated, because PA from sources other than lipogenesis from glucose contribute to the synthesis of PC-PA.

Table 2. Surfactant phosphatidylcholine kinetics after infusion of labeled PA and LLA

Patient	FSR of PA (%/h)	ST of PA (h)	Peak of PA (h)	Half-life of PA (h)	FSR of LLA (%/h)	ST of LLA (h)	Peak of LLA (h)	Half-life of LLA (h)
1	3.4	11.2	NA	NA	3.8	9.3	NA	NA
2	0.8	4.2	NA	NA	NA	NA	NA	NA
3	2.1	17.3	48	16.8	2.6	22.4	66	23.8
4	0.4	4.7	NA	NA	0.5	9.1	NA	NA
5	0.6	3.4	48	178	1.7	3.0	48	43.6
6	1.3	13.2	54	33.2	NA	2.5	18	144
7	2.1	8.4	36	33.8	2.1	16.4	36	37.3
8	0.7	7.4	60	77.9	2.0	7.3	60	47.5

FSR = fractional synthesis rate, ST = secretion time, peak = time of maximal enrichment, PA and LLA = palmitic acid and linoleic acid in surfactant PC, respectively.

In the present study, by infusing labeled PA, we measured the surfactant PC synthesized by the type II cells using plasma PA as metabolic precursor, but we could not calculate the surfactant PC-PA from *de novo* synthesis from glucose in the type II cells. Our patients also received an infusion of LLA, which is an essential fatty acid and cannot be endogenously synthesized. We used LLA to estimate the total PC surfactant production. The incorporation of labeled PA and LLA in surfactant PC, namely secretion time, began 9 h after the start of the isotope infusion. This does not suggest a different processing of PA *versus* LLA during the *de novo* synthesis of surfactant PC, which is consistent with *in vitro* studies.[24] The secretion time corresponds to the time required by the alveolar type II cells to take up the fatty acids from plasma lipids and to process and excrete them as PC into the alveolar space in addition to the time required by surfactant PC to reach the tracheal tree. Slightly shorter times have been reported in 10-d-old lambs that received [^{3}H]palmitate as a bolus.[17]

We reported longer secretion times (19.4 ± 2.3 h) in preterm infants with RDS treated with exogenous surfactant who received an i.v. infusion of [U-^{13}C]glucose as precursor for surfactant PC palmitate.[10] Whether this difference is determined by the type of precursor used (fatty acids *versus* glucose), the different patient characteristics, (critically ill infants *versus* small preterm infants with RDS), or dilution of the labeled PC that could have occurred via recycling in the type II cells after exogenous surfactant in the preterm infants with RDS is currently being studied. The time of maximal isotopic enrichment of the fatty acids in surfactant PC was approximately 2 d for both PA and LLA; these figures are comparable with the animal data in newborn lambs and rabbits.[17,22,25]

Although the patients studied constitute a very heterogeneous group, the incorporation rate of PA in surfactant PC was consistently lower than the respective incorporation rate of LLA in surfactant PC. The mean incorporation rate of PA in surfactant PC was 34.2 ± 24.8% per d of the PC PA pool size, which is approximately one half of the incorporation of LLA (50.8 ± 26,0% per d). Therefore, the infants in the study synthesized one third of their surfactant PC-PA pool from plasma PA and one half of their surfactant PC-LLA pool from plasma LLA each d. Lower FSR for PA than for LLA could be explained by the *de novo* synthesis of palmitate from other substrates than from fatty acids. In the case of LLA, this process cannot occur in animals because LLA is an essential fatty acid and there is no endogenous synthesis of LLA. In animals, Surfactant PC palmitate, derived from plasma fatty acids, represents only approximately 50% of the total PA incorporated in surfactant PC, whereas the other 50% is derived from glucose,[20,26] lactate, ketone bodies, and glutamate.[27,28] Despite that LLA represents only 2 to 3% of the total fatty acids of surfactant PC, its incorporation rate could be a more reliable measure of the rate of surfactant PC synthesis than that of PA. Therefore, according to this estimate, approximately 50% of the surfactant PC pool was synthesized on average by our group of patients in 1 d, which is in agreement with data in rabbits.[17,26]

The half-life of surfactant PC ranged between 1 and 7.4 d, with infants with the most severe respiratory failure having the shortest PA half-lives (patient 3, 6 and 7). The limited number of infants studied does not allow us to draw any firm conclusion, but the wide range suggests a different behavior of surfactant kinetics in our patients. In term newborn rabbits that received a bolus of [^{3}H]PA i.v., the half-lives of surfactant PC yielded comparable results ranging between 2 and 5.7 d.[17] Furthermore, the half-lives of PC-PA and of PC-LLA exhibited rather different values in the individual patients. In patient 5, the half-lives of PA and LLA were 178 and 43.6 h, respectively; in patient 6, we found the reverse and values were 33.2 and 144 h, respectively. We do not have an explanation for these finding because most of the animal work has been focused on the metabolism of disaturated PC and little attention has been paid to the metabolism of polyunsaturated fatty acids. Long half-lives of PC-PA could result from a reduced surfactant PC catabolism and/or could be associated to an "active" recycling. The latter process is known to exhibit a strong preference for saturated fatty acids.[29] Also, longer half-lives of PC-LLA could represent an inhibition of remodeling of unsaturated PC to saturated PC.[24] A larger proportion of surfactant PC-PA, synthesized via *de novo* lipogenesis from glucose, could also contribute to shorter half-lives during periods of increased surfactant synthesis. We have recently observed increased incorporation of palmitate from glucose in preterm baboons who have been treated prenatally with corticosteroids in comparison with baboons who did not receive steroids.[30]

In summary, this report describes a method for the measurement of surfactant synthesis and turnover *in vivo* in humans. These preliminary results in critically ill infants are consistent with data obtained from animal experiments and suggest that surfactant synthesis is a slow process. The use of stable isotope labeled fatty acids such as LLA and PA and of labeled glucose will expand the understanding of surfactant metabolism in humans. This will improve the rational use of exogenous surfactant in infants with lung injury.[8,9]

ACKNOWLEDGEMENTS

The authors thank the nurses of the Pediatric and Neonatal Intensive Care Unit of the University of Padua for their enthusiasm and their collaboration during the study. The authors also thank Ingrid Luijendijk for her indispensable contribution to the preparation of the albumin-FA solution.

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Chapter 5

Metabolism of Endogenous Surfactant in Premature Baboons and Effects of Prenatal Corticosteroids

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Summary

We studied the synthesis of surfactant and the effect of prenatal betamethasone treatment *in vivo* in very preterm baboons. Ten pregnant baboons were randomized to receive either betamethasone (Beta) or saline (Control) 48 and 24 h before preterm delivery. The newborn baboons were intubated, treated with surfactant, and ventilated for 6 d. They received a 24-h infusion with the stable isotope [U-^{13}C]glucose as precursor for the synthesis of palmitic acid in surfactant phosphatidylcholine (PC).

Palmitic acid in surfactant PC became enriched 27±2 h after the start of the isotope infusion and was maximally enriched at 100±4 h. The fractional synthesis rate of PC-palmitate in the Beta group (1.5±0.2% per d) was increased by 129% above Control (0.7±0.1% per d), ($p<0.02$, Mann-Whitney U-test). The absolute synthesis rate of PC in the Beta group (1.6±0.3 μmol/kg/d) was increased by 128% above Controls (0.7±0.2 μmol/kg/d) ($p<0.02$).

These data show that the synthesis of endogenous surfactant from plasma glucose as precursor is a slow process. It is shown for the first time *in vivo*, that prenatal glucocorticosteroids stimulate the synthesis of surfactant PC in the very premature baboon.

Am. J. Respir. Crit. Care Med. 1999; 160: 1481-1485

INTRODUCTION

Little is known about surfactant metabolism after preterm delivery of humans or animals. In term and preterm monkeys and lambs with surfactant deficiency, the pool sizes of surfactant in the alveolus increase slowly postnatally.[1,2] Recovery from respiratory distress syndrome (RDS) in monkeys is associated with larger increases in lung pool sizes of disaturated phosphatidylcholine (DSPC) than nonrecovery from RDS.[3] In the preterm human, the accumulation of alveolar surfactant is slow, as shown by a slow postnatal increase in surfactant phosphatidylcholine (PC) concentration in tracheal aspirates,[4] and low synthesis rates of surfactant PC.[5]

The improved pulmonary function of previously unstressed preterm animals after treatment with prenatal glucocorticoids results primarily from structural changes of the lungs that include increased lung gas volume, decreased perilobular connective tissue, decreased alveolar wall thickness,[6] and induction of antioxidant enzyme activities.[7] Improved pulmonary function can also result from increased amounts of surfactant. *In vitro*, glucocorticoids increase the activity of a number of enzymes of the surfactant synthetic pathway and increase surfactant phospholipid and protein synthesis, and lamellar body appearance in human fetal lung explants.[8-10] However, in most *in vivo* studies with preterm rabbits, preterm lambs, and very preterm baboons, treatment with betamethasone 48 h before delivery does not increase surfactant pool sizes of the alveolar lavage and total lung.[2,11,12] We reported large increases in surfactant PC pools after birth in both the lung tissue and alveolar pools of preterm ventilated baboons during the first days of life, and pool sizes were not significantly increased by prenatal glucocorticoids.[2] As pool size depends on both synthesis and clearance of surfactant, the measurement of the pool size is not equivalent to the measurement of the production rate. Therefore, it is currently unclear whether prenatal glucocorticoids increase surfactant production after preterm delivery.

We studied postnatal endogenous surfactant metabolism in the very premature baboon and tested the hypothesis that prenatal corticosteroids influence the synthesis of endogenous surfactant *in vivo*. These studies are comparable to our recent report of endogenous surfactant metabolism in preterm human infants[5] because we used the same stable non-radioactive isotope, [U-^{13}C]glucose, as a precursor for the synthesis of palmitic acid in surfactant PC.

METHODS

Animal Treatment and Postnatal Care

Animal care, fetal treatments, deliveries, and the postnatal studies were performed at the Southwest Foundation for Biomedical Research (San Antonio, TX) as reported previously.[2] All procedures were reviewed and conformed with AAALAC guidelines. Pregnancies were dated on the basis of cycle dates and growth parameters determined by prenatal ultrasounds performed at estimated fetal gestational ages of 70 and 100 d. At 123 ± 2 d gestation (term is 185 d) the pregnant baboons were randomly assigned to receive 6 mg of betamethasone (Celestone Soluspan®; Schering Pharmaceuticals, Kenilworth, NJ) or saline by intramuscular injection 48 and 24 h before delivery. At delivery, the pregnant baboons were sedated with ketamine (10 mg/kg, IM), intubated and anesthetized with 1.5% halothane. The preterm fetuses were delivered by cesarean section at 125 ± 2 d gestation and intubated. The newborns received surfactant (100 mg/kg) by tracheal instillation (Survanta®; donated by Ross Products, Columbus, OH). At birth, animals received parenteral fluids containing glucose, amino acids, multivitamins and appropriate electrolytes intravenously; they were not fed, and did not receive lipids.

To study the endogenous surfactant PC-palmitate production, all newborn baboons received a constant intravenous infusion of the stable isotope [U-^{13}C]glucose for 24 h, starting immediately after birth (t=0), at a rate of 0.17 mg/kg/min. Arterial blood (0.5 ml) was drawn at 0, 12, 18, and 24 h, for determination of plasma glucose enrichment. Tracheal aspirates were obtained every 12 h during the study period of 6 d. After instillation of 0.5 ml saline into the endotracheal tube, aspiration was performed with a 5F catheter, and the aspirate was frozen. At 144 h (6 d), the animals were killed with pentobarbital. Alveolar wash was performed in situ by filling the lungs with 0.9% NaCl at 4°C and recovering the fluid by syringe.[2] The lavage procedure was repeated five times. The lungs were removed, weighed, and homogenized and aliquots were frozen.

Analytical Procedures

The tracheal aspirates, alveolar washes, lung homogenates, and plasma glucose samples were processed as described previously.[5] In brief, blood was collected and directly centrifuged to separate plasma and cells. Plasma was delipidated with chloroform and methanol.[13] The water fraction was passed over anion- and cation-exchange resin. The glucose in the eluate was derivatized to an aldonitril pentacetate derivative.[14]

After thawing, the tracheal aspirate and alveolar wash were vortexed and centrifuged at 450 x g for 10 min at 4°C, and the pellet was discarded. From the tracheal aspirate, alveolar wash, and lung homogenate, lipids were extracted.[15] PC was isolated from the lipid extract by thin layer chromatography.[16] The PC was transmethylated to form fatty acid methyl esters.[17] The saturated (Sat) PC pool size was measured in the alveolar wash and lung homogenate by treatment of the lipid fractions with osmium tetroxide.[18] Saturated PC was isolated by column chromatography using alumna, and quantified by phosphorus assay.[19]

Determination of enrichment

The ^{13}C-enrichment of plasma glucose and of palmitate in surfactant PC was measured by gas chromatography-combustion interface-isotope ratio mass spectrometry (GC-CI-IRMS) (VG Isotech, Middlewich, Cheshire, UK) as previously described.[5] For glucose, 1μl was injected on a 25 m x 0.25 mm, 0.11 μm HT-5 capillary column (Scientific Glass Engineering, Victoria, Australia), with a split ratio of 30:1. The oven temperature was isothermal at 220°C. For PC-palmitate analysis, 1μl was injected on a 30 m Omega wax column (Supelco, Zwijndrecht, The Netherlands). The injection and oven temperature were 45°C for 2 min and raised at 15°C/min to 175°C, held for 15 min at 175°C, and subsequently increased by 2°C/min to 240°C. The enrichment was expressed in atom per cent excess (APE), which represents the increase in the percentage of carbon-13 atoms in total carbon dioxide from the combusted compounds above baseline enrichment (before isotope infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms added during derivatization.

Calculations

Calculations were performed as described previously.[5] for palmitic acid only as this is by far the most abundant fatty acid in PC. Tissue-bound and alveolar surfactant were regarded as one pool because studies in newborn rabbits showed that recycling is ~16 times faster than the *de novo* synthesis and clearance.[20]

The first appearance of enrichment was defined as the time delay between the start of the [U-^{13}C]glucose infusion and the detection of enriched palmitic acid in surfactant PC. The first appearance of enrichment was calculated by plotting the regression line for the linear increase of the enrichment *versus* time curve, and extrapolating it to baseline enrichment.

The fractional synthesis rate (FSR) of palmitic acid was expressed as the percentage of the total surfactant PC palmitate pool synthesized from glucose per day. It was calculated by

dividing the slope of the linear increase of enrichment of PC palmitate by the steady state enrichment of plasma glucose.[5,21]

The absolute synthesis rate (ASR) of surfactant PC from glucose only, was calculated by multiplying the FSR by the total lung Sat PC pool size. The value for the total lung Sat PC pool was the Sat PC pool size measured at birth in seven similar baboons (~38 μmol/kg Sat PC) plus the amount of Sat PC in the exogenous surfactant given to the animals at birth (~68 μmol/kg Sat PC).[2]

Data Analysis

Data are presented as mean ± standard error. The non-parametric Mann-Whitney U-test was applied to compare groups. A value of $p<0.05$ was accepted as significant.

RESULTS

The six control baboons and the four baboons exposed prenatally to betamethasone (Beta group), had similar birth weights: 394 ± 65 and 340 ± 27 g, respectively. The two groups had similar PO_2 and PCO_2 values while on similar ventilatory settings as reported previously.[2]

The ^{13}C-enrichment of plasma glucose reached similar steady states in all baboons during the period of isotope infusion (3.29 ± 0.38 in Controls and 3.29 ± 0.16 APE in the Beta group). The mean curves for ^{13}C-enrichment of palmitic acid in surfactant PC recovered from tracheal aspirates for both groups are shown in figure 1.

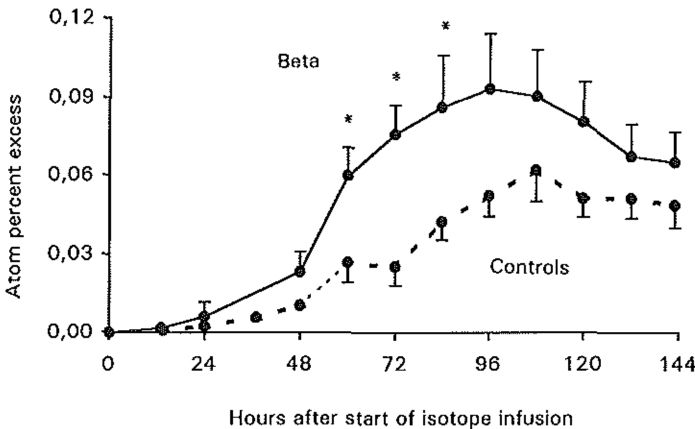
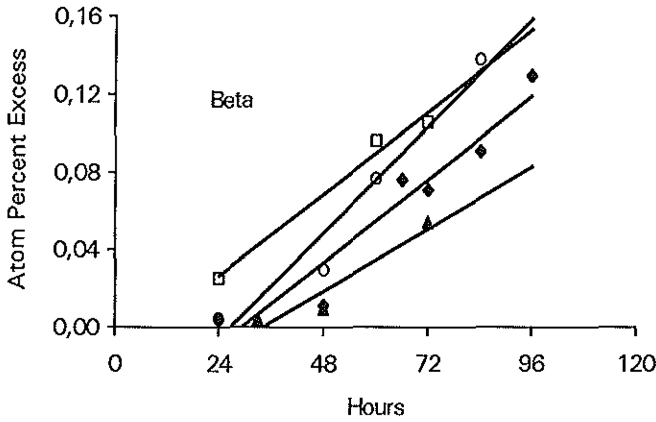


*Figure 1. ^{13}C-labeling of palmitic acid in surfactant phosphatidylcholine (PC) after a 24-h [U-^{13}C]glucose infusion. Pregnant baboons received either prenatal betamethasone or saline. When error bars are not visible, they coincide with the markers. The increased synthesis of PC-palmitate after betamethasone is shown by the increased incorporation of carbon-13 during the first days of life (*p<0.05).*

The first appearance of enrichment was similar at 27.6 ± 2.4 h for Controls and 26.0 ± 4.5 h for the Beta group (table 1). The ^{13}C-enrichment of PC-palmitate increased linearly in both groups and the enrichment was significantly higher in the Beta group as compared with the Control group during the interval from 48 to 96 h ($p<0.05$). The FSR was significantly increased in the Beta group (1.5 ± 0.2% per d) compared with Controls (0.7 ± 0.1% per d, $p<0.02$, table 1) as shown by the increased slopes of the enrichment *versus* time curves of PC-palmitate in the Beta group compared to the slopes in the Control group (fig. 2).



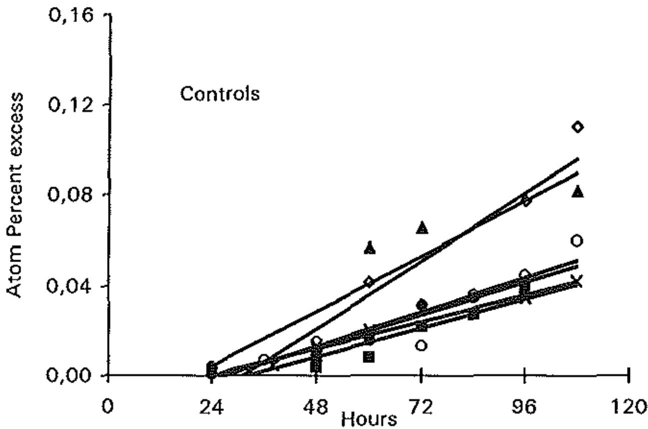


Figure 2. Pregnant baboons received either prenatal betamethasone (Beta) or saline (Controls). The regression line of ^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine (PC) for each preterm baboon is shown. As plasma glucose enrichment was identical in both groups, the increased FSR is directly represented by the increased slopes in the Betamethasone group com-pared to the Control group.

The ASR of Sat PC from glucose was higher in the Beta group (1.6 ± 0.3 μmol/kg/d) compared to the Control group (0.7 ± 0.1 μmol/kg/d, $p<0.02$). On d 6, the total lung Sat PC pool size (alveolar wash plus lung tissue) was similar for the Control and Beta groups (table 1). The time of maximal enrichment tended to be earlier in the Beta group (90 ± 8 h) than in the Control group (107 ± 3 h, $p=0.08$). The enrichments at 6 d in tracheal aspirates, alveolar washes, and lung homogenates were similar for the individual animals, and the mean values for the Control and Beta groups were also similar (tracheal aspirates, 0.052 ± 0.030; alveolar washes, 0.050 ± 0.023; and lung homogenates, 0.056 ± 0.026 APE). This implies that the enrichment of surfactant PC palmitate measured from tracheal aspirates is a good reflection of surfactant PC in the alveoli and tissue and is in agreement with a fast recycling of surfactant phospholipids.[20]

Table 1. Kinetics of surfactant PC palmitate in relation to prenatal betamethasone

	First appearance of enrichment (h)	Maximal enrichment (h)	FSR (%/d)	Absolute synthesis rate (μmol/kg/d)	Lung Sat PC pool on d 6 (μmol/kg)
Control group	27.6±2.4	107±3	0.7±0.1	0.7±0.1	186±32
Beta group	26.0±4.5	90±8	1.5±0.2	1.6±0.3	199±13
p-value	NS	0.08	0.02	0.02	NS

Ten pregnant baboons were randomized to prenatal betamethasone or saline. After very preterm delivery, baboons received a 24-h [U-^{13}C]glucose and ^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine (PC) was measured for 6 d. Kinetic parameters were calculated using enrichment versus time curves from plasma glucose and surfactant PC-palmitate. Saturated PC pool size was measured after killing on d 6. Statistical analysis to compare groups was performed with the Mann-Whitney U-test.

Discussion

An understanding of surfactant metabolism is necessary to optimize treatment of the very premature human infant with surfactant deficiency and possibly other diseases with decreased surfactant function. We infused the stable isotope [U-^{13}C]glucose in very premature baboons for 24 h and measured the ^{13}C-incorporation into palmitic acid in PC in the alveolar compartment. The ^{13}C-labeled PC increased slowly, reached the maximal value more than 4 d after the start of the isotope infusion, and remained high at d 6. These data show that the endogenous synthesis and secretion of PC is a slow process and that endogenously synthesized surfactant remains in the lung for a long time in the preterm primate. Five days after birth, the baboons also received an intravenous bolus of radioactive palmitate labeled with [^{3}H] and the specific activity in surfactant PC was measured at killing 24 h later, as described.[2] The percentage of surfactant PC secreted to the alveoli was only ~7.5% in 24 h. This slow rate of secretion is compatible with the kinetics calculated from the stable isotope data. A slow metabolism of surfactant has been described in nonprimate animals. In studies with newborn rabbits and lambs, maximal alveolar enrichments were found 35 to 60 h after a single injection of radiolabeled palmitic acid.[20,22,23] In term newborn sheep and term newborn rabbits, half-lives of [^{3}H]palmitate in surfactant PC were ~11.6 d and 2 to 4.5 d, respectively.[20,24,25]

In comparison with human preterm infants, the first appearance of enrichment in the alveolar compartment was significantly later in the baboons (~27 h *versus* ~19 h).[5] The time of maximal enrichment in steroid-treated baboons (~90 h) was comparable to the time of maximal enrichment in steroid-treated human preterm infants (~70 h). In the present study, the absolute production of Sat PC from glucose in the Beta group of ~1.6 μmol/kg/d, was lower than ~4.3 μmol/kg/d in preterm human infants treated with prenatal corticosteroids and exogenous surfactant.[5] These comparisons suggest a slower surfactant synthesis from glucose in the preterm baboon as compared with the human preterm infant, but do not show the contributions of other precursors. These baboons were more immature than the preterm human infants, which may explain the lower synthesis rates. The effects of gestational age or degree of lung immaturity have not been evaluated by these techniques in either preterm humans or baboons. We also found that prenatal corticosteroids significantly stimulated the incorporation of palmitate derived from glucose into PC: the FSR in the Beta group was increased by ~129% relative to Controls and the absolute production rate was increased in

the Beta group by ~128% relative to Controls (table 1). The PC-palmitate tended to be maximally enriched earlier in the Beta group (~90 h) than in the Controls (~107 h, $p<0.08$, table 1, figure 1), which is also compatible with stimulated carbon-13 incorporation from plasma glucose into PC-palmitate by betamethasone.

In vitro studies support the idea that glucocorticoids enhance surfactant synthesis.[8-10] Glucose incorporation into surfactant PC in perfused rat lungs is increased by betamethasone.[26] However, data on pool size measurements as an indication of surfactant synthesis *in vivo* are conflicting. In some studies with newborn rabbits, preterm monkeys, and lambs chronically catheterized *in utero*, corticosteroid treatment 2 to 3 d before delivery increased surfactant PC pool sizes in alveolar lavage and lung tissue significantly after birth.[27-30] In contrast, other studies in large animals show no prenatal steroid-induced increase of the alveolar[9,31-34] or total lung Sat PC pool size after birth.[9,31-33] Ballard *et al.* and Ikegami *et al.* did not find an increase in total lung Sat PC pool size at birth lambs when glucocorticoids were administered 2 to 4 d before preterm delivery.[11,31] However, when steroids were administered one week before delivery, the total lung Sat PC pool sizes at birth increased significantly.[11,31] In the present study, we did not measure surfactant PC pool sizes at birth. We demonstrated that the process of synthesis and secretion are slow and that the production rate of Sat PC from glucose in preterm newborns is low compared to the total Sat PC pool on d 6. Therefore, the pool size will not increase significantly until several days after exposure to betamethasone. This low synthesis rate relative to the pool size may explain why most studies find long delays between prenatal glucocorticoid treatment and increased surfactant PC pools.[11,31]

The dosage and exposure time to steroids, the species (48 h exposure in a rat is relatively long compared to lambs or baboons), and the experimental conditions that could cause secondary stress to the fetus also may contribute to discrepancies in the various reports of glucocorticoids on surfactant metabolism.

In the present study, there was no effect of prenatal betamethasone treatment on Sat PC pool size in total lung at d 6 (table 1). Probably physiological changes after birth and therapeutic interventions such as ventilation have larger effects on surfactant metabolism than do prenatal glucocorticoids. Such effects are consistent with the findings by Seidner *et al.*, who showed that the lung tissue Sat PC pool size was larger in the very preterm baboon after 6 d of ventilation (125 d gestation plus 6 d ventilation) than in either the near term baboon (175 d gestation) or the adult baboon.[2] A theoretical explanation for the fact that we did not find increased pool sizes on d 6 could be that prenatal betamethasone stimulates both synthesis and clearance of surfactant as shown by Fiascione *et al.* in preterm rabbits.[35] However, In the present study the clearance of exogenous labeled surfactant was not increased.[2]

In the type II pneumocyte, glucose is a primary precursor for fatty acids used for the synthesis of surfactant PC. This is especially true in a state of relative fatty acid deficiency, as in the present study.[36,37] The baboons did not receive any lipids during the entire study period, and the fat stores in these very premature baboons are minimal. Sanders at al. showed that the conversion of glucose to palmitic acid is a fast process.[38] Other substrates for surfactant PC are present as well, such as intracellular glycogen, fatty acids, lactate, pyruvate, and ketone bodies. The overall kinetics for surfactant PC presented here, and the increased synthesis rates after glucocorticoids, are measured from glucose only and may not represent total surfactant synthesis. The synthesis rates are valid from this precursor, but probably underestimations; a more direct precursor of PC-palmitate such as palmitic acid may yield more accurate estimations of absolute synthesis rates but ignores the importance of lipogenesis in the type II cell. It is, in the present study, unclear whether prenatal glucocorticosteroids stimulate the use of other precursors and how they influence glucose enrichment in the type II cell.

In vitro, steroids increase glycogenolysis in the type II cell, which is associated with synthesis of surfactant PC; whether this occurs in the living primate has not been studied. If prenatal steroids increased glycogenolysis in the type II cell during the period of labeled glucose incorporation into surfactant PC palmitate, than the glucose as a precursor would be diluted, resulting in a lower enrichment of surfactant PC palmitate after steroids. We found, however, increased ^{13}C-enrichment of surfactant PC palmitate. It is unlikely that glycogen depletion had already occurred before birth, as in a few baboons no glycogen depletion was found by electron microscopy after 6 d of ventilation.[2] Glucose can be used to describe surfactant metabolism, to evaluate differences between groups, and to study effects of interventions and clinical conditions. In addition, glucose can be used safely and easily in critically ill preterm human infants.

In conclusion, the data show that endogenous surfactant synthesis and turnover are slow processes. The endogenous synthesis of surfactant PC from glucose is stimulated within 48 h by prenatal betamethasone after delivery of the very premature baboon.

ACKNOWLEDGMENTS

The authors thank Ingrid H. T. Luijendijk, Ad de Bruijn, and Annemiek de Raadt, Erasmus University Rotterdam, Department of Pediatrics, for excellent technical assistance.

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Chapter 6

The Effect of Prenatal Corticosteroids on Endogenous Surfactant Synthesis in Premature Infants Measured with Stable Isotopes

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Summary

Most *in vitro* studies show that (prenatal) corticosteroids stimulate the synthesis of surfactant phosphatidylcholine (PC) but studies in animals are controversial.

Whether prenatal corticosteroids stimulate the surfactant PC synthesis in humans has not been studied. We studied the endogenous surfactant PC synthesis in relation to prenatal corticosteroid treatment in 27 preterm infants with respiratory distress syndrome. Infants received a 24-h infusion with the stable isotope [U-^{13}C]glucose starting ~5 h after birth. The ^{13}C-incorporation into palmitic acid in surfactant PC from serial tracheal aspirates and in plasma triglycerides and phospholipids was measured by isotope-ratio mass spectrometry. Premature infants had received either zero (n = 11), one (n = 4), or two doses (n = 12) of prenatal betamethasone (12 mg intramuscularly). The fractional synthesis rate (FSR) of surfactant PC from glucose was 1.7 ± 0.3%/d without corticosteroid treatment, 2.9 ± 1.4%/d with one dose and 5.8 ± 1.3%/d after two doses of prenatal corticosteroids. Using multiple regression analysis, the FSR of surfactant PC increased with 40% (confidence interval: 7 to 82%/d, $p < 0.02$) per dose of corticosteroids and doubled after two doses corticosteroids. The ^{13}C-enrichment of plasma triglycerides and phospholipids was not increased by corticosteroids.

These data show that prenatal corticosteroid treatment stimulates surfactant synthesis in the preterm infant.

Am. J. Respir. Crit. Care Med. in press

INTRODUCTION

After very premature delivery, many infants develop respiratory distress syndrome (RDS) caused by primary surfactant deficiency among other factors. Prenatal corticosteroid administration to women at risk for premature delivery reduces the incidence and severity of RDS as well as mortality in preterm infants.[1] Prenatal corticosteroids improve pulmonary function in preterm animals with RDS by stimulating structural development,[2] induction of antioxidant enzymes,[3] and decreasing vascular permeability.[4] Improved pulmonary function can also result from increased amounts of surfactant in the alveolar space. In many *in vitro* studies corticosteroids increase the activity of the enzymes for the synthesis of surfactant lipids[5-7] and increase surfactant protein synthesis.[6,8-10] In *in vitro* experiments with lung slices and isolated type II cells, corticosteroids increase the incorporation of radiolabeled precursors into surfactant PC, reflecting increased PC synthesis.[5,7,11-13] Despite these strong indications for corticosteroid stimulation, most studies in animals do not show increased surfactant PC pool sizes or increased precursor incorporation after prenatal corticosteroid treatment,[2,4,14-20] some studies reported increased pool sizes[20-22] and one study found decreased alveolar surfactant pool sizes after a single dose of prenatal betamethasone.[23]

The amount or concentration of surfactant in the alveolar space does not adequately reflect surfactant synthesis because they depend on the amount secreted to and cleared from the alveolus. In a randomized study in preterm baboons it was recently found that surfactant PC synthesis from glucose was stimulated by two doses of prenatal corticosteroids.[24] Whether prenatal corticosteroids stimulate the surfactant PC synthesis in preterm infants with primary surfactant deficiency has not been studied. We recently reported data on surfactant metabolism in six preterm infants using a novel and safe technique.[25] The present clinical study is the first implementation of our recently developed method. With this technique, using stable isotopes, the hypothesis was tested whether prenatal corticosteroids stimulate surfactant synthesis in critically ill preterm infants with RDS.

METHODS

Subjects and study design

Consecutive infants requiring mechanical ventilation were studied starting directly after birth. Inclusion criteria were: gestational age <32 wk, intubation for RDS as defined clinically and by chest radiograph,[26] and written parental informed consent. Exclusion criteria were: congenital infection, maternal diabetes, and chromosomal abnormality. Patients received a constant i.v. infusion of the stable isotope [U-^{13}C]glucose (Campro Scientific, Veenendaal, The Netherlands) for 24 h at 0.17 mg/kg/min. The start of the study (t=0) was defined by the start of the isotope infusion. This was 5.3 ± 0.5 h after birth. The total glucose intake was 5.7 mg/kg/min including nonlabeled glucose, the infants did not receive any lipids the first 48 h of the study. Before and during the glucose infusion, 1 ml arterial blood was drawn every 6 h for the determination of ^{13}C-enrichment of plasma glucose and of palmitic acid in plasma triglycerides, phospholipids, and in the free fatty acid fraction.

Exogenous surfactant (Survanta®; Abbott Laboratories, North Chicago, IL) was administered endotracheally at t=0, at a dose of 100 mg/kg phospholipids, if the mean airway pressure exceeded 7.5 cm of water, or if the inspiratory oxygen fraction was higher than 0.40. Infants received a second dose 6 h later if the criteria were still met. The attending neonatologist administered a third dose if appropriate within 20 h from the second dose. Tracheal aspirates were obtained every 6 h during the time that the infant was intubated. The tracheal suctioning procedure was performed during routine patient care and did not deviate from the normal clinical protocol.[25] Tracheal aspirates were immediately placed at -20°C,

until further processing. No tracheal suction was done within 6 h after surfactant administration. Total parenteral nutrition including lipids was started at t=48 h. Infants were grouped according to the number of doses of prenatal corticosteroids that their mother received (one dose is 12 mg dexamethasone intramuscularly). When delivery at admission was expected immediately, corticosteroids were not given (= Group 0). When delivery was not expected immediately, the first dose corticosteroids was given (= Group 1). A second dose corticosteroids was given 24 h after the first dose if delivery had not occurred (= Group 2). The study was approved by the local medical ethics committee.

Table 1. Patients' characteristics

	Birth weight (g)	Gestational age (wk)	RDS grade (I-IV)	Doses of surfactant	Days of ventilation
Group 0 [11]	1035 (640-2030)	29.4 (25.9-32.0)	3.5 (1-4)	2.0 (1-3)	5.5 (1.0-51)
Group 1 [4]	878 (590-1800)	26.6 (25.6-31.3)	3.0 (2-3)	2.0 (1-3)	8.5 (5.0-17)
Group 2 [12]	905 (715-1270)	27.9 (25.6-30.3)	2.5 (0-4)	1.5 (0-2)	6.8 (1.0-39)
0 *vs* 2, p-value	0.5	0.3	0.1	0.02	0.8

Analytical procedures

Surfactant PC palmitate isolation. The tracheal aspirates were processed as described before.[25] In short, cells were removed by centrifugation, internal standard was added, organic extraction was used to isolate surfactant lipids,[27] and surfactant PC was isolated by TLC.[28] The PC was derivatized,[29] and the fatty acid methyl esters were stored at -20°C. Tracheal aspirates containing visible blood were not analyzed.

Surfactant PC concentration. The fatty acid methyl esters were analyzed by gas-chromatography (GC) and the amount calculated using the internal standard.[30] The concentration of surfactant PC in the epithelial lining fluid (ELF) was calculated by correction for dilution of the ELF during endotracheal suction with normal saline: dilution factor = $[urea]_{serum}/[urea]_{tracheal\ aspirate}$.[31]

Blood samples. Collected blood was directly centrifuged to separate cells and plasma. By organic extraction plasma was divided in a water fraction containing glucose and an organic solvent fraction containing lipids.[32] The glucose was isolated using anion and cation exchange resin.[25] The glucose was derivatized to an aldonitril pentacetate derivative.[33] From the plasma lipid fraction, triglycerides, phospholipids, and free fatty acids were isolated by TLC using Kieselgel plates (Merck, Darmstadt, Germany). The solvent system contained heptane/diisopropylether/acetic acid 60/40/3, v/v/v). The triglycerides, phospholipids, and free fatty acids were derivatized,[29] and the fatty acid methyl esters were stored at -20°C.

Determination of enrichment. The ^{13}C-enrichment of palmitic acid in surfactant PC, triglycerides, phospholipids, free fatty acids, and of plasma glucose was measured by gas chromatography-combustion interface-isotope-ratio mass spectrometry (GC-CI-IRMS) (VG Isotech, Middlewich, Cheshire, UK).[25,29] The enrichment is expressed in atom per cent excess (APE), which represents the increase in percentage of carbon-13 atoms in total carbon dioxide from the combusted compounds above baseline enrichment (before isotope infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms added during derivatization.

Calculations

As palmitic acid is by far the most abundant fatty acid in surfactant PC, calculations were performed for this fatty acid only.[25]

The first appearance of enrichment was defined as the time delay between the start of the [U-^{13}C]glucose infusion and the detection of ^{13}C-enriched palmitic acid in surfactant PC. The first appearance of enrichment was calculated by plotting the regression line for the linear increase of the enrichment *versus* time curve, and extrapolating it to baseline enrichment.[25,34]

The fractional synthesis rate (FSR) of surfactant PC is the percentage of the total surfactant PC pool synthesized from glucose per day. The FSR was calculated by dividing the slope of the linear increase of enrichment of PC palmitate by the steady state ^{13}C-enrichment of plasma glucose.[25,34]

The half-life of surfactant PC was calculated by exponential curve fitting of the downslope of the enrichment *versus* time curve.[25]

Data analysis

Data are presented as mean ± SEM or as median (range). Comparisons between the largest groups of 0 (n = 11) and 2 (n = 12) doses of prenatal steroids were made using the Mann-Whitney U-test. To adjust for potential confounding factors in the evaluation of the number of doses of steroids regarding enrichment, multiple regression analysis was used. In this analysis the FSR had to be transformed logarithmically in order to obtain normally distributed data.

RESULTS

Twenty-seven infants were included, 11 in Group 0 (no prenatal corticosteroids), four in Group 1 (one dose), 12 in Group 2 (two doses of prenatal corticosteroids) (table 1). The patient groups were comparable regarding gestational age, birth weight, and the fraction of infants which were adequate or small for gestational age. The RDS grade and number of days of ventilation were similar between groups (table 1). The ^{13}C-enrichment of plasma glucose reached steady state in all infants between t = 6 and 24 h and was similar in all groups (Group 0: 2.65 ± 0.15; Group 1: 2.28 ± 0.09; Group 2: 2.43 ± 0.14 APE). Infants in Group 2 required a significantly lower number of doses of surfactant compared to Group 0 (1.5 [0 to 2] and 2.0 [1 to 3], respectively, p = 0.02). In total 468 tracheal aspirates were analyzed.

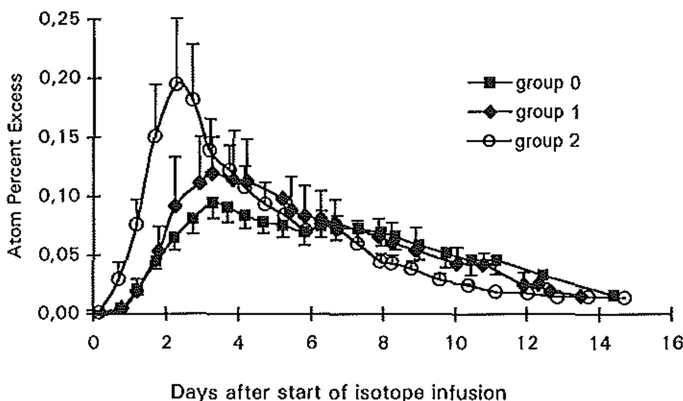

Figure 1. ^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine (PC). Ventilated preterm infants received a 24-h [U-^{13}C]glucose infusion as precursor for PC-palmitate. Preterm infants were exposed to zero, one, or two doses of prenatal corticosteroids. The incorporation of carbon-13 into PC during the first days of life is increased after betamethasone.

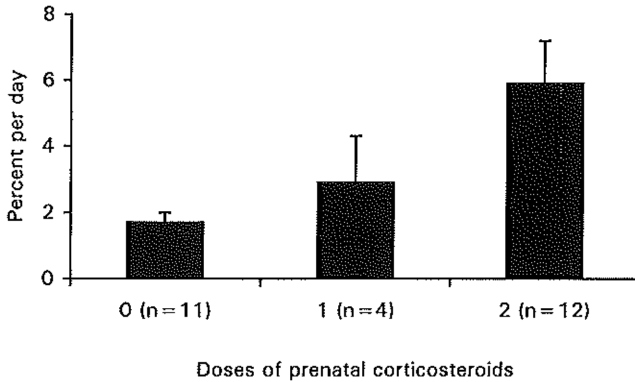

Figure 2. The fractional synthesis rate (FSR) of surfactant phosphatidylcholine (PC). Infants received. [U-^{13}C]glucose i.v. and the ^{13}C-enrichment of surfactant PC palmitate was measured. The FSR was significantly stimulated by the treatment with prenatal corticosteroids with 40% per dose ($p<0.02$).

The ^{13}C-enrichment of palmitic acid in surfactant PC in the three groups is shown in figure 1. The increased ^{13}C-enrichment in the corticosteroid treated groups compared to Group 0 is evident. The kinetic parameters were calculated from these curves and shown in table 2. The first appearance of ^{13}C-enrichment and half-life of surfactant PC were similar in the three groups. There was a trend towards earlier maximal enrichment in Group 2 compared to Group 0 (59.4 ± 4.0 and 76.9 ± 7.8 h, $p=0.07$). The FSR of palmitic acid in surfactant PC from glucose increased significantly by the administration of prenatal corticosteroids (fig. 2). By univariate analysis between Group 0 and Group 2, the surfactant synthesis significantly increased by corticosteroids (1.7 ± 0.3 *versus* 5.9 ± 1.3%/d, $p=0.0002$).

Exogenous surfactant dilutes the labeled surfactant PC derived from endogenous synthesis. Therefore, we corrected for the number of doses of surfactant administered using multiple regression analysis. This analysis also allowed to correct for the effect of gestational age. The mean FSR increased linearly on a logarithmic scale, with an increase of 40% (95% confidence interval: 7 to 82%, $p<0.02$) per dose of corticosteroids administered prenatally. This is an increase of 96% after two doses prenatal corticosteroids.

The liver can use the infused [U-^{13}C]glucose for lipogenesis which results in labeled plasma lipids.[25] The type II pneumocyte can use palmitic acid from plasma triglycerides, phospholipids, and free fatty acids for surfactant PC synthesis. So, an increased labeling of surfactant PC after prenatal corticosteroids could in theory result from increased ^{13}C-enrichment of plasma lipids from synthesis by the liver after corticosteroids with subsequent uptake by lung. To study this possibility, we measured the ^{13}C-enrichment of palmitic acid in plasma triglycerides, phospholipids, and in the free fatty acid fraction. The palmitic acid in triglycerides and phospholipids became enriched within 6 h after the start of the [U-^{13}C]glucose infusion (fig. 3A and 3B). The enrichment of free palmitic acid was too low (much lower than that of surfactant PC palmitate) to be measured accurately and was therefore only performed in several patients (data not shown). There was no statistically significant difference between the groups regarding ^{13}C-enrichment of palmitic acid in plasma triglycerides and phospholipids. These data show that the increased enrichment of surfactant PC palmitate after prenatal steroids is due to increased endogenous synthesis of surfactant PC, and is not a reflection of plasma lipid enrichment.

Table 2. Kinetics of surfactant phosphatidylcholine

	First appearance of enrichment (h)	Time of maximal enrichment (h)	Half-life (h)	FSR % per d	Concentration of PC (mg/ml ELF)
Group 0 [11]	18.7±2.8	76.9±7.8 [7]	98.8±8.3 [5]	1.70.3	2.4 (0.4-14.3) [5]
Group 2 [4]	21.3±2.2	76.8±7.6 [4]	105±10.9 [3]	2.91.4	2.9 (1.7-4.2) [2]
Group 2 [12]	17.4±3.0	59.4±4.0 [8]	86.0±9.9 [9]	5.91.3	8.1 (0.9-37.5) [9]
0 *vs* 2, p-value	0.26	0.07	0.34	0.0002	0.19

Twenty-seven preterm infants received either zero, one, or two doses of prenatal corticosteroids. The fractional synthesis rate (FSR) and time of first appearance of enrichment were measured in all infants. Time of maximal enrichment, half-life, and the PC concentration were measured in the number of infants mentioned between brackets [n].

The surfactant PC concentration at birth (before surfactant administration) was 2.4 (0.4 to 14.3) in Group 0, 2.9 (1.7 to 4.2) in Group 1, and 8.1 (0.9 to 37.5) mg/mL ELF in Group 2. Although the concentration tended to be higher in Group 2, this was not significant, probably due to the small number of infants. The concentration of surfactant PC was measured in only 16 of the 27 patients because some infants received exogenous surfactant as therapy already before informed consent was obtained for this study.

Discussion

This study describes for the first time in humans the relation between the exposure to prenatal corticosteroids and *de novo* surfactant PC synthesis. Preterm infants received [U-^{13}C]glucose as a precursor for the synthesis of palmitic acid in surfactant PC. We found a significantly increased surfactant PC synthesis from glucose with a linear increase of 40% per dose of prenatal corticosteroids (fig. 2). This implies that administration of two doses of corticosteroids to the mother at risk of preterm labor as is generally accepted[35] doubles the production of surfactant PC from glucose in the preterm infant. This finding shows that stimulated general lung maturation is accompanied by increased surfactant PC production.

These findings are in line with many *in vitro* studies that show induction of enzymes for surfactant synthesis and increased precursor incorporation after prenatal corticosteroids.[11,12] Glucose incorporation into surfactant PC in organotypic cultures of type II cells is increased by betamethasone.[12] In a study by Kessler *et al.* in premature baboons, a 72-h treatment with prenatal dexamethasone did not increase radioactive palmitate incorporation in lung lipids, but shifted the incorporation to disaturated PC in lung lavage fluid 3 h after birth.[20] We recently found that in very preterm baboons which received exogenous surfactant and were ventilated for 6 d, surfactant PC synthesis from glucose approximately doubled after two doses prenatal corticosteroids, which is comparable to the findings in the present study.[24]

Data on surfactant pool sizes in animal studies as an indication of surfactant synthesis *in vivo* are conflicting; some studies find increased pool sizes[20-22] but most studies do not find an increased surfactant pool in the lung after corticosteroids.[2,4,14-19] The concentration surfactant PC in the ELF tended to increase after two doses of corticosteroids at birth (table 2). The concentration of PC was measured in only 16 from the 27 patients because some infants received exogenous surfactant already before informed consent was obtained. In a study by Kari *et al.* in human preterm infants, prenatal dexamethasone had no significant effect on the surfactant concentration in ELF.[36] Ballard *et al.* and Ikegami *et al.* did not find

an increased total lung saturated PC pool size at birth in preterm lambs when corticosteroids were administered 2 to 4 d before preterm delivery. However, when corticosteroids were administered one week before delivery, the total lung saturated PC pool at birth had increased significantly.[16,21] The discrepancies between increased surfactant synthesis and not significantly elevated surfactant pools or concentrations in our and other studies can be explained by the low synthesis rate relative to the pool size.

This hypothesis is supported by the other kinetic parameters (table 2). The long delay between the start of the stable isotope infusion and the first enrichment in surfactant PC palmitate show that the synthesis of palmitate, intracellular processing, and secretion of surfactant to the alveolar space is a slow process. The delay between the start of the isotope infusion and the time of maximal enrichment was long in all groups. The time to reach maximal enrichment and half-life of surfactant PC tended to be shorter in Group 2 which is compatible with stimulated surfactant metabolism. The long half-lives of ^{13}C-enrichment show that endogenous synthesized surfactant remains in the lungs for many days and is diluted slowly by newly synthesized unlabeled surfactant. The half-lives for phosphatidylglycerol (~105 h) and sphingomyelin (~97 h) in preterm infants treated with Survanta® were comparable to our data.[37] The half-live of phosphatidylglycerol was ~30 h in preterm infants treated with human amniotic extracted surfactant.[38] In the newborn rabbit, the half-life of surfactant PC was ~57 h when labeled with palmitic acid and ~136 h when labeled with choline.[39] Increased clearance of labeled surfactant from the lung shortens half-life and more recycling of components of surfactant prolongs half-life. These variables were not measured and can play a role in other studies as well. The type of surfactant studied and the label used also influence half-lives measurements.[37,39]

In the present study, we measured the synthesis of palmitic acid of surfactant PC from the precursor glucose only. The type II cell has, besides glucose, several other sources for the synthesis of surfactant PC, like palmitic acid, intracellular glycogen, lactate, and ketone bodies.[40] Glucose is, however, an important substrate for the fatty acids of surfactant PC.[41-43] certainly in relative fatty acid deficiency like in these infants as intravenous feeding with lipids started 48 h after the start of the isotope infusion. The type II cell can use palmitic acid from the plasma free fatty acid fraction, from plasma triglycerides, and probably phospholipids for the synthesis of surfactant PC.[42]

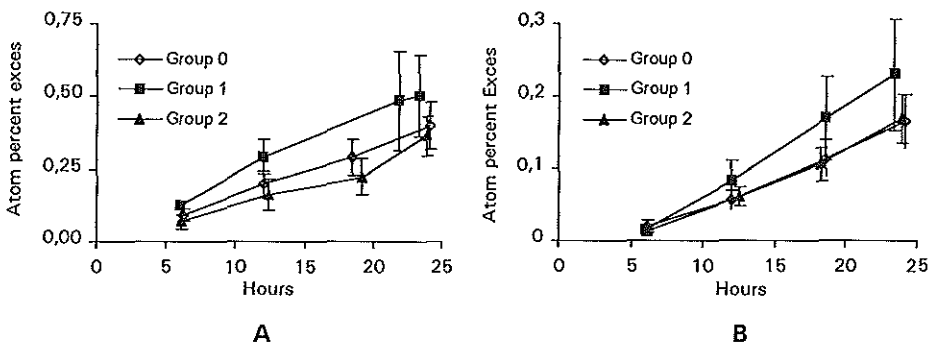

Figure 3. The ^{13}C-enrichment of plasma triglycerides (A) and of plasma phospholipids (B). After a 24-h [U-^{13}C]glucose infusion. The ^{13}C-enrichment was measured in palmitic acid in these plasma lipids. The ^{13}C-enrichment of plasma triglycerides and phospholipids did not increase by prenatal corticosteroids.

The lung fibroblast accumulates triglycerides and transfers these lipids to the type II pneumocyte, and this process is stimulated *in vitro* in rat lung by glucocorticoids.[44] An increased ^{13}C-enrichment of surfactant PC could therefore be a reflection of increased ^{13}C-enrichment of plasma lipids due to stimulated lipogenesis in the liver after prenatal steroids.

We measured the ^{13}C-enrichment of palmitic acid in plasma free fatty acids, triglycerides and phospholipids in all groups. As shown in figures 3A and 3B the ^{13}C-enrichment of triglycerides and phospholipids did not change after prenatal steroids and can not lead to increased ^{13}C- incorporation in surfactant in the corticosteroid treated groups. The ^{13}C-enrichment of palmitic acid in the free fatty acid fraction was too low to be measured accurately (much lower than that of surfactant PC), and was therefore only measured in some patients (data not shown).

Thus, the increased incorporation of ^{13}C from glucose into surfactant PC found in Groups 1 and 2 does not reflect the enrichment of plasma lipids, but reflects increased surfactant phospholipid synthesis from glucose. The increased FSR of surfactant PC reflects the principle that prenatal corticosteroids increase surfactant phospholipid production in the preterm infant, but it is unclear what the total absolute increase is as the contribution of the other precursors is unknown.

The total surfactant pool and the pools of the metabolic intermediates are unknown, but influenced by the treatment with large amounts of exogenous surfactant. We corrected by multiple regression analysis for the potential confounding effects of the number of doses of exogenous surfactant. As a result of the way of inclusion of the patients, the different groups could have been subject to different durations of prenatal stress. The impact of the prenatal stress is, however, not known, and is a limitation of the study. It is, however, ethically not possible to perform this study in preterm infants in a randomized fashion, as administration of prenatal corticosteroids is an established therapy to improve the outcome in the premature infants.

In summary, this study shows for the first time that treatment with prenatal corticosteroids increases surfactant PC production from the important precursor glucose in the preterm infant with severe RDS. The data show that endogenous surfactant metabolism is a slow process and that stimulation of synthesis by steroids increases alveolar pool sizes probably only after many days. Therefore, the increased surfactant synthesis after the generally advized corticosteroids treatment,[35] probably plays only a minor role in the reduced incidence of RDS and improved outcome. The accelerated development of the surfactant system is part of the enhanced integrated development of the lung.

ACKNOWLEDGEMENTS

We thank Daphne J. M. T. Janssen, Department of Pediatrics, Sophia Childrens' Hospital Rotterdam, The Netherlands for excellent technical assistance and the nursing staff, residents and neonatologists for their help and support with this study.

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Chapter 7

Treatment with Exogenous Surfactant Stimulates Endogenous Surfactant Synthesis in Premature Infants with Respiratory Distress Syndrome

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Summary

Treatment of preterm infants with respiratory distress syndrome (RDS) with exogenous surfactant has greatly improved clinical outcome. Some infants require multiple doses and it has not been studied whether these large amounts of exogenous surfactant disturb endogenous surfactant metabolism in humans. We studied endogenous surfactant metabolism in relation to different amounts of exogenous surfactant, administered as rescue therapy for RDS.

Preterm infants were intubated and mechanically ventilated for respiratory insufficiency. They received a 24-h infusion with the stable isotope [U-^{13}C]glucose starting 5.3 ± 0.5 h after birth. The ^{13}C-incorporation into palmitic acid in surfactant phosphatidylcholine (PC) isolated from serial tracheal aspirates was measured. Infants received either zero ($n=5$), one ($n=4$), two ($n=15$), or three ($n=3$) doses of Survanta® (100 mg/kg) when clinically indicated. Using multiple regression analysis, the absolute synthesis rate (ASR) of surfactant PC from plasma glucose increased with 1.3 ± 0.4 mg/kg/d per dose of Survanta® ($p=0.007$) (mean ± sem). The ASR of surfactant PC from glucose was increased by prenatal corticosteroid treatment with 1.3 ± 0.4 mg/kg/d per dose corticosteroid ($p=0.004$), and by the presence of a patent ductus arteriosus with 2.1 ± 0.7 mg/kg/d ($p=0.01$).

These data are reassuring and show that multiple doses of exogenous surfactant for RDS in preterm infants are tolerated well by the developing lung and stimulate endogenous surfactant synthesis.

Crit. Care Med. in press

INTRODUCTION

Pulmonary surfactant deficiency at birth is the main cause of neonatal respiratory distress syndrome (RDS). Administration of exogenous surfactant rapidly augments the alveolar surfactant pool size and acutely decreases the degree of respiratory failure and increases the survival rate in neonates with RDS.[1] However, some infants do not respond or respond only temporarily to surfactant therapy, and require retreatment with surfactant. A sustained clinical improvement is probably depending on a sufficient endogenous surfactant production, among other factors.

Feedback inhibition of endogenous surfactant production could occur as one treatment dose of 100 mg phospholipids/kg contains 10 to 20 times the amount of phosphatidylcholine (PC) present in the alveoli in preterm infants with RDS,[2,3] and 5 to 10 times the amount of alveolar PC in the adult rabbit.[4] *In vitro* studies showed that the addition of surfactant or lavage fluid inhibited the incorporation[5] of labeled precursors into disaturated PC and decreased secretion[6] of surfactant lipids by type II cells, and Miles *et al.* hypothesized the presence of a negative feedback mechanism *in vitro*.[7] In another *in vitro* study, however, the incorporation of [^{3}H]choline was stimulated and the incorporation of [^{3}H]acetate was not affected by administration of phospholipids.[8] Oetomo *et al.* showed in adult rabbits *in vivo* an increased synthesis and secretion of PC after treatment with exogenous natural rabbit surfactant.[9] After instillation of Survanta® only the secretion was stimulated, but not precursor incorporation.[9] Others reported no change in surfactant metabolism *in vivo* by surfactant treatment.[10,11]

Whether surfactant administration in human preterm infants influences endogenous surfactant metabolism has not been studied. After exogenous surfactant administration, the concentrations of PC remains elevated for many days.[12,13] However, it is not possible to distinguish between the exogenous and endogenous surfactant in those studies, so the contribution of newly synthesized surfactant could not be estimated. Furthermore the surfactant synthesis is not reflected adequately by the measurement of pool sizes or concentrations alone.

We tested the hypothesis that the administration of exogenous surfactant influences endogenous surfactant synthesis in preterm infants. The stable isotope [U-^{13}C]glucose was used as a precursor for palmitic acid in surfactant PC to measure endogenous synthesis after various doses of exogenous surfactant used as rescue therapy for RDS.

METHODS

Subjects

All infants were admitted to the neonatal intensive care unit of the Sophia Children's Hospital. Inclusion criteria were: primary intubation and mechanical ventilation directly after birth, gestational age <32 wk, and written parental informed consent. Exclusion criteria were: congenital infection, maternal diabetes, and chromosomal abnormality. The study was approved by the Medical Ethics Review Board of the University Hospital/Erasmus University Rotterdam. Patients received a constant infusion of the stable isotope [U-^{13}C]glucose (Campro Scientific, Veenendaal, The Netherlands) for 24 h at 0.17 mg/kg/min. The start of the isotope infusion defined $t=0$ (5.3 ± 0.5 h after birth). The total glucose intake was 5.7 mg/kg/min. The infants did not receive any lipids during the first 48 h of the study. Before and during the glucose infusion, 1 ml arterial blood was drawn every 6 h for the determination of ^{13}C-enrichment of plasma glucose. RDS was defined clinically and by chest radiograph.[14] Exogenous surfactant (Survanta®, Abbott Laboratories, North Chicago, IL, USA) was administered endotracheally at $t=0$, at a dose of 100 mg/kg phospholipids, if the mean airway

pressure exceeded 7.5 cm of water, or if the inspiratory oxygen fraction was higher than 0.40. Infants received a second dose 6 h later if the criteria were still met. A third dose was administered within 20 h from the second dose if respiratory failure persisted.

Tracheal aspirates were obtained every 6 h during the time that the infant was intubated. The tracheal suctioning was performed during routine patient care and did not deviate from the normal clinical protocol. Tracheal aspirates were immediately placed at -20°C, until further processing. No tracheal suction was done within 6 h after surfactant administration. Total parenteral nutrition including lipids was started at t=48 h. A patent ductus arteriosus (PDA) was considered significant for this study when clinical suspicion was confirmed by echo and when treatment with indomethacine was necessary. Infants were grouped according to the number of doses of exogenous surfactant received (Group 0 received no surfactant; Group 1 received one dose; Group 2, two doses; Group 3, three doses).

Analytical procedure

Surfactant PC palmitate isolation. The tracheal aspirates were processed as described before.[15] In short, cells were removed by centrifugation, organic extraction was used to isolate surfactant lipids,[16] and surfactant PC was recovered by thin layer chromatography.[17] The PC was derivatized,[18] and the fatty acid methyl esters were stored at -20°C. Tracheal aspirates containing visible blood were not analyzed.

Blood samples. Collected blood was directly centrifuged to separate cells and plasma. Plasma was delipidated with chloroform and methanol.[19] The glucose was isolated using anion and cation exchange resin,[15] and derivatized to an aldonitril pentacetate derivative.[20]

Determination of enrichment. The ^{13}C-enrichment of palmitic acid in surfactant PC and of plasma glucose was measured by gas chromatography-combustion interface-isotope ratio mass spectrometry (GC-CI-IRMS) (VG Isotech, Middlewich, Cheshire, UK).[15,18] The enrichment is expressed in atom per cent excess (APE), and represents the increase in the percentage of carbon-13 atoms in total carbon dioxide from the combusted compounds above baseline enrichment (before infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms added during derivatization.

Calculations

As palmitic acid is by far the most abundant fatty acid in surfactant PC, calculations were performed for this fatty acid only.[15] The tissue-bound and alveolar surfactant was regarded as one pool as the recycling is ~16 times faster than *de novo* synthesis and clearance.[4,10,21] In preterm baboons that received [U-^{13}C]glucose, the ^{13}C-enrichment of PC-palmitate in alveolar lavage, tracheal aspirate and lung tissue was similar on d 6 after birth.[22] This shows that alveolar and lung tissue surfactant (= total surfactant pool) can be considered as one metabolic pool for the calculations.

The first appearance of enrichment was calculated by plotting the regression line for the linear increase of the enrichment *versus* time curve of surfactant PC palmitate, and extrapolating it to baseline enrichment.[15]

The fractional synthesis rate (FSR) of palmitic acid in surfactant PC is the percentage of the total PC-palmitate pool synthesized from glucose per day. The FSR was calculated by dividing the slope of the linear increase of enrichment of surfactant PC palmitate by the steady state enrichment of plasma glucose.[15] In the present study the total surfactant pool sizes were different due to different amounts of exogenous surfactant administered. To correct for this we calculated the *absolute synthesis rate* (ASR) of surfactant PC.

The ASR was calculated by multiplying the FSR with the total lung surfactant PC pool. We did not measure the total surfactant PC pool in these infants but estimated the total PC pool. For the preterm infants without RDS, the total pool size was considered to be equal to the

total lung pool (alveolar wash plus lung tissue) found in 140 d preterm baboons without RDS as this is the closest model of the human; this pool size was 39 mg PC/kg body weight.[23] For the infants with RDS, this pool size was halved (19 mg PC/kg) as many studies have shown that the surfactant concentration[3] and amounts in total lung found in postmortem studies in RDS[24] is approximately half the amount in premature infants without RDS. For the infants that received exogenous surfactant for RDS, the pool size was considered to be the sum of endogenous surfactant (19 mg/kg) and the amount from exogenous surfactant (one dose of Survanta® =87 mg PC/kg).

The half-life of surfactant PC was calculated by exponential curve fitting of the downslope of the enrichment *versus* time curve.[15]

Data analysis

Data are presented as mean±sem or as median (range). In evaluating the effects of surfactant administration on the ASR, while adjusting for the effects of prenatal corticosteroids, multiple regression analysis was used. Using this method also other potential confounders were taken into account. A value of $p<0.05$ (two-sided) was regarded as significant.

RESULTS

Twenty-seven infants were included, five in Group 0 (no surfactant treatment), four in Group 1 (one dose of surfactant), 15 in Group 2 (two doses of surfactant), and 3 in Group 3 (3 doses of surfactant) (table 1). The patient groups were comparable regarding gestational age and birth weight. All infants in Group 0 had received a full course of prenatal dexamethasone (two doses between 7 d and 24 h before birth). In Group 0, one infant had RDS grade two and one infant had grade one, three infants had mild respiratory insufficiency with apnea's without pulmonary pathology on chest radiograph (table 1). In Group 2 and 3, most infants had severe RDS.

The ^{13}C-enrichment of plasma glucose was in steady state in all infants between t=6 and 24 h and was similar in all groups (Group 0: 2.22±0.08; Group 1: 2.36±0.12; Group 2: 2.55±0.13; Group 3: 2.71±0.48 APE).

In total 468 tracheal aspirates were analyzed. The ^{13}C-enrichment of surfactant PC palmitate in the four groups is shown in figure 1. The more exogenous surfactant was administered the lower the enrichment of PC palmitate was, due to massive dilution of label by the unlabeled surfactant.

Table 1. Patient characteristics

	Group 0	Group 1	Group 2	Group 3
Birth weight (g)	845 (715-1270)	848 (690-2030)	1080 (590-1800)	920 (640-1610)
Gestational age (wk)	27.7 (26.0-28.4)	27.4 (26.0-32.0)	28.0 (25.6-31.4)	29.7 (27.4-31.0)
Doses prenatal steroids	2.0 (2-2)	1.0 (0-2)	1.0 (0-2)	0 (0-0)
RDS grade (0-4)	0.0 (0-2)	2.5 (1-3)	3.0 (2-4)	4.0 (3.0-4.0)
Patent ductus arteriosus	2	1	10	3
Duration of ventilation	7.0 (2-39)	2.0 (1.0-12)	6.0 (1.5-31)	22 (3.0-31)

Patient characteristics according to the number of doses of surfactant. RDS=respiratory distress syndrome. Data in median (range).

The time of first appearance of enrichment (table 2) was found to increase significantly ($p<0.01$) with increasing doses of exogenous surfactant (increase: 4.6 ± 1.6 h per dose). This is probably due to dilution of label by exogenous surfactant. The time of maximal enrichment and the half-life could not be measured in all infants because some infants were already extubated. For the evaluable subjects, the time of maximal enrichment and the half-life tended to increase (both $p<0.10$) when more surfactant was administered (table 2).

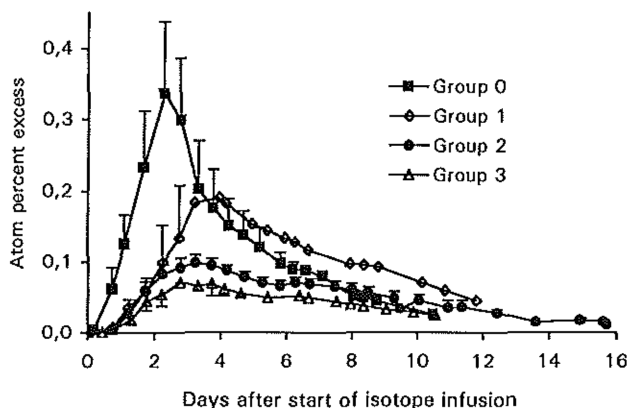

Figure 1. ^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine after a [U-^{13}C]glucose infusion. Infants in Group 0 received no exogenous surfactant, in Group 1 one dose, in Group 2 two doses, and in Group 3 three doses of surfactant.

The FSR decreased significantly ($p<0.001$) with increasing doses of exogenous surfactant (table 2). The FSR is directly calculated from the linear increase in figure 1 (see Methods) and therefore can not be used to directly compare synthesis rates of surfactant PC between the groups due to dilution by exogenous surfactant. By calculating the absolute synthesis rates, groups with different pool sizes can be compared (ASR = FSR times pool size). Allowing for the effects of prenatal corticosteroids using multiple regression analysis, the mean ASR was found to increase significantly in a linear fashion with increasing amounts of surfactant (figure 2 and table 3). This analysis also showed that the ASR increased significantly with the presence of PDA and decreased with advancing gestational age (table 3).

Discussion

Although the administration of surfactant has become routine treatment of neonatal RDS, there is little information whether large amounts of exogenous surfactant influence endogenous surfactant metabolism, and whether feedback mechanisms operate. *In vitro* and animal studies give conflicting results regarding this issue and studies in humans have not been performed. The present study reports the effects of surfactant therapy on endogenous surfactant metabolism. Surfactant synthesis was measured by using glucose labeled with the stable isotope ^{13}C as a precursor for surfactant PC-palmitate, and was related to the number of doses of exogenous surfactant.

The endogenous surfactant PC synthesis from glucose increased by ~1.3 mg/kg/d per dose of exogenous surfactant. This implies that exogenous surfactant stimulates endogenous surfactant PC production and that the presence of a negative feedback mechanism is unlikely. Although the increase of ~1.3 mg/kg/d per dose surfactant is small compared to the surfactant pool sizes after treatment with exogenous surfactant, the importance of this finding is that endogenous surfactant synthesis is not suppressed by surfactant treatment.

Table 2. Kinetics of surfactant PC in relation to doses of exogenous surfactant

	Group 0	Group 1	Group 2	Group 3
FSR (%/d)	8.9±2.4	3.3±1.4	2.5±0.3	1.7±0.6
ASR (mg/kg/d)	2.7±0.8	3.5±1.4	4.8±0.7	4.8±1.8
First appearance of enrichment (h)	11.2±3.3	15.7±3.9	21.8±2.1	22.8±3.4
Time of maximal enrichment(h)	53±3.8 [3]	65 [1]	73±4.9 [13]	72±17 [2]
Half-life (h)	70±15 [3]	98 [1]	99±6.5 [10]	99±26 [2]

Group 0= no surfactant, Group 1= one dose, etc. The fractional synthesis rate (FSR), absolute production rate (ASR), and time of first appearance of enrichment were measured in all infants. The time of maximal enrichment and the half-life were measured in the number of infants indicated between brackets [n]. Per dose surfactant, the ASR increased with 1.3±0.4 mg PC/kg/d (p=0.007), the time of first enrichment increased with 4.6±1.6 per dose surfactant (p<0.01), the time of maximal enrichment and the half-life tended to increase (both p<0.1).

These findings are in agreement with increased precursor incorporation after incubation with surfactant components in isolated type II cells[8] and in primary cultures of type II cells.[25] In healthy adult rabbits *in vivo*, administration of surfactant to the left lung only resulted in increased incorporation of radiolabeled plasma palmitic acid in the left but not in the right lung.[9] In preterm ventilated lambs, surfactant treatment did promote [^{3}H]palmitic acid incorporation into surfactant PC, after correction for increased pool size due to exogenous surfactant.[10] A negative effect of exogenous surfactant has been reported in *in vitro* studies. These studies used surfactant preparations containing surfactant protein A (SP-A),[5-7] and Dobbs *et al.* showed a specific inhibition of surfactant secretion by SP-A.[6] In the present study Survanta® which does not contain SP-A was used. This could explain the discrepancy between our study and the studies showing inhibition of endogenous surfactant synthesis.

Other components of Survanta® could have a stimulating effect on endogenous synthesis, like phosphatidylglycerol (4% of phospholipids). Phosphatidylglycerol has been shown to stimulate surfactant synthesis *in vitro* by stimulating the rate limiting enzyme CDP-phosphocholine cytidylyltransferase.[25,26] By the administration of Survanta large amounts of palmitic acid are delivered to the lung, as a degradation product and as free palmitate (7% of total lipids). Palmitic acid has been shown to stimulate enzymes involved in surfactant synthesis and to increase the surfactant PC synthesis in rat lung explants.[27]

Table 3. Absolute synthesis rate of PC

Variable	Effect on ASR (mg PC/kg/d)	P-value
Exogenous surfactant	+1.3±0.4 per dose	0.007
Prenatal corticosteroids	+1.3±0.4 per dose	0.004
Gestational age	-0.4±0.2 per week	0.03
PDA	+2.1±0.7 yes *versus* no	0.01

Multiple regression analysis of the effects of parameters on absolute synthesis rate (ASR) of surfactant PC palmitate from glucose. Regression coefficients±standard error. PDA=patent ductus arteriosus.

Others, however, found no increased surfactant PC synthesis by palmitic acid *in vitro*.[25,28] The increased surfactant PC synthesis from glucose in the present study could be induced by the recycling of certain components of exogenous surfactant which could stimulate the surfactant metabolic pathway. An alternative explanation for increased surfactant synthesis could be that pulmonary improvement after surfactant therapy is accompanied by increased lung surfactant synthesis.

The time of first appearance and time of maximal enrichment were longer in the groups that received surfactant compared to Group 0. They were both longer probably due to dilution by exogenous unlabeled surfactant. The half-lives calculated tended to be shorter in Group 0, but the number of measurements were rather small. The half-lives are comparable to those as reported before.[15]

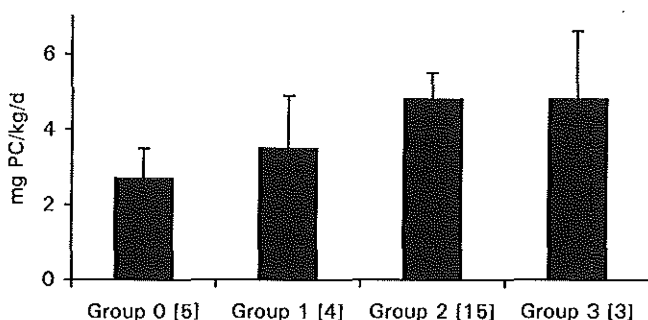

Figure 2. Absolute synthesis rate (ASR) of surfactant PC from plasma glucose according to the number of doses of surfactant. Infants in Group 0 received no exogenous surfactant, in Group 1 one dose, in Group 2 two doses, and in Group 3 three doses of surfactant. The number of subject between brackets [n]. Mean ± SEM

The ASR of surfactant PC was increased by prenatal corticosteroids with 1.3 mg/kg/d per dose corticosteroids (table 3). This implies that after two doses of corticosteroids, the ASR increased with 2.6 mg/kg/d which is three times higher than in preterm baboons who received two doses of prenatal corticosteroids.[22] In preterm infants with RDS the FSR increased with ~100% after two doses of prenatal betamethasone,[29] in that report, however, the ASR had not been calculated. Although the ASR increased in the present study, it is unlikely that this increase plays a major role in decreasing the incidence and severity of RDS at birth. Probably other effects of prenatal corticosteroids like stimulated structural development, induced antioxidant enzymes, and decreased vascular permeability play a more important role as some of these factors occur within 15 h after corticosteroid treatment in preterm lambs.[30]

When recovery from RDS occurs several days after birth, pulmonary vascular resistance falls, which can lead to the clinical appearance of a significant PDA. The use of exogenous surfactant could accelerate pulmonary improvement accompanied by earlier occurrence of PDA.[31] This could explain why surfactant synthesis increased in the presence of PDA. It could be that the PDA leading to pulmonary congestion and decreased pulmonary function stimulates surfactant synthesis as a compensation mechanism. A compensation mechanism in a situation of surfactant dysfunction could be suggested as meconium inhibits surfactant function *in vitro*[32] but stimulates surfactant secretion in isolated rat alveolar type II cells.[33] However, in preterm infants with RDS and with or without PDA, the percentage disaturated PC, lecithin/sphingomyelin ratio, percentage phosphoinositol and phosphatidylglycerol in tracheal aspirates were similar.[34]

We found that the surfactant synthesis rate decreases with advancing gestation (table 3). Although this decrease is small (0.4 mg/kg/d per week GA), it is in contrast to current belief

that surfactant synthesis increases with maturation. The gestational age of the infants studied was, however, maximally 32 weeks, while the concentration and lecithin / sphingomyelin ratio in amniotic fluid has been found to increase later than 32 weeks.[35] It could be that surfactant synthesis is relatively constant up to approximately 32 weeks, and increases afterwards. Other reasons for decreased ASR with gestation could be that smaller infants use relatively more glucose for PC-palmitate synthesis. The contribution of substrates used for surfactant phospholipid synthesis changes during development due to changes in substrate availability.[36] Fatty acids synthesized *de novo* from glucose account for 30 to 60% and 20 to 40% of total lipids synthesized by the type II cell in adult and newborn rats, respectively.[37-39] Another explanation could be that older infants have already produced larger surfactant PC pools before birth, resulting in more dilution of labeled surfactant during the first days of life. As intubation and ventilation were inclusion criteria, we included a selected group of older patients requiring ventilation. An alternative is therefore, that older infants had relatively more severe pulmonary pathology than the younger infants resulting in lower surfactant synthesis rates.

The ASR was calculated using estimated surfactant total pool sizes but the surfactant pool size in Group 0 could be smaller or larger. A larger pool size in Group 0 would increase the ASR and at a certain pool level the ASR would not be lower than in the surfactant treated groups. In order to study the strength of our conclusions, we calculated the maximal pool size of surfactant PC for Group 0 that would still show that exogenous surfactant increases endogenous production, this was a value of 57 mg/kg. The maximal pool size of surfactant PC for Group 0 associated with the finding that exogenous surfactant does not suppress endogenous production was ~250 mg/kg (Group 0 *versus* Groups 1, 2, and 3 together). These calculated surfactant PC pool sizes are probably not so high *in vivo* as total lung surfactant pool PC sizes are 40 to 50 mg/kg in preterm baboons,[23] preterm lambs,[40] and preterm rabbits.[41] Therefore, our conclusions hold even though the surfactant PC pool size in Group 0 could be higher.

In the surfactant treated infants, the ASR would be lower than calculated by us when exogenous surfactant would leave the lung resulting in smaller surfactant pool sizes. It is, however, not likely that the pool size decreased significantly after surfactant treatment. In 3-day-old rabbits, total lung surfactant pool sizes remained absolutely constant for 3 d after increasing the pool three- to five-fold by exogenous surfactant.[42] Seidner *et al.* found in preterm ventilated baboons which received 100 mg/kg Survanta at birth, that total lung Sat PC pool sizes were higher on d 6 of life than the sum of the endogenous pool and the amount administered.[23] This could suggests that in the preterm infant the total surfactant pool size remains constant or increases after surfactant treatment during the first days of life. Glatz *et al.* measured a loss of only ~25% of a treatment dose of exogenous surfactant in newborn lambs and rabbits in 48 h.[13] Others showed that a fixed percentage of multiple doses exogenous surfactant is lost from the lung in 3-day-old rabbits.[43] If the ASR is calculated in the present study under the premise that the total PC pool size decreased with 25% due to loss of exogenous surfactant, than the ASR increased linearly per dose surfactant (1.0 mg/kg/d per dose, $p<0.01$).

To study endogenous surfactant metabolism, we used the stable isotope [U-^{13}C]glucose as precursor. Other substrates available for the synthesis of surfactant PC palmitate include palmitic acid from plasma lipids, intracellular glycogen, lactate, and ketone bodies.[44] These substrates have been used as radioisotopes in animals and *in vitro*, and recently surfactant metabolism in older infants was studied using [U-^{13}C]palmitate.[45] Glucose is, however, an important substrate for the fatty acids of surfactant PC,[37,46,47] certainly when lipids are not available, and has been reported to account for 20 to 40% of the *de novo* synthesized fatty acids in the type II cell in newborn rats.[37-39] In order to achieve low levels of plasma lipids during the measurement of the synthesis of surfactant PC palmitate, the preterm infants

received i.v. lipid feeding 48 h after the start of the isotope infusion. However, the calculation of the total synthesis rates by us are underestimations as the contribution of other precursors to the surfactant phospholipids is unknown.

In summary, treatment of the preterm infant with RDS using exogenous surfactant as rescue therapy stimulates endogenous surfactant synthesis from the important precursor glucose. It is reassuring that the presence of a negative feedback mechanism of endogenous surfactant metabolism is unlikely. The data show that the absolute production of surfactant PC is moderately increased after prenatal corticosteroids. These data show that the combined use of prenatal steroids and postnatal surfactant have additive effects on endogenous surfactant metabolism. The presence of a PDA was correlated with increased endogenous surfactant synthesis, but it is unclear which mechanism could explain this finding.

ACKNOWLEDGMENTS

The authors express their sincere thanks to Wim van den Berg (Internal Medicine II, Erasmus University Rotterdam, The Netherlands) for support and technical advice, to Babette van Beusekom and Jack Tauladan (medical students, Erasmus University, Rotterdam, The Netherlands) for technical assistance, and to the nursing staff, the residents, and the neonatologists for their help and support with this study.

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CHAPTER 8

Changes in the Fatty Acid Composition Reflect the Metabolism of Surfactant Phosphatidylcholine in Human Preterm Infants

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Summary

Surfactant composition and turnover were studied in human preterm infants (<32 weeks gestation, n=28). Infants received either 0, 1, 2, or 3 doses of Survanta® (~85% palmitic acid, PA). During the period of intubation, tracheal aspirates were collected and the surfactant PC was analyzed by gas chromatography.

Exogenous surfactant increased the PA immediately to ~82%. Subsequent endogenous surfactant PC synthesis (~62% PA) diluted the alveolar surfactant. This dilution resulted in a decrease in PA percentage with a half-life of 18.2, 78.2, and 95.0 h after 1, 2, or 3 doses, respectively. Infants received intravenous lipids (~53% linoleic acid, LA). Linoleic acid (considered as a label as it is an essential fatty acid) became incorporated in surfactant PC after a delay of ~3 d. Without intravenous lipids there was no increase of LA in surfactant PC. Exogenous surfactant increased the alveolar surfactant PC concentration for 2 d after 1 dose of surfactant but for more than a week after 2 doses.

The slow dilution of exogenous surfactant by endogenous surfactant and the delayed incorporation of plasma lipids into endogenous PC suggest a slow synthesis of surfactant PC. The surfactant deficiency in premature infants with severe respiratory distress syndrome is reversed for more than a week with 2 doses of surfactant. The composition of intravenous feeding in the neonatal period influences surfactant composition and potentially function.

Submitted

INTRODUCTION

Respiratory distress syndrome (RDS) remains a major cause of morbidity and mortality in the premature infant, although surfactant substitution therapy has greatly improved outcome. Several issues regarding surfactant therapy remain unclear such as optimal dosing, timing, composition of surfactant, mode of administration, and effect on endogenous surfactant production.[1-4] Some infants do not respond or require retreatment with multiple doses.[5] These different clinical responses in individual patients might be related to differences between infants in the synthesis and clearance of surfactant, and might be important variables in the recovery from RDS. To better understand the pathophysiology of RDS and to optimise treatment a thorough understanding of surfactant metabolism is essential. Although some studies have focussed on surfactant metabolism, there is still very little known about surfactant synthesis and clearance in preterm infants. Surfactant is a mixture of lipids (~90%) and specific proteins (~10%). The main surface tension lowering component is dipalmitoylphosphatidylcholine (DPPC, containing 2 palmitic acids).

Surfactant synthesis and clearance have been described in animals using substrates labeled with radioactive isotopes[6,7] but this approach is not acceptable in preterm infants. Hallman *et al.* reported that the concentration of surfactant phosphatidylcholine (PC) in tracheal aspirates increases slowly over several days in infants with RDS and reaches values as found in infants without RDS after ~5 d.[8] Recently, a new method with stable isotopes for studying surfactant metabolism in humans has become available.[9] In the preterm infant, the synthesis rate of surfactant PC from glucose is very low.[9] Only few studies have assessed the effect of exogenous surfactant therapy in RDS on PC concentrations in premature infants. Treatment with 1 dose of human amniotic fluid surfactant containing surfactant protein A increased the PC and surfactant protein A concentrations for more than a week.[8] The surfactants used today, however, are derived from organic lipid extraction usually with specific lipids added, but lack surfactant protein A. The different composition could result in different clearance rates.[10] Ashton *et al.* found increases of the DPPC:palmitoyl-oleoyl-PC ratio (DPPC:POPC ratio) in tracheal aspirates after administration of artificial surfactant with a subsequent decrease over several days.[11] The half-lives of phosphatidylglycerol and sphingomyelin from the airways after tracheal instillation of surfactant have been estimated but the half-life of the PC has not been measured.[10,12]

We studied general surfactant PC metabolism in preterm infants. Infants received exogenous surfactant which was regarded as "labeled" because it contains 85% palmitic acid (PA) in PC while endogenous surfactant contains 62% PA in PC. The time to reach initial ("baseline") PA percentage in PC after exogenous surfactant administration is a reflection of endogenous surfactant PC synthesis. Infants received intravenous (i.v.) lipids containing 53% linoleic acid (LA). The LA was regarded as a label as it is an essential fatty acid which can not be synthesised by the human body. The delay between the start of i.v. LA administration and the incorporation of LA in surfactant PC was used as a measure of surfactant PC synthesis. Surfactant clearance was studied from the decrease in surfactant concentration in the epithelial lining fluid.

METHODS

Subjects

The studies were approved by the medical ethics committee of the Erasmus University Rotterdam/University Hospital Rotterdam. Patients were included if they were <32 weeks of gestation and intubated at birth for respiratory insufficiency, without congenital infection, and after written parental informed consent. RDS was defined clinically and radiographically, and

graded.[13] Patients received exogenous surfactant (Survanta®, Abbott Laboratories, North Chicago, IL, USA) endotracheally at a dose of 100 mg/kg phospholipids, if the mean airway pressure exceeded 7.5 cm of water, or if the inspiratory oxygen fraction was higher than 0.40. Infants received a second dose 6 h later if the criteria were still met. A third dose was administered within 20 h from the second dose if respiratory failure persisted. Infants were grouped according to the number of doses of exogenous surfactant received (Group 0 received no surfactant; Group 1 received one dose; Group 2, two doses; Group 3, three doses). For Group 0 the study started at 5 h after birth, and the administration of the first dose of surfactant (~5 h after birth) defined the start of the study for Group 1, 2, and 3. Survanta® contains a higher percentage of DPPC (~85% PA) than natural human surfactant. The fatty acid composition of PC in Survanta® was measured to be: C14:0:2.1±0.2 (myristic acid, MA); C16:0 (PA): 84.7±1.1; C16:1ω7:1.4±0.1; C16:1ω9:0.8±0.1; C18:0:3.0±0.0; C18:1ω7:0.4±0.1; C18:1ω9:6.24±0.9; 18:2ω6 (linoleic acid, LA):0.5±0.1 weight percent.

Tracheal aspirates were obtained before surfactant therapy and then every 6 h as long as the infant was intubated. No tracheal suctioning was done within 6 h after surfactant administration. The tracheal suctioning procedure was performed during routine patient care and did not deviate from the normal clinical protocol. Twenty seconds after 0.5 ml normal saline was instilled into the tracheal tube, suction was performed beyond the tip of the endotracheal tube, the suction catheter was then flushed with normal saline. Tracheal aspirates were immediately placed at -20°C.[14] As energy source, only glucose i.v. was administered initially, and 48 h after the start of the study, parenteral feeding was started. The parenteral feeding consisted of lipids (Intralipid® 20% Pharmacia & Upjohn, Woerden, The Netherlands, 2.9 g/kg/d containing ~53% LA, ~11% PA, and ~0.1% MA) and amino acids (Primène® 10% Clintec, Montargis, France, 2.4 g/kg/d). The first day of parenteral feeding half of these amounts were given.

Baboon studies

In our neonatology unit, all preterm infants receive total parenteral nutrition including lipids. Because we did not deviate from the normal clinical protocol for this study, we did not include a group of preterm infants without i.v. lipids. Instead, we used the very premature baboon as a model for RDS.[15] Preterm baboons (125±2 d) were delivered, intubated, and received 1 dose of Survanta® (100 mg/kg) immediately after birth. Blood was collected at t=0, t=0.75, t=1, and t=6 d. Tracheal aspirates were collected every 12 h until killing on d 6. The baboons did not receive any lipids during the study. The clinical data of the baboons have been described by Seidner *et al.*[15]

Table 1. Patients' characteristics

Group (n=)	Gestation (wk)	Birth weight (g)	RDS grade	Days ventilation
0 (5)	27.8±1.1	909±214	0.4±0.9*	16.7 (2-63)
1 (5)	29.0±2.9	1083±541	2.0±1.4	4.6 (2-12)
2 (14)	28.3±2.1	1098±352	3.2±0.7	11.3 (1-51)
3 (4)	28.1±2.1	1003±422	3.8±0.5	23.8 (8-38)

*Patients were attributed to a group based on the number doses of surfactant required. Mean±SD, day of ventilation: mean (range). *Patients without RDS were classified as grade 0.*

Analytical procedure

Tracheal aspirates. After thawing, normal saline was added up to a total volume of 2.6 ml, and from this sample urea was determined using a highly sensitive urease-based kit. The aspirate was vortexed and centrifuged at 450 x g for 10 min at 4°C, and the supernatant was recovered.[14,16] A known amount of internal standard (PC-$(C17)_2$) was added, and the lipids were extracted.[17] PC was isolated from the lipid fraction by thin layer chromatography (TLC) using Kieselgel 60 TLC plates (Merck Darmstadt, Germany) and a Camag Linomat IV (Merck Darmstadt, Germany). The solvent system contained chloroform, methanol, 2-propanol, KCl 25%, and triethylamine (10/3/8.3/2/6, v/v/v/v/v).[18] The PC was derivatized,[19] and the fatty methyl esters extracted with hexane and stored at -20°C. Tracheal aspirates containing visible blood were not analyzed (15 samples).

Blood samples. After collection the blood from the baboons was directly centrifuged to separate cells and plasma. The lipids from plasma were extracted according to Folch.[20] Triglycerides (TG) and phospholipids (PL) were isolated by TLC. The solvent system contained heptane, diisopropylether, acetic acid (60/40/3, v/v/v). The triglycerides and phospholipids were derivatized,[19] the fatty acid methyl esters were stored at -20°C.

Determination of composition and concentration

Fatty acid composition of surfactant PC, plasma TG and plasma PL, and amount of surfactant PC were analyzed by gas-chromatography (GC) (5890 series II, Hewlett-Packard, Amstelveen, The Netherlands)[19,21] with a variability of reinjection of the same sample of less than 2%. When one sample was diluted a 100 times, the maximal coefficient of variation was 4% between the undiluted and diluted sample. This variability is low and less than the biologic variability. The concentration of surfactant PC in the epithelial lining fluid (ELF) was calculated by correcting for dilution of the ELF during endotracheal suction: dilution factor = $[\text{urea}]_{\text{serum}}/[\text{urea}]_{\text{aspirate supernatant}}$.[8]

Data analysis

Data are presented as mean ± standard error of the mean (sem), unless stated otherwise. The urea was measured after addition of saline to the aspirate (see *analytical procedure*) and therefore could be very low and variable. The dilution factors were: Group 0: 98.9 ± 10.8 (12-653) mean ± sem (range); Group 1: 93.0 ± 7.8 (28-278); Group 2: 119 ± 8.8 (7-906); Group 3: 138 ± 12 (8-557). There was no correlation between the urea dilution factor and the PC concentration in ELF (data not shown), and therefore, the sampling procedure probably had no influence on the PC concentrations in the tracheal aspirates. The one-way-ANOVA test was applied to compare groups. A value of $p < 0.05$ (two tailed) was accepted as significant.

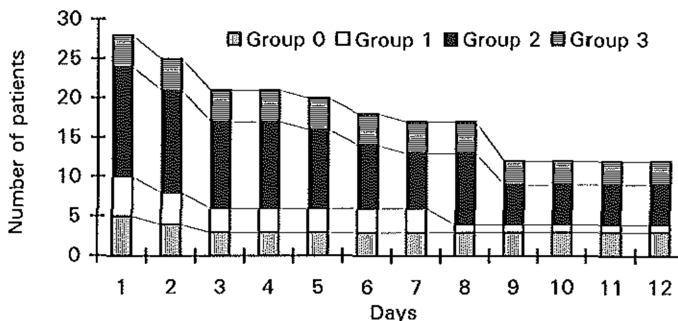

Figure 1. The number of patients included in the study per day. Day 1 starts at ~5 h after birth.

RESULTS

The patient characteristics are presented in the table and the number of patients intubated during the study in figure 1. From 28 patients 498 tracheal aspirates were analyzed. Five infants did not require surfactant (= Group 0) of whom 3 infants had mild respiratory insufficiency with apneas without pulmonary pathology on chest radiograph, and no cardiac disease, pulmonary infection, sepsis, or any other major disease. One infant in Group 0 had RDS grade 1 and one infant had grade 2. Five infants required 1 dose (Group 1), 14 infants required 2 doses (Group 2), and 4 infants required 3 doses of surfactant (Group 3). Gestational age and birth weight were similar in all groups (table). The severity of RDS was significantly less in Group 0 compared to Group 2 and 3 ($p<0.01$). The number of patients included per group on each day is shown in figure 1.

Group 0 had higher concentrations of surfactant PC in ELF at birth than Groups 1, 2, and 3 together (19.8 ± 5.1 *versus* 2.2 ± 0.6 mg/ml ELF, respectively, $p<0.00001$) (fig. 2). The PC concentration in Group 0 decreased significantly ($p<0.05$) on d 3 and 4, and increased afterwards. In Groups 1, 2, and 3 the surfactant PC concentrations were similar before surfactant administration. In Groups 1 and 2, treatment with surfactant increased the PC concentration to values as measured in Group 0. After the administration of one dose of surfactant, the PC concentration decreased already by d 2 to reach low values by d 3, while in group 2 the PC concentration did not decrease for more than 11 d (fig. 2).

In Group 3 the PC concentrations increased much higher than in Groups 0, 1, and 2 but returned to low levels (~4 mg/ml) by d 6 (see legends of figure 2). The effect of 4 times per d endotracheal suctioning has probably little effect on total surfactant pool size as we measured the total surfactant PC removed during 10 d with suctioning every 6 h to be only ~3 mg.

At birth, infants in Group 0 had a significantly higher percentage of palmitic acid (PA) in PC when compared to Groups 1, 2, and 3 combined (62.2 ± 1.1 *versus* 55.8 ± 1.5, $p=0.05$) (fig. 3). There was no difference between males and females in any group. The percentage of PA in PC in Group 0 remained constant from birth up to d 8 (fig. 3). In the groups receiving exogenous surfactant (~85% PA in PC), the percentage of PA in PC increased to values exceeding 82% (fig. 3). The percentage of PA in PC in the tracheal aspirates decreased due to endogenous surfactant synthesis with a lower percentage of PA and disappearance of exogenous surfactant. During the first 3 d after surfactant administration in Group 1, the PA percentage decreased to baseline with a half-life of ~18 h, but then increased again to a slightly higher constant level (fig. 3).

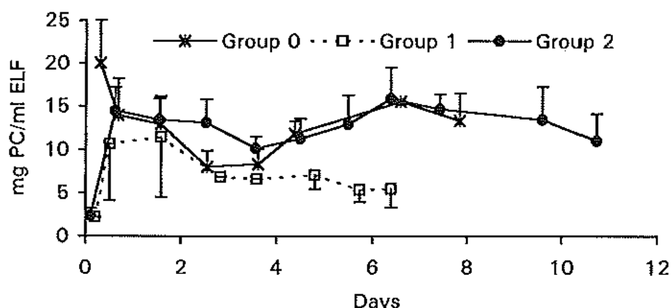

Figure 2. Surfactant phosphatidylcholine concentrations [PC] in epithelial lining fluid. The [PC] in Group 0 decreased significantly on d 2 to 4 ($p<0.05$), and the decrease in Group 2 on d 2 to 4 was not significant. Because the [PC] in Group 3 increased to very high values, the curve of Group 3 is not depicted for clarity of the figure. In Group 3, the [PC] before surfactant was 1.6 ± 0.9 mg/ml ELF, and increased after 3 doses surfactant to 41.0 ± 28.5 mg/ml on d 1.5, and decreased to 4.5 mg/ml on d 6 and remained constant afterwards. Results were pooled at 12 h intervals. When error bars are not visible, they are small and within the size of the data symbols.

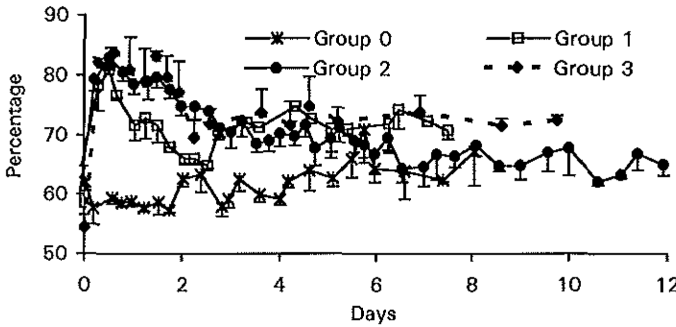

Figure 3. Percentage of palmitic acid (PA) in surfactant phosphatidylcholine (PC) from sequential tracheal aspirates. In Groups 1, 2, and 3 the percentage of PA in tracheal aspirates increased after exogenous surfactant (~85% PA) and decreased due to dilution by endogenous surfactant synthesis, and clearance of exogenous surfactant. In Group 0 the percentage PA was significantly higher than in Groups 1, 2, and 3 at t=0. The percentage PA was significantly higher in Groups 1, 2, and 3, than in Group 0 after exogenous surfactant until d 6. When error bars are not visible, they are small and within the size of the data symbols.

The decrease of the percentage of PA in PC in Group 2 had a half-life of 78.2 h. For the half-life calculation, the average percentage of Group 0 was considered as the new baseline (62% PA in surfactant PC) for Group 1 and 2. The PA percentage in Group 3 decreased to a baseline of ~70% PA in surfactant PC with a half-life of ~95 h.

The percentages of the main fatty acids (except PA) in PC in Group 2 are shown in figure 4. The LA (18:2ω6) percentage (and all other fatty acids) in surfactant PC decreased due to surfactant administration with a high percentage of PA and subsequently increased due to the disappearance of exogenous surfactant and the endogenous PC synthesis.

The increase of LA in PC could coincide with –and thereby obscure– specific incorporation of plasma LA into surfactant PC. As both LA and myristic acid (C14:0, MA) percentages in PC are influenced equally by exogenous surfactant, we revealed the specific the increase in LA by calculating the ratio of the percentages of LA to MA; the LA:MA ratio (fig. 5). We choose MA as the comparator for LA as both fatty acids are esterified at the sn-2 position of PC. Moreover, MA is very low in Intralipid® and is present in endogenous surfactant. Three days after the start of the i.v. feeding with lipids, the LA:MA ratio in PC in Group 0 and Group 2 started to increase above baseline value (fig. 5). The data for Group 1 and 3 are not shown, but the same trend was present in these groups as well. The LA:MA ratio of surfactant PC, and plasma TG and PL in the baboons which did not receive intravenous lipids remained constant (fig. 6).

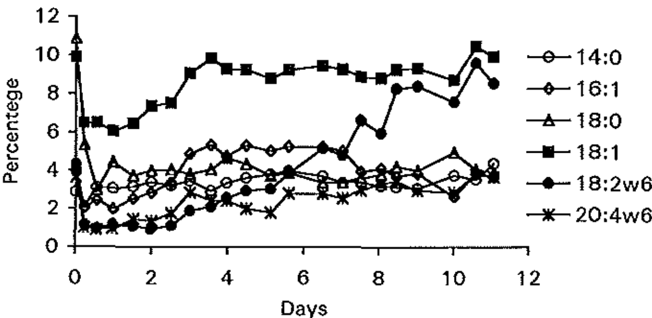

Figure 4. Percentage of the minor fatty acids in surfactant phosphatidylcholine (PC) in Group 2. After exogenous surfactant, the percentages of the fatty acids decreased due to the increase of the palmitic acid (PA) percentage. The percentages increased again due to the decrease of the PA percentage. Only 18:2ω6 increased above baseline. Results were pooled at 12 h interval.

Discussion

This study describes in critically ill preterm infants the concentration of surfactant PC and the percentages of the different fatty acids in PC after surfactant administration and i.v. lipids during the first 11 d of life. Changes in surfactant PC concentration and composition with time give information on surfactant synthesis and clearance. Our data show that the endogenous synthesis and secretion of surfactant is a slow process. This is shown by a slow dilution of exogenous surfactant by endogenous surfactant (fig. 3) and a slow incorporation of LA from plasma into surfactant PC (fig. 5).

This study suggests that two doses but not one dose of exogenous surfactant are sufficient to increase surfactant concentration for long periods. Furthermore, the data are in line with previous studies in which infants who develop RDS have biochemical evidence of lung immaturity.[8,12,22-24]

In the present study, infants that did not require surfactant therapy (Group 0) had ~8 times higher PC concentrations in the ELF at birth and a significantly higher PA percentage in PC as compared to infants that required surfactant therapy. Others have found 4[22] and 7 times[8] higher concentrations of PC in tracheal aspirates in infants without RDS than in infants with RDS at birth. After one and two doses of surfactant, the concentration of PC reached values as in Group 0. The decreased PC concentration of Group 0 on d 3 and 4 could reflect insufficient surfactant production. The following increased PC concentration could be explained by stimulation of synthesis by the introduction of intravenous feeding. The provided fatty acids could stimulate PC synthesis with LA incorporation into PC with subsequent acyl remodelling.[25,26] However, the PC concentration in the tracheal aspirate is only an indirect reflection of the synthesis. In Group 1, the PC concentration decreased by d 2 to reach low values by d 3. This is in concord with the observation that one dose of surfactant accomplishes a transient decrease in ventilatory requirement.[27] In Group 2, the concentration did not decrease at all after surfactant treatment for 11 d (fig. 1). This slow catabolism of exogenous surfactant in our study is in agreement with findings in animal studies. In 3-d-old rabbits that received a trace amount of labeled PC, the clearance was ~16%/d.[28] In term newborn sheep, after tracheal instillation of PC labeled with [^{3}H]PA, the half-life of PC was ~11 d.[29] In term newborn rabbits who received [^{3}H]PA i.v., alveolar [^{3}H]PC had a half-life of >2 to 5.7 d.[30,31] Others have found indications of a slow clearance of exogenous surfactant in human preterm infants. After treatment with one dose human amniotic fluid surfactant which contained SP-A, the PC concentration in tracheal aspirates did not decrease for 1 week.[8]

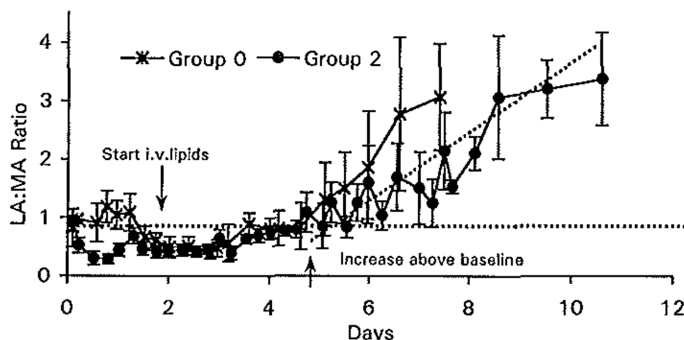

Figure 5. Ratio of linoleic acid (LA) to myristic acid (MA) in surfactant phosphatidylcholine PC. The horizontal dashed line indicates the baseline value of the LA:MA ratio. The oblique dashed line is the slope of the increasing ratio. On d 5 in both groups, the LA:MA ratio started to increase above baseline and indicates a specific incorporation from plasma LA into surfactant PC.

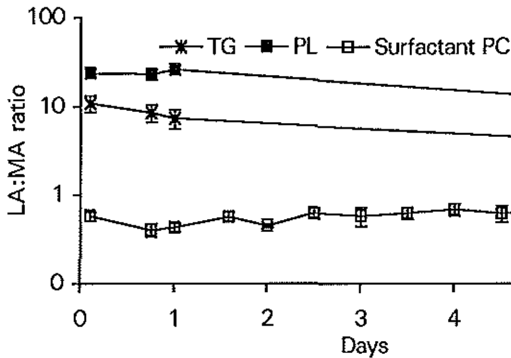

Figure 6. Ratio of linoleic acid (LA) to myristic acid (MA) in plasma triglycerides (TG), plasma phospholipids (PL), and in surfactant phosphatidylcholine (PC) in 10 preterm ventilated baboons. The LA:MA ratio did not increase in plasma TG and PL, and in surfactant PC because the baboons did not receive Intralipid®.

Treatment with two doses synthetic surfactant increased the ratio of DPPC:POPC for 4 d, illustrating slow clearance of exogenous DPPC.[11] In another study in preterm infants that received one or two doses Survanta®, Sat PC concentrations were increased up to ~4 d.[22]

The time necessary for plasma lipids to be taken up by the type II cell, incorporated into surfactant PC, intracellularly processed and secreted to the alveoli was estimated from the interval between initiation with i.v. lipids containing 53% LA and a specific increase of the LA percentage in surfactant PC. LA is an essential fatty acid and can not be synthesised *de novo* by the type II cell and the liver. Therefore, the LA was derived from exogenous sources and thus can be used as a label. This specific increase was expressed as a LA:MA ratio in order to minimize effects of surfactant therapy (see *Results*). In Group 0 and 2 the LA:MA ratio increased above baseline value 3 d after the start of the lipid infusion (fig. 5). This interval is long which could mean that surfactant synthesis and accumulation is slow, and plasma fatty acids are incorporated slowly in surfactant PC in preterm infants. In newborn rabbits and sheep there was a slow incorporation of radiolabeled plasma PA into PC and a maximal specific activity only after ~32 and ~42 h, respectively.[32,33] When term infants with a postnatal age of ~50 d received LA labeled with the stable isotope ^{13}C intravenously, the ^{13}C-label was first seen in LA in surfactant PC after ~10 h.[34] This is faster than the 3 d in the present study, probably because in older infants precursor incorporation and surfactant synthesis are enhanced, as is the case in animal studies.[32,33] Because we could not include a group of infants without Intralipid®, we used the preterm baboon that did not receive i.v. lipids as a model for the preterm infant without i.v. feeding. The fact that the LA:MA ratio in plasma TG and PL, and in surfactant PC did not increase (fig. 6) strongly suggests that the increased LA:MA ratio in the preterm infants is related to i.v. lipid administration. These data show that the composition of the feeding influences surfactant composition and potentially function.

The delay of specific incorporation of LA from parenteral lipids could also be related to uptake by the liver before delivery to the lungs as lipoproteins. Whether and to which extend liver metabolism plays a role in the delay of plasma LA incorporation into surfactant PC is unclear, and therefore the delays calculated by us are probably maximal values. It is, however, unlikely that a significant part of the delay of ~3 d is due to incorporation of fatty acids from Intralipid® into lipoproteins in the liver, as this is a much faster process and lipid metabolism in the premature is in extreme flux.[35]

At birth, the PA percentage in PC was significantly higher in Group 0 than in Groups 1, 2, and 3, illustrating a more mature surfactant composition in infants that did not require surfactant therapy. The PA percentage in PC increased significantly after surfactant therapy (~85% PA in Survanta® PC). An indication of endogenous surfactant synthesis and secretion can be

appreciated from the dilution of exogenous surfactant by endogenous surfactant with ~62% PA in PC. In the present study, the dilution is expressed as a half-life of percentage of PA towards a new baseline. The fact that the percentage in all groups remained higher than in Group 0 indicates significant recycling of exogenous surfactant and a slow *de novo* surfactant synthesis. The long half-lives of the PA percentage in surfactant PC Groups 2 and 3 are comparable with the half-life of ~113 h of PA in surfactant PC in preterm infants that received [U-^{13}C]glucose intravenously.[9] The half-life was longer in Group 3>2>1 (95, 78, and 18 h, respectively), probably because of the larger pool size of surfactant PC when more doses surfactant were administered. Our data are in agreement with studies in preterm lambs that received radiolabeled surfactant. The specific activity in these preterm lambs decreased slowly, indicating that dilution from unlabeled endogenous secretion was low.[36] Half-lives of other surfactant phospholipid components after exogenous surfactant have been measured in human preterm infants. Half-lives for phosphatidylglycerol were ~30 h with human amniotic fluid surfactant,[12] ~43 h with Alveofact®,[10] and ~4.4 d with Survanta®.[10] Half-life of sphingomyelin was calculated to be ~4 d.[10] The half-life of the major phospholipid PC has not been measured before.

Some animal studies suggest that the surfactant pool size remains stable because endogenous synthesis can compensate for surfactant clearance. Seidner *et al.* found in ventilated preterm baboons which received 100 mg/kg Survanta® at birth, that total lung surfactant PC pool sizes were higher at killing on d 6 of life than the sum of the endogenous pool and the amount administered.[15] In newborn rabbits that received surfactant treatment and were time-killed until d 3 of life, the total lung surfactant PC pool did not change for 3 d.[37] It is a limitation of the current study that we can not measure total surfactant pool size at multiple time points in these infants, and therefore can not calculate absolute values of surfactant synthesis and clearance.

We measured concentration and composition of surfactant in aspirates after deep endotracheal suctioning because it gave us the opportunity to do frequent sampling. Recently the surfactant phospholipids from tracheal aspirates and bronchoalveolar lavage were shown to have the same composition of the phospholipids and PC fatty acids.[38] We measured the different fatty acids in PC, but the measurement of the complete spectrum of individual molecular species of PC by HPLC as described by Postle *et al.*, can provide more detailed information about PC metabolism and the acyl remodelling of PC to DPPC.[25,26,39] Although Groups 1 and 3 are small, the data are in line with the conclusions from Group 0 and 2. Differences between the groups should, however, be regarded with some caution. Despite the mixed diagnoses in Group 0, the results apply to preterm infants that did not require exogenous surfactant at birth. Indeed, surfactant concentrations at birth were higher than in the other groups.

In conclusion, the composition of intravenous feeding in the neonatal period influences surfactant composition and possibly function.[40,41] The delayed incorporation of plasma lipids into endogenous surfactant PC and the slow dilution of exogenous surfactant by endogenous surfactant indicate that premature infants with RDS synthesise surfactant PC at a low rate. Surfactant deficiency is reversed adequately for more than one week by administration of 2 doses of surfactant.

ACKNOWLEDGEMENTS

The studies in baboons were performed by S. R. Seidner, Southwest Foundation for Biomedical Research, San Antonio, TX, in collaboration with M. Ikegami and A. H. Jobe Pulmonary Biology, Children's Hospital Medical Center, Ohio. All laboratory analysis were performed at the Erasmus University Rotterdam, The Netherlands. We thank the nursing staff, residents, and neonatologists for their help with the study.

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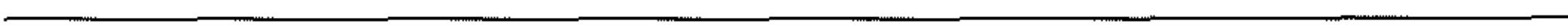
Chapter 9

General Discussion



INTERPRETATION AND IMPLICATIONS OF THE STUDIES

A major problem after premature delivery is the deficiency of adequate amounts of surfactant leading to respiratory distress syndrome (RDS). The surfactant synthetic pathway matures during gestation and alveolar surfactant concentrations in the alveoli start to increase after 20 to 24 weeks. The alveolar surfactant pool at birth in infants with RDS is 5 to10 mg PC/kg and increases afterwards.[1] From animal studies it is known that the synthesis of surfactant is slow and in humans the concentration of surfactant in the alveoli increases slowly after birth. In the studies performed in infants in the past, surfactant analysis of sequential tracheal aspirates was the only available method for the evaluation of surfactant production in humans.[2-4] Although this method provides information on quality and concentration of surfactant, it does not give information on endogenous surfactant synthesis and catabolism.

We developed a method employing the safe nonradiating stable isotopes to study surfactant metabolism (chapter 3). We measured in preterm infants with RDS that the endogenous labeling of surfactant PC palmitate from plasma glucose started after ~18 h and that the labeled surfactant disappeared from the alveoli with a half-life of >100 h.[5] This slow labeling reflects a low synthesis rate of endogenous surfactant (fractional synthesis rate, FSR, from glucose ~2.7%/kg/d). This finding is agreement with studies performed in animals using radioactive precursors and with studies on surfactant concentrations in preterm infants.[3,6-9] In older critically ill infants, the synthesis rate of surfactant PC was much higher when studied with the intravascular precursors [U-^{13}C]palmitic acid (~34%/kg/d) and [U-^{13}C]linoleic acid (~50%/kg/d) than in the study in premature infants with [U-^{13}C]glucose as precursor (chapter 4).[10] Thus it could be that free fatty acids are a more important precursor for the fatty acids of surfactant PC than *de novo* fatty acid synthesis from glucose. However, one has to consider that, when comparing the studies between the preterm infants and the older infants that the two studies were significantly different. Presence or absence of RDS, differences in the diet, the labeled precursor, the measured surfactant component, the age, the surfactant pool size have impact on study results. In piglets, it was recently shown that plasma free fatty acids are the primary source of the fatty acids surfactant PC as compared to *de novo* fatty acid synthesis from acetate.[11]

The observation that in preterm infants endogenous surfactant remains in the alveoli for long times (half-life ~100 h) is in contrast to studies in preterm infants with exogenous surfactant where half-lives are shorter (~36 h).[12] It could be that the use a plasma precursor ([U-^{13}C]glucose) to label endogenous surfactant prolongs the half-life of endogenous surfactant by ongoing synthesis (chapter 3). This is, however, not certain as we also found in critically ill infants that the half-life of endogenous labeled surfactant (with plasma [U-^{13}C]palmitic and linoleic acid) was only ~40 h (chapter 4).[10] Furthermore, Griese *et al.* found similar half-lives of surfactant components (phosphatidylglycerol and sphyngomyelin) of ~100 h.[4] It would, therefore, be interesting to study in the same individual the disappearance of an exogenous label and an endogenous label simultaneously.

Prenatal corticosteroids are nowadays routinely used to reduce the risk of RDS.[13] It is currently unclear whether the beneficial effects of prenatal corticosteroids are related to the surfactant system. *In vitro* studies show increased activities of the enzymes involved in surfactant synthesis. In some animal studies increased labeled precursor incorporation and increased surfactant pool sizes have been observed after prenatal corticosteroids. However, many studies in animal studies show no effect on surfactant pool size. In preterm infants, prenatal corticosteroids did not increase surfactant concentration in the epithelial lining fluid.[14] In a randomized study in baboons (chapter 5) and in a nonrandomized study in preterm infants (chapter 6) prenatal corticosteroids increased the surfactant PC synthesis from plasma glucose (FSR of PC doubles after 2 doses). An increased labeling of palmitic acid in surfactant PC after prenatal corticosteroids could in theory result from increased ^{13}C-

enrichment of plasma lipids from synthesis by the liver after corticosteroids with subsequent uptake by lung. We found no difference in labeling of plasma triglycerides and phospholipids between treated and control groups in the preterm infants. Thus the increased enrichment of surfactant PC palmitate after prenatal steroids was probably due to increased endogenous synthesis of surfactant PC, and is not a reflection of plasma lipid enrichment. In the free fatty acid fraction, the enrichment was so low that the fatty acids could not be a precursor for the higher enriched surfactant PC fatty acids. This suggestion is in contrast to the findings of Martini *et al.* in piglets who showed that free fatty acids are more important than *de novo* fatty acid synthesis.[11] Probably, in the premature infant with little fatty acid supply during the first three days after birth as in our studies, *de novo* fatty acid synthesis in the type II cell is more important than lipid uptake from plasma. When fatty acids are available as in the older children (chapter 4) and in the piglets,[11] lipid uptake may become more important.

This increased synthesis from glucose after prenatal corticosteroids is rather small and will probably lead to small increases in surfactant pool size. In addition to improved lung development after prenatal corticosteroids as shown by others,[15,16] the increased surfactant synthesis could play a role in the prevention of RDS. A smaller increase in surfactant pool size is necessary to prevent the development of RDS than to treat RDS. The study in preterm infants was not randomized as randomization in this patient population is not possible anymore as prenatal corticosteroids are routine treatment.

Exogenous surfactant administered at birth for RDS is distributed homogeneously throughout the lung, mixes with the endogenous surfactant pool, and reduces surface tension directly. The amounts of exogenous surfactant administered are large compared to the endogenous surfactant pool (10 to 20 times). As many feedback mechanisms operate in the human to regulate metabolism, others studied the influence of exogenous surfactant on endogenous surfactant synthesis *in vitro* and in animals. *In vitro* studies mainly described inhibition of precursor incorporation after surfactant administration. In rabbits the precursor incorporation was increased after surfactant administration.[17] Such studies have not been performed in humans. We found that exogenous surfactant stimulated the endogenous surfactant synthesis significantly, which is in agreement with the studies in rabbits (chapter 7). Although the increases found by us appear to be low (~1.3 mg/kg/d PC from glucose), it is reassuring that endogenous synthesis is not suppressed and that the presence of a negative feedback mechanism is unlikely.

LIMITATIONS OF THE STUDIES

The studies in the infants on the effects of prenatal corticosteroids and exogenous surfactant were not randomized as it is ethically not possible to randomize these patients for prenatal corticostertoids and exogenous surfactant. Furthermore, this kind of experiments is very expensive and intensive; it is difficult to study large series. Correction for some variables was performed by using strict inclusion criteria and multiple regression analysis.

We used tracheal aspirates to collect alveolar surfactant PC. In most animal studies, bronchoalveolar lavage is used, but this method cannot be used for frequent sampling in the studied population. All tracheal aspirates were handled similarly in all studies. Therefore, any inaccuracy would have occurred in all patient groups probably to the same extent. The tracheal aspirates were centrifuged (10 min at 450 x g) at a greater speed than in most studies (normally 10 min at 150 x g). This high speed was chosen to pellet any degraded cells due to freezing and thawing after collection of the aspirate. Loss of surfactant by this procedure does not influence the measurement of ^{13}C-enrichment in surfactant PC palmitate. The ^{13}C-enrichment is measured by mass spectrometry; by direct measurement of the $^{13}C/^{12}C$ ratio in

palmitic acid of surfactant PC. This ratio is not influenced by sampling size or loss during preparation.

We concentrated on the palmitic acid metabolism of surfactant PC. The PC molecule comprises different fatty acids, choline, glycerol and phosphate. *In vitro* studies showed a different metabolism for the different components which could be the case in our studies as well. We found for example different synthesis rates (FSR) of surfactant PC from plasma palmitic acid and plasma linoleic acid. This should be considered with the interpretation of the results. It is, however, unknown to which extent the metabolism of the different components of PC differ in infants. For proper functioning of surfactant, other phospholipids and surfactant proteins are necessary as well. The metabolisms of the components are probably different and therefore, the metabolism of PC palmitate does not necessarily represent overall surfactant metabolism. There is probably no overall surfactant metabolism.

We measured the incorporation of the ^{13}C molecules derived from labeled plasma glucose and palmitic acid. The palmitic acid in the type II cell is also synthesized from other substrates like glycogen, intracellular palmitate, ketone bodies, glycerol, and lactate. The synthesis measured by us is therefore not the total surfactant PC synthesis but an underestimation. In fact, calculation of the fractional synthesis rate is only possible from the enrichment of the direct precursor. The direct precursor in the type II cell for palmitic acid synthesis is acetyl-CoA. The enrichment of acetylCoA cannot be measured, and therefore the calculations were performed from plasma precursors enrichments in all groups in the same way.

When labeled surfactant PC is newly synthesized, it is diluted by the unlabeled surfactant pool. The ^{13}C-enrichment of PC in tracheal aspirates is thus dependent on the size of the total surfactant PC pool size. The comparison of labeling of surfactant is thus only correct when total surfactant PC pool sizes are similar. We corrected for differences in pool size by multiple regression analysis for doses of surfactant (chapter 6) and by multiplying the FSR with the amount of surfactant administered (chapter 7).

It is not entirely clear how to interpret the calculated half-life of ^{13}C-enriched surfactant PC. Synthesis of unlabeled surfactant after stopping isotope infusion decreases the enrichment of alveolar surfactant. Loss of labeled surfactant from the lung decreases the labeled surfactant pool size which leads to more rapid dilution of the label by unlabeled endogenous surfactant. In the patients that received exogenous surfactant, the decrease of enrichment is also related to the decrease in pool size after unlabeled surfactant administration. As all processes occur simultaneously, a short half-life could have two explanations: a rapid loss of exogenous surfactant, the negative scenario, or a rapid synthesis of newly unlabeled surfactant, the positive scenario.

Studies using labeled components to study surfactant metabolism can give very different results, which can be related to the labeled substrate used, the route of administration, the surfactant pool studied (e.g. alveolar or lung tissue), and the developmental stage of the subject. These different experimental conditions make direct comparisons between studies difficult and should always be considered carefully.

Directions for future research

The study of the metabolism of the specific surfactant proteins would be an enormous contribution to the overall understanding of surfactant metabolism. The proteins are essential for normal lung function in terms of alveolar gas exchange, but also for the natural defence against inhaled micro-organisms. Studies require significant amounts of labeled exogenous aminoacids, for example leucine.

The metabolism of PC has been studied by us using labeled glucose and fatty acids. The data give valuable information but methodological problems remain. The use of a more direct

precursor could be used to reflect more directly surfactant metabolism. An example could be the use of labeled choline, as this component is not synthesized in large amounts by the body and is merely derived from feeding. Therefore, after equilibration with alveolar and lung tissue, the plasma enrichment of choline could be comparable with enrichment in the type II cell.

As choline is an essential substance for PC synthesis, and parenteral feeding of the preterm infants does not contain choline, there could possibly be a relative choline deficiency, hampering surfactant synthesis in the critical period of RDS. The effect of choline supplementation on surfactant synthesis in the preterm infant would be interesting to study.

It would be interesting to study the relative contribution of *de novo* fatty acid synthesis of palmitic acid in PC from glucose or the contribution from plasma palmitic acid by infusion of labeled glucose and palmitic acid simultaneously in one individual.

It is not entirely clear how to interpret the calculated half-life of surfactant PC. The decrease of enrichment is related to newly synthesised unlabeled PC, and the catabolism and clearance from the lung. Synthesis of unlabeled surfactant after stopping isotope infusion decreases the enrichment of alveolar surfactant. Loss of labeled surfactant from the lung decreases the labeled surfactant pool size, which leads to more rapid dilution of the label by unlabeled endogenous surfactant. To study this, it would be necessary to administer an intravenous labeled precursor and a labeled exogenous surfactant in the same patient.

Dexamethasone administered in the immediate postnatal period to preterm infants with RDS reduces the severity of lung disease and the incidence of bronchopulmonary dysplasia.[18] It is not known whether this beneficial effect is related to the stimulation of lung maturation including surfactant synthesis. It would be interesting to study whether the development of BPD and the beneficial effects of postnatal dexamethasone are related to surfactant metabolism.

There are indications of a disturbed surfactant metabolism in several other neonatal lung diseases for which currently exogenous surfactant is not routinely used. Such diseases include congenital surfactant protein B deficiency,[19-22] congenital diaphragmatic hernia,[23] meconium aspiration syndrome,[24-28] sepsis with pneumonia or ARDS, severe respiratory insufficiency requiring extra corporal membrane oxygenation.[29,30] It would be a challenge to study surfactant metabolism in these diseases in infants with the use of the methods that have been discussed in the current thesis.

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Chapter 10

Summary

Samenvatting

Chapter 1 introduces the function, composition, and metabolism of pulmonary surfactant and some questions regarding surfactant metabolism.

The studies in this thesis were performed to:
1. develop and use a novel method to study surfactant metabolism in preterm and older infants.
2. study endogenous surfactant synthesis in relation to prenatal glucocorticosteroids.
3. study the influence of surfactant therapy on endogenous surfactant metabolism.
4. study surfactant composition and concentration after surfactant therapy.

Chapter 2 reviews the literature on surfactant metabolism with emphasis on preterm infants. Primary surfactant deficiency is a major cause of respiratory distress syndrome (RDS) in the preterm infant. Surfactant is a mixture of lipids (90%) and specific proteins (10%). Phosphatidylcholine (PC) accounts for ~70% of the surfactant lipids. Surfactant is synthesized and stored as lamellar bodies in the alveolar type II pneumocyte. Surfactant is secreted to the alveolar space and prevents alveolar collapse by reducing the surface tension at the air-liquid interface. Surfactant is cleared from the alveoli mainly by reuptake by the type II cell and is recycled to a large extend, or can be cleared from the lungs. During fetal lung development the synthetic pathways of surfactant phospholipids mature leading to increased surfactant pool sizes and improved composition. The endogenous *de novo* synthesis and secretion of surfactant PC seem to be slow processes. Therefore, the lungs of the premature infant seem not to be able to adapt rapidly to extra-uterine life which requires a considerable alveolar surfactant pool and therefore exogenous surfactant administration is necessary to rapidly augment surfactant pools in RDS. After surfactant administration the concentration of alveolar surfactant remains elevated for many days. Some *in vitro* and animal studies suggest the presence of a negative feedback mechanism of exogenous surfactant to regulate endogenous surfactant synthesis. However, data from preterm infants show that multiple doses of exogenous surfactant do not suppress endogenous surfactant synthesis.

Corticosteroids increase the activity of the enzymes for surfactant synthesis *in vitro*. Data on surfactant pool sizes after corticosteroids from animals studies are conflicting. In preterm infants surfactant synthesis is stimulated by prenatal corticosteroids, but synthesis rates are low in general, which allows alveolar pool sizes to increase only several days after stimulation by corticosteroids.

Chapter 3 describes a study in preterm infants with respiratory insufficiency who received at birth a 24-h infusion with the stable isotope [U-^{13}C]glucose as a precursor for palmitic acid of surfactant PC. During the entire period of intubation, tracheal aspirates were collected to obtain serial time points to measure the incorporation of the stable isotope ^{13}C into PC palmitate. PC palmitate became enriched after ~19 h and reached maximum enrichment ~70 h after the start of the isotope infusion. The fractional synthesis rate (FSR) was calculated to be ~2.7%/d, which is the percentage of the total surfactant PC pool synthesized from glucose per day. The labeled PC-palmitate disappeared with a half-life of ~113 h.

In conclusion, these data show that endogenous surfactant synthesis and clearance are slow processes. This method is used further in next chapters to study surfactant metabolism.

Chapter 4 describes endogenous surfactant metabolism in infants requiring mechanical ventilation for a variety of diseases. Surfactant deficiency has been implicated in the pathophysiology of adult respiratory distress syndrome. We used the intravenous tracers [U-^{13}C]palmitic acid and [U-^{13}C]linoleic acid. Infants received a 24-h constant infusion with both tracers simultaneously. Surfactant PC became labeled with ^{13}C-palmitic acid and ^{13}C-linoleic acid after ~10 h. The time of maximal enrichment was ~47 h for both tracers. The FSR of surfactant PC

from palmitic acid ranged from 0.4 to 3.4%/h and the FSR from linoleic acid ranged from 0.5 to 3.8%/h. The half-lives of palmitate-labeled PC ranged from 17 to 178 h and the half-lives from linoleic acid labeled PC ranged from 24 to 144 h.

There was a large variability in metabolic parameters of surfactant between patients probably reflecting the diversity in diseases of the subjects studied.

Chapter 5 describes surfactant metabolism in very premature baboons and reports on the effects of prenatal corticosteroids on surfactant synthesis. Pregnant baboons were randomized to receive either betamethasone (beta) or placebo (control). After preterm delivery at 67% of term gestation, baboons were intubated and received surfactant for RDS. [U-^{13}C]glucose was used as a tracer to study surfactant PC metabolism. Palmitic acid in surfactant PC became enriched after ~27 h and was maximally enriched at ~100 h. The fractional synthesis rate of PC-palmitate in the beta group (~1.5%/d) was increased by 129% above control (~0.7%/ d). The total lung pool sizes of PC were not increased in the beta group as compared to the control group on day 6. We hypothesize that factors after birth like ventilation stimulate surfactant synthesis and overpower the effects of prenatal steroids.

These data show that the synthesis of endogenous surfactant PC from plasma glucose is a slow process and that prenatal corticosteroids stimulate the synthesis of surfactant PC in the very premature baboon.

Chapter 6 shows the effect of prenatal corticosteroid treatment on endogenous surfactant synthesis in preterm infants. Pregnant women at risk of preterm delivery had received either zero ($n=11$), one ($n=4$), or two doses ($n=12$) of prenatal betamethasone (12 mg intramuscularly). After birth, 27 infants received [U-^{13}C]glucose as a precursor for palmitate in surfactant PC, plasma phospholipids, and triglycerides. The FSR of surfactant PC from glucose was ~1.7%/d without prenatal corticosteroid treatment, ~2.9%/d with one dose and ~5.8%/d after two doses of prenatal corticosteroids. The PC concentration in the epithelial lining fluid tended to be higher in the corticosteroid-treated groups. The enrichment of palmitic acid of plasma triglycerides and phospholipids from glucose was not increased by corticosteroids.

In conclusion, the increased enrichment of surfactant PC palmitate is not a reflection of increased liver lipogenesis. These data show that treatment with prenatal corticosteroids stimulates surfactant synthesis in the preterm infant. However, the increased synthesis is probably too slow to increase the surfactant pool size rapidly.

Chapter 7 deals with the impact of exogenous surfactant on endogenous surfactant synthesis in preterm infants. Some *in vitro* and animal studies suggest that exogenous surfactant regulates endogenous surfactant synthesis by a feedback mechanism. Preterm ventilated infants received a 24-h infusion with the stable isotope [U-^{13}C]glucose starting ~5 h after birth. The ^{13}C-incorporation into palmitic acid of surfactant phosphatidylcholine (PC) isolated from serial tracheal aspirates was measured. Infants received either zero ($n=5$), one ($n=4$), two ($n=15$), or three ($n=3$) doses of Survanta® (100 mg/kg) when clinically indicated. The absolute synthesis rate (ASR) of surfactant PC from plasma glucose increased with ~1.4 mg PC/kg/dose of Survanta® ($p=0.007$). Using multiple regression analysis prenatal corticosteroid treatment increased the ASR from plasma glucose with ~1.4 mg PC/kg/dose ($p=0.01$). The presence of a patent ductus arteriosus was associated with an increase of the ASR from plasma glucose with ~2.1 mg PC/kg ($p<0.05$). The ASR decreased with ~0.4 mg PC/kg/week advancing gestational age ($p=0.03$).

These data are reassuring and show that in preterm infants, that multiple doses of exogenous surfactant for RDS do not down regulate but even stimulate endogenous surfactant synthesis.

Chapter 8 presents a study on the concentration and composition of surfactant obtained from serial tracheal aspirates from preterm infants and preterm baboons both with respiratory distress. Infants requiring surfactant (one, two, or three doses) had significantly lower surfactant PC concentrations than infants that did not require surfactant (2.2±0.6 *versus* 19.8±5.1 mg/ml ELF, respectively, $p<0.00001$). The percentage PA in PC increased after exogenous surfactant (containing ~85% palmitic acid in PC), and then decreased with a half-life of 18, 78, and 95 h after one, two, and three doses of surfactant, respectively. This decrease in palmitic acid percentage in tracheal aspirates was due to dilution by endogenous surfactant containing a lower percentage of palmitic acid. Three days after the start of parenteral feeding containing ~53% linoleic acid, there was a specific increase in linoleic acid percentage in surfactant PC in the preterm infants. Preterm baboons that did not receive any lipids were used as control group for the infants who all received intravenous lipids. In the baboons, the linoleic acid percentage did not increase in the plasma triglycerides and phospholipids, nor in surfactant PC.

It is concluded that the composition of parenteral feeding influences surfactant PC composition and possibly function. The delayed incorporation of plasma lipids into endogenous surfactant PC and the slow dilution of exogenous surfactant by endogenous surfactant suggest a slow synthesis of surfactant PC.

Samenvatting

Hoofdstuk 1, Introduktie. Surfactant wordt in de longen gemaakt en uitgescheiden naar de longblaasjes (=alveoli) die met lucht zijn gevuld. Het surfactant in de longblaasjes is nodig om de longblaasjes te ontplooien bij de inademing en open te houden bij de uitademing. Surfactant kan deze functie vervullen doordat het de oppervlaktespanning tussen het water en de lucht in de longblaasjes verlaagt. Bij te weinig surfactant in de longblaasjes zullen de longblaasjes samenvallen en daardoor de opname van zuurstof en de afgifte van kooldioxide ernstig belemmeren. Tekort aan werkzaam surfactant direkt bij de geboorte is de belangrijkste reden van het Respiratoir Distress Syndroom (RDS), ook wel hyaliene membraan ziekte (HMZ) genoemd, bij te vroeg geboren kinderen.

Het onderzoek beschreven in dit proefschrift heeft de volgende vraagstellingen:
1. kan een methode ontwikkeld worden om de produktie en afbraak (metabolisme) van surfactant te bestuderen bij te vroeg geboren en oudere kinderen.
2. hebben corticosteroïden die voor de geboorte van het kind (= prenataal) werden toegediend invloed op de produktie van surfactant na de geboorte.
3. wordt de eigen (= endogene) produktie van surfactant beïnvloed als aan het kind surfactant via de beademingsbuis direkt in de longen wordt toegediend.
4. hoe verlopen de concentratie en samenstelling van surfactant in de longblaasjes nadat surfactant aan het kind is toegediend. Zegt dit iets over het metabolisme van surfactant.

Hoofstuk 2 is een overzicht van de literatuur over het metabolisme van surfactant met name bij te vroeg geboren kinderen. Met de aanmaak, afbraak en hergebruik wordt het metabolisme van surfactant bedoeld. Surfactant bestaat voornamelijk uit vetten (90%), voornamelijk (±70%) fosfatidylcholine (FC) en eiwitten (5 tot 10%). Het wordt gemaakt en opgeslagen als lamellaire structuren, in de type II cellen in de longen. Nadat het surfactant is uitgescheiden naar de longblaasjes verspreid het zich als een zeer dunne laag over het totale longoppervlak.

Na een aantal ademhalingscycli wordt surfactant afgebroken en grotendeels door de type II cell weer opgenomen en hergebruikt; een klein deel wordt afgevoerd.

Tijdens de foetale ontwikkeling van het kind ontwikkelt het enzymsysteem dat betrokken is bij de aanmaak van surfactant, waardoor de hoeveelheid toeneemt en de samenstelling van het surfactant in de longblaasjes verbetert. Daardoor zijn de longen van het voldragen kind bij de geboorte goed geschikt voor lucht-ademhaling. De aanmaak van surfactant fosfatidylcholine en de uitscheiding naar de longblaasjes zijn waarschijnlijk langzame processen. De longen van het te vroeg geboren kind lijken daardoor niet in staat te zijn zich snel aan te passen aan het leven buiten de baarmoeder; want voor gasuitwisseling is een voldoende hoeveelheid surfactant noodzakelijk. Daarom is het bij sommige te vroeg geboren kinderen nodig na de geboorte surfactant aan de longen toe te dienen (exogeen surfactant). Nadat exogeen surfactant is toegediend, blijft de concentratie in de longblaasjes verhoogd gedurende meerdere dagen. In enkele studies met geïsoleerde type II cellen en dieren wordt de eigen aanmaak van surfactant geremd door exogeen surfactant toe te dienen. In een studie bij te vroeg geboren kinderen lijkt er geen negatief terugkoppelingsmechanisme te bestaan.

In geïsoleerde type II cellen en weefselkweken blijkt dat glucocorticosteroïden de aktiviteit van enzymen die nodig zijn voor de aanmaak van surfactant verhogen. In studies met dieren blijkt echter niet altijd dat de hoeveelheid surfactant ook toeneemt na behandeling met glucocorticosteroïden. In te vroeg geboren kinderen lijkt echter wel de aanmaak van surfactant gestimuleerd te worden. Echter, de aanmaak blijft toch een langzaam proces, waardoor het enkele dagen duurt voordat de hoeveelheid surfactant significant is toegenomen.

Hoofdstuk 3 beschrijft een studie in te vroeg geboren kinderen met hyaline membraan ziekte. Het metabolisme van surfactant werd bestudeerd door stabiele isotopen in de vorm van een 24-uurs infuus met [U-^{13}C]glucose toe te dienen en vervolgens de inbouw van het ^{13}C-isotoop in surfactant te vervolgen. Het ^{13}C isotoop in de glucose wordt door de longen gebuikt om de vetzuren van surfactant fosfatidylcholine te maken. Als dit ^{13}C wordt aangetoond in surfactant is het surfactant "verrijkt". Eenvoudig gesteld geldt dat hoe meer ^{13}C in surfactant FC gemeten wordt, des te hoger de aanmaak van surfactant FC is.

Surfactant kan uit slijm vanuit de longblaasjes worden geïsoleerd. Dit slijm wordt routinematig bij alle beademde kinderen om de paar uur uit de beademingsbuis gezogen om deze doorganklijk te houden. Het materiaal verkregen door uitzuigen wordt een trachea-aspiraat genoemd. Bij de kinderen in de studies van dit proefschrift werden niet meer trachea-aspiraten afgenomen dan voor de normale patiëntezorg nodig was. De snelheid van inbouw en verdwijning van ^{13}C is een maat voor de surfactant-aanmaak en verdwijning van surfactant. Het infuus met [U-^{13}C]glucose werd ongeveer 5 uur na de geboorte gestart en na 24 uur gestopt. Zolang het kind beademd werd, kon slijm uit de longen worden verkregen waaruit surfactant fosfatidylcholine werd geïsoleerd. Het belangrijkste vetzuur in fosfatidylcholine, palmitinezuur (C16:0), werd verrijkt met ^{13}C ongeveer 19 uur na start van het infuus.

De verrijking was maximaal na ongeveer 70 uur. De fractionele synthese snelheid was ongeveer 2,7% per dag. Dit betekent dat 2,7% van de totale hoeveelheid surfactant fosfatidylcholine per dag uit glucose wordt aangemaakt. Het surfactant fosfatidylcholine verdween uit de longblaasjes met een half-waardetijd van ongeveer 113 uur.

Dit betekent dat endogeen surfactant langzaam wordt aangemaakt en langzaam uit de longblaasjes verdwijnt. Deze nieuwe en veilige methode is gebruikt om het metabolisme van surfactant te bestuderen zoals beschreven in de volgend hoofdstukken.

Hoofdstuk 4. Tekort aan surfactant is niet alleen beschreven bij te vroeg geboren kinderen, maar ook bij bij oudere kinderen met respiratoir falen. Daarom werd in deze groep kinderen het metabolisme van surfactant bestudeerd. De tracers [U-^{13}C]palmitinezuur en [U-^{13}C]linolzuur werden tegelijkertijd via een infuus gegeven gedurende 24 uur tegelijkertijd. Deze

vetzuren kunnen door de long direkt gebruikt worden voor de opbouw van surfactant fosfatidylcholine; daardoor wordt het fosfatidylcholine verrijkt met ^{13}C. Na toediening van isotoop was het surfactant fosfatidylcholine verrijkt na ongeveer 10 uur onafhankelijk van de gemeten tracer (palmitinezuur of linolzuur). Het tijdstip van maximale verrijking was voor beide tracers gelijk en was ongeveer 47 uur. De fractionele synthese snelheid varieerde van 0,4 tot 3,4% per uur vanuit palmitinezuur en 0,5 tot 3,8% per uur vanuit linolzuur. De half-waardetijd varieerde voor palmitinezuur-verrijkt surfactant van 17 tot 178 uur en voor linolzuur-verrijkt surfactant van 24 tot 144 uur.

Conclusie: de variabiliteit van het metabolisme van surfactant is aanzienlijk en hangt mogelijk samen met het ziektebeeld.

Hoofdstuk 5 beschrijft het metabolisme van surfactant in te vroeg geboren bavianen en de invloed van corticosteroïden op de aanmaak van surfactant. Corticosteroïden voor de geboorte (= prenataal) werken als stress wat de aanmaak van surfactant zou kunnen stimuleren. Zwangere bavianen kregen, gerandomiseerd, of corticosteroïden tijdens de zwangerschap of fysiologisch zout (placebo). Bij deze bavianen werd bij 67% van de normale zwangerschapsduur een keizersnede verricht waardoor te vroeg geboren bavianen verkregen werden voor deze studie. De bavianen werden beademd en kregen surfactant-therapie. [U-^{13}C]glucose werd via een infuus gedurende 24 uur toegediend als bouwstof voor vetten in surfactant fosfatidylcholine. Surfactant fosfatidylcholine werd verrijkt na ongeveer 27 uur en was maximaal verrijkt na ongeveer 100 uur. De factionele synthese snelheid van fosfatidylcholine in de placebo-groep was 0,7% per dag en in de groep met corticosteroïden 1,5% per dag. De totale hoeveelheid surfactant in de longen was na zes dagen even groot in beide groepen.

Wij hypothetiseren dat de totale hoeveelheid surfactant FC op dag 6 niet vergroot was na corticosteroïden omdat andere stress-faktoren in beide groepen na de geboorte zoals beademing het stimulerende effekt van corticosteroïden teniet doen. Deze data tonen aan dat het metabolisme van surfactant in de te vroeg geboren baviaan langzaam is en dat de aanmaak van surfactant gestimuleerd wordt door toediening van corticosteroïden voor de geboorte.

Hoofdstuk 6 beschrijft het effekt van prenatale corticosteroïden op de aanmaak van surfactant in te vroeg geboren kinderen. Kinderen kregen voor de geboorte via de moeder 0 keer (n=11), 1 keer (n=4) of 2 keer (n=12) prenataal corticosteroïden (12 mg betamethasone intramusculair aan moeder toegdiend). Na de geboorte kreeg ieder kind [U-^{13}C]glucose intraveneus als bouwstof voor vetzuren in surfactant fosfatidylcholine. De [U-^{13}C]glucose kan ook worden gebruikt door de lever voor het maken van fosfolipiden en triglyceriden die vervolgens in het bloed circuleren.

Het surfactant FC was meer verrijkt met ^{13}C na corticosteroïden. De fractionele synthesesnelheid van surfactant fosfatidylcholine uit glucose was 1,7; 2,9 en 5,8% per dag na 0, 1 en 2 doses prenatale corticosteroïden, respectievelijk. Er was een trend van verhoogde concentratie van surfactant in de longblaasjes na corticosteroïden.

De [U-^{13}C]glucose in het bloed wordt ook door de lever gebruikt om plasma-fosfolipiden en -triglyceriden van te maken. Het zou kunnen dat de lever door corticosteroïden meer plasmafosfolipiden en -triglyceriden lipiden gaat maken en dat dus meer ^{13}C-verrijkte lipiden circuleren. Deze lipiden zouden door de long kunnen worden opgenomen uit het bloed en worden gebruikt voor de aanmaak van surfactant. Op die manier zou een toegenomen verrijking van surfactant na corticosteroïden veroorzaakt kunnen worden door toegenomen omzetting van glucose in lipiden door de lever met daarna opname door de long met omzetting in surfactant, zonder toename van aanmaak van surfactant. De verrijking van triglyceriden en fosfolipiden in bloed was echter gelijk in de drie groepen. Dit geeft aan dat de toegenomen verrijking in sur-

factant na corticosteroïden niet het gevolg is van toegenomen aanmaak van lipiden in de lever, maar van toegenomen aanmaak van surfactant in de long.

De conclusie is dat behandeling met corticosteroïden voor de geboorte de eigen aanmaak van surfactant FC stimuleert. Echter, de toename van aanmaak is waarschijnlijk klein, waardoor een significante toename van de hoeveelheid surfactant enkele dagen duurt. Prenatale corticosteroïden hebben echter toch een positieve invloed op de ziekte en overleving van het kind als ze binnen 24 uur voor de geboorte worden toegediend. Waarschijnlijk is het positieve effekt van prenatale corticosteroïden meer een gevolg van toegenomen structurele ontwikkeling van de long dan een toegenomen hoeveelheid surfactant.

Hoofdstuk 7 beschrijft het effekt van toediening van surfactant (exogeen) op de eigen aanmaak (endogeen) van surfactant. In enkele studies met geïsoleerde type II cellen en dieren wordt de eigen aanmaak van surfactant geremd door exogeen surfactant toe te dienen, en wordt de aanmaak via een negatief feedback-mechanisme gereguleerd. Te vroeg geboren kinderen kregen een 24-uurs infuus met [U-^{13}C]glucose. De ^{13}C inbouw in palmitinezuur van surfactant fosfatidylcholine werd gemeten als maat van endogene aanmaak van surfactant fosfatidylcholine. Beademde kinderen kregen geen surfactant, 1 dosis, 2 doses of 3 doses surfactant omdat dit op klinische gond geïndiceerd was. De absolute produktiesnelheid van surfactant fosfatidylcholine vanuit plasma glucose steeg met ongeveer 1,4 mg/kg per dosis surfactant ($p=0.01$). De absolute synthesesnelheid steeg met ongeveer 1,4 mk/kg per dosis prenatale corticosteroïden.

Bij kinderen is voor de geboorte een bloedvat (ductus Botalli) aanwezig tussen de slagader naar de longen (longslagader) en de grote lichaamsslagader (aorta). De ductus Botalli moet na de geboorte sluiten om problemen te voorkomen. In deze studie bleek dat als de ductus Botalli niet sloot binnen enkele dagen na de geboorte en klinische problemen gaf, de aanmaak van surfactant hoger was. Deze bevinding was niet verwacht en kunnen wij niet verklaren. De aanmaak van surfactant nam af met ongeveer 0,4 mg/kg per week met het toenemen van de zwangerschapsleeftijd.

De conclusie is dat de eigen aanmaak van surfactant niet wordt onderdrukt door behandeling met surfactant, en juist enigszins wordt gestimuleerd.

Hoofdstuk 8 beschrijft de vetzuursamenstelling en de concentratie van surfactant fosfatidylcholine bij te vroeg geboren kinderen en bavianen met respiratoir distress syndroom. Kinderen kregen 0 tot 3 doses exogeen surfactant, de bavianen kregen 1 dosis. Bij kinderen die surfactant nodig hadden, werden significant lagere surfactant-concentraties gevonden direct na de geboorte dan bij kinderen die geen surfactant nodig hadden ($2{,}2 \pm 0{,}6$ en $19{,}8 \pm 5{,}1$, repectievelijk, $p<0{,}00001$).

Het percentage palmitinezuur in surfactant fosfatidylcholine in het trachea-aspiraat steeg nadat exogeen surfactant (met ongeveer 85% palmitinezuur) was toegediend. Na deze steiging daalde het percentage palmitinezuur met een half-waarde tijd van 18, 78 en 95 uur na 1, 2 of 3 doses surfactant, respectievelijk. Deze daling werd veroorzaakt door verdunning van het exogene surfactant door endogeen surfactant met een lager percentage palmitinezuur.

Drie dagen na de start met intraveneuze vetten met ongeveer 52% linolzuur, werd een specifieke steiging in het percentage linolzuur in surfactant gevonden. Bij de bavianen die geen intraveneuze vetten kregen bleef het percentage linozuur in het bloed constant. Daarom werd geen toename van linolzuur in surfactant bij de bavianen gevonden.

De conclusie is dat intraveneuze voeding invloed heeft op de samenstelling van surfactant en daardoor ook mogelijk de werking beïnvloed. De vertraagde inbouw van linolzuur uit bloed in surfactant en de trage verdunning van het surfactant in de longen door eigen aanmaak toont aan dat de endogene aanmaak van surfactant fosfatidylcholine een langzaam proces is.

List of Publications

Papers

Bunt JEH, Zimmermann LJI, Wattimena JLD, Beek RHT van, Sauer PJJ, Carnielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotopes. *Am. J. Respir. Crit. Care Med.* 1998; 157 (3): 810-814

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ABSTRACTS

Bunt JEH, Gavilanes AWD, Reulen J, Blanco CE, Vles JSH.The influence of hypoxemia on neuronal activity continuously registrated by the cerebral function monitor (CFM) in newborn piglets. *Pediatr. Res. 1995; 36: 43A.*

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Dankwoord

Mijn grootste dank gaat uit naar de ouders van de deelnemende kinderen die belangeloos hebben meegewerkt. Gelukkig zagen zij in dat niet-therapeutisch onderzoek bij wilsonbekwamen van belang is.

De verpleging van de neonatale intensive care heeft alle trachea-aspiraten verzameld. Ik dank jullie hartelijk voor de hulp en ondersteuning van dit onderzoek. Het is belangrijk dat onderzoek wordt gedaan bij deze kleine patiënten en zonder jullie is dat niet mogelijk.

I would like to thank the Sophia Foundation for Medical Research for making this project financially possible.

Lieve pap en mam, Harry en Annette, zonder jullie liefde, stimulering, troost en pakken slaag zou ik dit proefschrift nooit geschreven hebben. Zonder jullie zou ik nooit de kracht hebben gehad mijn studies te voltooien en tevreden te zijn met wie ik ben. Ik hou van jullie.

Dr. Luc Zimmermann, beste Luc, heel hartelijk bedankt voor de afgelopen jaren. Ik wil je bedanken voor de gedreven en serieuze discussies die we hebben gevoerd. Onze passie voor dit onderzoek en flexibiliteit pasten goed bij elkaar. Wij zaten regelmatig tot diep in de nacht en vroege ochtend de resultaten te beoordelen. We vonden de data mooi, maar wat betekenen ze? Uren achter de computer, moe naar huis, maar wel weer een abstract geschreven. Bedankt voor het plezier dat we hebben gehad, hier in Rotterdam en tijdens congresbezoek waar we altijd een hotelkamer deelden en soms zelfs een bed. Soms met wat te veel biertjes in een kroeg kletsen over het onderzoek en het leven, het hoorde erbij. Je Nederlands is er op vooruit gegaan! Bedankt voor je persoonlijke begeleiding.

Dr. Virgilio Carnielli, beste Virgilio ik dank je voor de kritische vragen en ondersteuning in het begin in Rotterdam, later vanuit Italië. Jouw creativiteit gevoed door kennis is van groot belang geweest voor dit project. Je harde werken is altijd een voorbeeld maar ook schrikbeeld geweest. Ik wens je veel geluk in Engeland en hoop dat Paola spoedig bij je zal zijn.

Prof. dr. P. J. J. Sauer, beste Pieter ik dank je voor het vertrouwen dat je in mij had en mij deze AIO-positie aanbood. Na Virgilio vertrok ook jij helaas uit Rotterdam. Ondanks de afstand Rotterdam-Groningen, bleef je continue steun en aandacht belangrijk. Je kennis over stabiele isotopen was in het begin erg belangrijk en geruststellend; tenminste iemand die het begreep. Ik begrijp het nu ook. Bedankt.

Alan H. Jobe, MD, PhD, dear Alan, it has been a honour for me that I got the chance to work with you. The ease with which you interpreted my data is exceptional; your critiques invaluable. I enjoyed very much the visit to your lab in Cincinnati and the warm welcome at your place. I would like to thank you for coming to Rotterdam to participate in the discussion of my thesis.

Dear Machiko Ikegami, MD, PhD, and Steven Seidner, PhD, I would like to thank you for giving me the opportunity to participate in the baboons studies. It was a pleasure to work with you.

Prof. dr. H. A. Büller en Prof. dr. B. Lachman wil ik hartelijk bedanken voor de bereidheid in de kleine commissie zitting te nemen, de beoordeling van het manuscript en deel te nemen aan de discussie van mijn thesis. Prof. dr. H. A. Büller dank ik voor het optreden als secretaris.
Ik wil graag de professoren H. P. Haagsman (Utrecht) en S. Bambang Oetomo (Groningen) bedanken voor hun komst naar Rotterdam en hun bijdrage aan de oppositie. Prof. dr. J. N. van den Anker dank ik voor de bereidheid mijn thesis te bediscussiëren.

Een groot deel van de afgelopen jaren heb ik doorgebracht in het wetenschappelijk laboratorium Kindergeneeskunde. Ik wil graag Darcos Wattimena, Janine den Ouden, Ad de Bruijn, Ingrid Luijendijk, Annemiek de Raad en Paul Adam bedanken voor hun bijdrage aan het onderzoek. Darcos, jou kennis van de apparatuur was van uiterst belang voor het welslagen van ons onderzoek. Bedankt voor de gezelligheid, vooral op tijden dat mensen normaliter niet werken. Luc en ik hebben vaak gebeden dat je niet onder de tram zou komen.

Janine, van jou heb de Bligh en Dyer, dunne laag chromatografie en derivatisering geleerd. Ik heb er jaren *plezier* van gehad. Beste Ad we zullen doorgaan...Wim van den berg van Interne Geneeskunde II bedank ik voor de oprechte interesse in mijn onderzoek en de steun van de afgelopen jaren. Dr. Peter Hermans wil ik bedanken voor zijn steun aan dit onderzoek.

Het Algemeen Klinisch Chemisch Lab (voorheen CKCL) wil ik bedanken voor de snelle bepaling van de ureumconcentratie in de trachea-aspiraten.

Ik bedank het befaamde "Surfactant Laboratorium in Utrecht" onder leiding van Prof. dr L. M. G. van Golde voor de grote gastvrijheid. Martin van Eyk, analist, we hebben nogal wat proeven gedaan en je hebt me veel geleerd. Ik vindt het erg jammer dat het niet gelukt is de ^{13}C-verrijking in de surfactant-eiwitten te meten en studies in mijn proefschrift op te nemen. Er wordt nog steeds aan gewerkt. Henk, Koos, Edwin, Bianca, Martin en Rob, bedankt voor de gastvrijheid en gezelligheid, ik was graag in jullie lab. Gaan we nog eens stappen? Edwin veel succes met je promotie, twee dagen voor die van mij. Doe je je haar in vlechtjes, de 14^{e}?

Dr. W. C. Hop, bedankt voor het feit dat je altijd op korte termijn een gaatje had (al snel meer dan een uur) om data te analyseren.

De afgelopen jaren hebben vijf medisch studenten hun afstudeer project onder mijn supervisie voltooid. Ik wil jullie bedanken voor het harde werken, vooral in het laboratorium. Ik vond het erg leuk om jullie in het lab verschillende analyses te leren. Jullie hebben mij veel werk uit handen genomen en veel gezelligheid gebracht. Dimitrie Bouwens en Ilaicha Ramnath, jullie zijn allebei getrouwd tijdens jullie stage. Zijn er al kids? Jack Tauladan, met jouw heb ik veel lol gehad, wil je nog lab komen doen, een lekker Bligh en Dyertje kan er altijd tussendoor! Simone ter Horst, de blonde uit Leiden die gewoon twee studies doet. Hoe was je buitenlandse stage? Babeeete van Beusekom, al aan de man? Ik hoop dat ik jullie binnenkort zie.

Als onderzoeker ben je soms eenzaam op pad. Het is dan ook extra belangrijk andere gelijkgestemden te ontmoeten, op het werk, in de kroeg, thuis, tijdens het AIO weekend of in Parijs met de Ropa Run. Ik wil jullie graag hier noemen om jullie te bedanken voor de goede gesprekken en/of het bier c.q. whisky. Bovenal mijn kamergenoten Dr. Anton Hulsman, Dr. Svati Patandin en Hestien Vreugdenhil. Dan ladies first: Margriet van Stuijvenberg, Ester de Kleijn, Nicolette Arends (Nico), Hettie Janssens, Saskia de Wildt, Marja Suur, Inez Bronsveld, Dr. Annemie Boehmer, Annemarie van Rossum, Inge van der Sluis, Ingrid Slobbe, Yvonne van Pareren, Dr. Hanneke Meijers-IJsselstijn, Hannerieke van der Hout, Karin Overweg, Rianne Oostenbrink, Dr. Manou Batstra, Maartje ten Berge. De heren: Laurens Koopman, Boris Koerts, Dr. Theo Sas, Dick Mul, Dr. Albert Wolkerstorfer, Marcel Sluijter, Aart Verwest, Dr. René Kornelisse

Mijn liefste collega en opvolgster Daphne Janssen. Lieve Daphne, ik ben je dankbaar voor de steun het vertrouwen die je me gaf. "Je kan het!" zei je vaak. Ik ben je dankbaar. 1999 was niet eenvoudig, maar wel spannend!. Twee dagen na de promotie vliegen we naar Guatamala. We moeten nog zoveel samen doen. Let the happiness in.

CURRICULUM VITAE

Jan Erik H. Bunt was born in Utrecht on June 29, 1968, from a Danish mother and Dutch father. He has one sister (Astrid, 1970, "paranymph" on june 16) and two brothers (Jasper and Marten, 1976, see cover of thesis). He completed secondary school (Athenaeum, Augustinianum Scholengemeenschap, Eindhoven) in 1987. He attended Medical School at the University Maastricht from 1987 until December 1994. While in Medical School, he took part in research and clinical care involving foot problems in patients with diabetes mellitus at the Academic Hospital Maastricht (with Nicolaas C. Schaper, MD, PhD, Dept. Medicine). He performed studies on cerebral function in preterm infants and newborn piglets at the Academic Hospital in Maastricht (with Carlos E. Blanco, MD, PhD and Danilo WD Gavilanes, MD, Dept. Pediatrics and with Hans. S. H. Vles, MD, PhD, Dept. Neurology). As a clinical student he visited for three months the Department of Pediatrics and Neonatology at the Rigshospital København in Denmark (heads: Henrik Hertz, MD, PhD, and Gorm Greisen, MD. PhD). After receiving his medical degree in December 1994, he worked for four years as research-fellow (Assistent In Opleiding, AIO). Under the supervision of Luc J. I. Zimmerman, MD, PhD, Virgilio P. Carnielli, MD, PhD, and Pieter J. J. Sauer, MD PhD, this research project lead to the current thesis. From April 1999, he worked as a resident in pediatrics not in training (Assistent Niet In Opleiding, AGNIO) at the Sophia Children's Hospital/University Hospital Rotterdam (heads: Hans A. Büller, MD, PhD, and Herman J. Neijens, MD, PhD). In this hospital, Jan Erik started his clinical pediatric residency in training (Assistent In Opleiding, AGIO) in January 2000. At present he works at the Sint Franciscus Gasthuis, Rotterdam (head: Ralph Spritzer, MD, PhD) to continue his clinical pediatric residency in training.