THE REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE IN FETAL TYPE II CELLS

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THE REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE IN FETAL TYPE II CELLS

De regulatie van CTP:phosphocholine cytidylyltransferase in foetale type II cellen

PROEFSCHRIFT

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Terwijl elk creatief denken door een belang van de denkende gestimuleerd wordt, worden gedachten nooit door belangen verminkt en verdraaid, maar alleen door belangen die onverenigbaar zijn met de waarheid als ontdekking van het wezenlijke in het bestudeerde object (E. Fromm: De zelfstandige mens)

En jij die rechtvaardigheid wilt verstaan, hoe zul je dit kunnen, tenzij je alle daden bekijkt in het volle licht (Kahlil Gibran: De profeet)

Maar wie handelt naar de waarheid, komt tot het licht, opdat het van zijn werken moge blijken, dat ze in God zijn verricht (Johannes Evangelie 3.21)

Voor Moniek,

Dries, Wim en Hanne

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Chapter 9 Summary and perspectives

List of Abbreviations

CTP:phosphocholine cytidylyltransferase	CT
phosphatidylcholine	PC
disaturated phosphatidylcholine	DSPC
dipalmitoylphosphatidylcholine	DPPC
oleic acid	OA
minimal essential medium	MEM
fibroblast-conditioned cortisol-containing medium	FCM
fibroblast-pneumocyte factor	FPF
respiratory distress syndrome	RDS
thyrothropin-releasing hormone	TRH
surfactant protein A	SP-A
surfactant protein B	SP-B
surfactant protein C	SP-C
congenital diaphragmatic hernia	CDH
severe left-sided (or bilateral) CDH, induced by maternal feeding of 100 mg Nitrofen on day 10 of gestation	d10-CDH
small right-sided CDH induced by maternal feeding of 60 mg Nitrofen on day 12 of gestation	d12-CDH

CHAPTER 1

INTRODUCTION

1.1 Clinical background

Respiratory distress syndrome (RDS), also known as hyaline membrane disease, is an important cause of neonatal and infant mortality (1). Together with congenital malformations, it is the leading cause of death in preterm infants (2) and is responsible for serious morbidity in survivors, associated with high costs to society (3).

Since 1959 it is known that RDS is caused by lung immaturity with concomitant surfactant deficiency (4). Numerous small studies and large multicenter trials have demonstrated decreased death rates and complications from RDS as a result of treatment with surfactant (5,6). Despite its success however, surfactant therapy is not a panacea. Recent meta-analyses from the available data do not show a consistent decrease in long term pulmonary complications such as bronchopulmonary dysplasia, nor in the major non-pulmonary complications such as intraventricular haemorrhage (5,6).

Postnatal surfactant treatment is clearly not a substitute for attempts to increase fetal lung maturation (6). Antepartum corticosteroid therapy is more effective than postnatal surfactant therapy in reducing mortality and complications from RDS (7). Currently, other ways to increase lung maturation are being studied. Clinical studies with maternal administration of a combination of thyrotropin-releasing hormone (TRH) and corticosteroids suggested a slight advantage over corticosteroids alone in the prevention of bronchopulmonary dysplasia (8,9), but a recent multicenter trial found a worse outcome after prenatal corticosteroids + TRH compared to corticosteroids alone (10). Thus, major problems remain. In the recent surfactant trials, only about 15-20% of the babies at risk of developing RDS could be given

antenatal steroids (11). Maternal administration of steroids is not without complications (12), can impair lung growth (13), and can cause long term adverse effects, potentially into adulthood, which are only recently being recognized (14). TRH, via an increase in thyroid hormones, seems to have some theoretical disadvantages in that it inhibits maturation of anti-oxidant enzymes at least in the rat (15,16), does not stimulate (or even inhibits) surfactant protein production (17-20), and inhibits fatty acid synthesis (21-23).

At the moment, the benefits of prenatal corticosteroids outweigh the disadvantages, but insufficient reduction in bronchopulmonary dysplasia is achieved in the entire group of very preterm infants, presently seen in neonatal intensive care units. Enhancing lung maturation seems more effective than surfactant therapy in reducing RDS and its complications, but faster, safer, more efficient, and more specific ways to increase lung maturation and surfactant production are clearly needed.

Therefore, it is necessary to understand more about the mechanisms of morphological and biochemical lung maturation and their regulation. More specific, a better understanding of the regulation of surfactant synthesis during lung development is needed.

1.2 Fetal lung development

It is now generally recognized that lung development can be subdivided into five stages (reviewed in 24). (1) In the *embryonic period* (3-6 weeks in human, 10-16 days in rat) the lung originates from a diverticulum of the ventral wall of the primitive gut and soon divides into two bronchial

buds. This endodermally derived epithelium later differentiates into both the respiratory epithelium lining the airways and the specialized epithelium that lines the alveoli. The lung bud grows into a mass of mesodermal cells from which blood vessels, smooth muscle, cartilage, and other connective tissues differentiate. Ectoderm contributes to the innervation of the lung. The two lung buds develop lobar buds which subsequently undergo progressive dichotomous branching. As in other organs, mutual interactions between epithelium and mesenchyme are essential for the sequential events of organogenesis. Mesenchyme has been demonstrated to play a determining role in the formation of the characteristic branching morphology. (2) In the *pseudoglandular period* (6-16 weeks in human, 16-19 days in rat) 16 to 25 generations of presumptive airways result from the repeated dichotomous branching. These ducts are surrounded by abundant mesenchyme, and in cross section the tissue resembles glandular tissue. The ducts end in terminal sacs, the presumptive alveolar ducts, which are lined by a columnar epithelium. (3) In the *canalicular period* (16-28 weeks in human, 19-20 days in rat) the functionally important respiratory or gas-exchanging portion of the lung becomes delineated. This period is characterized by the differentiation of the alveolar epithelium, a decrease in the relative amount of connective tissue in the lung and an increase in the number of blood vessels, with capillaries coming into closer contact with the primitive alveoli. Also, the first appearance of differentiated type II pneumocytes, the producers of surfactant, is noted. All these changes make that babies who are born towards the end of this period are potentially viable. However, the relatively small surface area for gas exchange, the still thick intersaccular septa and the high cuboidal epithelium may pose significant problems for gas exchange, especially if surfactant production is deficient and alveolar collapse occurs.

(4) In the *terminal saccular period* of lung development (28-36 weeks in human, 21-22 days in rat) the lungs are further prepared for air breathing after birth: the respiratory portion of the lung further differentiates. Respiratory bronchioles rapidly subdivide into an array of thin walled primitive alveolar ducts and primitive alveoli, which are lined by type II and flat type I pneumocytes in close contact with a rapidly proliferating capillary network. (5) In the *alveolar period* (in the human starting before term birth and continuing after birth, postnatal in rats) true alveoli are formed by indentations of the septal wall. The thinning of the walls continues and the amount of connective tissue decreases further. The number of alveoli increases up to eight years of life.

It is evident that the fetal lung morphogenesis, as described in the 5 phases, involves major structural changes which are associated with both *cell proliferation (growth)* and *cell differentiation*. *Lung growth* is regulated by physical factors and hormones. The effect of these factors may be mediated by intercellular interactions, extracellular matrix components and growth factors. The *cytodifferentiation* of the different lung cells is essential for adequate lung function. The regulation of the differentiation of the type II pneumocytes is especially interesting as they are the producers of pulmonary surfactant.

It can be concluded that, although the lung has no major functions before birth, its almost complete morphological and biochemical development and maturation before birth is crucial for survival immediately after birth. From the sequence of normal lung development, it is also easy to understand that very premature infants (<28 weeks and especially <26 weeks) with lungs in the canalicular stage of development, frequently have suboptimal gas exchange, even in the absence of the typical hyaline membrane disease.

1.3 Alveolar type II cells

During embryonic lung morphogenesis the walls of the lung primordium are lined with undifferentiated columnar epithelial cells, which later differentiate into prospective bronchial epithelium and prospective alveolar epithelium. The acinar tubules during the late pseudoglandular and early canalicular stages of lung development are lined with cuboidal epithelium. At this stage of development, these cells do not contain lamellar bodies, characteristic of mature type II cells. Nevertheless, they do express phenotypic features and possess antigenic determinants of mature type II cells (25-28). They are frequently called protodifferentiated type II cells or pre-type II cells. During the canalicular period of lung development, the rapid proliferation slows down and the pre-type II cells start to mature further. The most striking morphological feature is the decrease in glycogen content and at the same time the increase in number and size of lamellar bodies (29). These lamellar bodies are the intracellular storage pools of pulmonary surfactant.

It is now firmly established that the alveolar type II cells are the producers of surfactant (reviewed in 30). Several experimental models have been used to study surfactant synthesis such as whole animal studies in vivo, isolated perfused lung and lung slices in vitro, lung explants in culture and finally isolated type II cells in primary culture. Each model has certain advantages and disadvantages. For physiologic studies the intact animal is usually best suited. In vitro models however, overcome the problem of the possible indirect effect mediated via other organs on the lung. The lung consists of more than 40 cell types and only a small percentage of these are

type II cells. Thus, isolated type II cell studies are the only model to overcome this problem of cell heterogeneity, and offer many advantages over other models in the study of surfactant metabolism (13,30,31). In the studies described in this thesis, we elected to use primary cultures of fetal type II cells or pre-type II cells, because the aim of our studies was to investigate the regulation of an important regulatory enzyme in surfactant production within the cell. However, we have to realize that isolated type II cells in primary culture cannot be considered a physiologic model and that the data from such studies cannot be extrapolated directly to the whole animal and especially not to the human situation without further validation. Another problem with isolated type II cells is that they lack the interaction with other cells, which has been shown to be important in the regulation of surfactant synthesis by hormones, growth factors or other stimuli. Glucocorticoids have been shown to stimulate the production of fibroblast-pneumocyte-factor (FPF) in fibroblasts which in turn stimulates surfactant phospholipid production in type II pneumocytes. Hormones such as sex hormones and insulin affect surfactant production by means of an effect on FPF in fibroblasts.

We conclude that the choice of primary cultures of fetal type II cells to study the (intracellular) regulation of an important enzyme in surfactant production is a logical one. However, it is necessary to keep a good perspective of the value of these findings for the in vivo situation.

1.4 The composition and function of surfactant

The main function of surfactant is to decrease the surface tension at the air-liquid interface of the alveoli in a manner that depends on alveolar

surface area (32). The reduction of surface tension at this very extensive alveolar surface promotes lung expansion on inspiration and prevents lung collapse on expiration. Thus, surfactant plays a major role in the pressure-volume characteristics of the lung and in the gas exchange. The importance of surfactant is best illustrated by the respiratory distress syndrome where an inadequate amount and dysfunction of surfactant leads to an almost general alveolar collapse while the intratracheal administration of exogenous surfactant leads to a dramatic improvement in lung expansion and gas exchange.

It is generally assumed that two surfactant pools exist in the lung. One is the intracellular pool, which consists of the lamellar bodies, the characteristic organelles of type II cells. Lamellar bodies are secreted by type II cells into the second pool, the alveolar pool. In the alveolus, surfactant exists in many different morphological forms which are converted into each other (reviews 30,33). Lamellar bodies unravel to form tubular myelin which most likely is a direct precursor of the surfactant monolayer, the functional form of surfactant. "Used surfactant" then leaves the monolayer as small vesicular structures which are taken up again by the type II cells. A large part of this material can be re-used by the type II cell for secretion. Thus, surfactant recycling is at least as important as de novo synthesis of surfactant in the adult lung. In the newborn lung, recycling is quantitatively even much more important (about 15 times) than de novo synthesis (33).

The composition of surfactant is somewhat variable depending on the animal species. In general, it consists of about 90% lipid, 5-10% protein and small amounts of carbohydrate. Four specific surfactant-associated proteins have been described. Surfactant protein A (SP-A) is the most abundant surfactant-associated protein and makes up 3-4% of the surfactant mass. It is

a large collagen-like glycoprotein which is synthesized in type II cells but also in Clara cells of the respiratory bronchioles. The synthesis of SP-A is developmentally and hormonally regulated. SP-A plays a role in tubular myelin formation, in the regulation of re-uptake and secretion of surfactant by type II cells, in the adsorption of surfactant phospholipids at the air-liquid interface (together with SP-B and SP-C). Especially intriguing is its role in non-immunological pulmonary defence (32). Surfactant protein B (SP-B) and surfactant protein C (SP-C) are smaller and very hydrophobic proteins. Together they make up 1-2% of the surfactant mass. SP-B plays a role in the tubular myelin structure but the most important function of both SP-B and SP-C is to promote the phospholipid adsorption at the air-liquid interface in the alveoli (32). Surfactant protein D (SP-D) is a more recently characterized surfactant protein which has several characteristics and functions in common to SP-A (34). The surfactant lipids mainly consist of phospholipids (80-90%). Cholesterol is the major neutral lipid. Phosphatidylcholine (PC) represents about 80% of surfactant phospholipids and is thus by far the most abundant phospholipid and surfactant component (30,31,35). Approximately 60% of the PCs are fully saturated. This disaturated phosphatidylcholine is almost entirely dipalmitoylphosphatidylcholine, which is the main surface tension lowering component (36). Therefore adequate amounts of this surfactant component are essential for proper lung function immediately after birth. Phosphatidylglycerol is the second most abundant phospholipid in surfactant, accounting for 10% of total lipids in mature surfactant. However, in immature infants with RDS phosphatidylglycerol is almost absent. Phosphatidylglycerol and the other phospholipids help in the spreading of disaturated phosphatidylcholine at the alveolar lining, but their precise function is not completely resolved.

1.5 The regulation of CTP:phosphocholine cytidylyltransferase activity in fetal type II cells during development

PC is quantitatively the most important component of pulmonary surfactant and the disaturated form of PC is the main surface tension lowering component. Because adequate amounts of surfactant are essential for proper lung function immediately after birth, the production of surfactant PC increases towards the end of gestation (30,31,37). The CDPcholine pathway is the primary pathway for de novo PC synthesis in the developing lung (30,31,35) and the activity of this pathway has been shown to increase during late gestation (38). In the second and third chapter of this thesis, we will present evidence showing that the enzyme CTP:phosphocholine cytidylyltransferase (CT) is a rate-limiting step in the CDPcholine pathway in fetal type II cells, and that the activity of this enzyme increases with advancing gestation at the same time as the increase in surfactant PC synthesis. CT is also an important target for regulation by a variety of hormones that are known to affect surfactant lipid synthesis in the developing lung. Therefore, the regulation of the activity of CT is very important for the regulation of surfactant PC synthesis. However, it should be kept in mind that an overall metabolic pathway is never regulated under all circumstances by the activity of one single enzyme.

1.6 Aims

The aim of the investigations described in this thesis is to study the regulation of CT in maturing fetal rat type II cells at late gestation. These studies can help to better understand the regulation of surfactant synthesis and will eventually benefit sick human infants.

In Chapter 2, a review of the literature regarding the regulation of CT is presented, with emphasis on what is known in the developing lung and especially fetal type II cells. In the introduction of this review the CDP-choline pathway for de novo PC synthesis is briefly described and evidence is presented which demonstrates that CT is a rate regulatory step in this pathway. As knowledge is progressing fast, new developments have been made since some of our studies were performed. To give an updated overview, we therefore also included the studies described in this thesis.

In Chapter 3, the rate of PC synthesis from different precursors is studied in fetal type II cells during development and correlated with the activity of the three enzymes in the CDPcholine pathway and the subcellular distribution of CT.

In Chapter 4, it is investigated whether the increased CT in fetal type II cells during development is due to an increase in CT gene expression and CT protein levels.

In Chapter 5, the role of lipids in the developmental activation of

cytosolic and microsomal CT in fetal type II cells is studied.

In Chapter 6, the role of cAMP-dependent protein kinase in the developmental regulation of CT activity in fetal type II cells is investigated.

In Chapter 7, the role of protein phosphatases and protein kinase C in the developmental regulation of CT activity in fetal type II cells is studied.

In Chapter 8, the mechanisms of reduced surfactant PC in a rat model for congenital diaphragmatic hernia are investigated. PC synthesis and CT activity in fetal type II cells isolated from fetuses with congenital diaphragmatic hernia are studied in relation to type II cell-fibroblast interactions.

In Chapter 9, the results from the studies are summarized and overall conclusions and future perspectives are presented.

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CHAPTER 2

REVIEW:
REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLYL-TRANSFERASE ACTIVITY IN DEVELOPING FETAL LUNG

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submitted

2.1 Introduction

The mechanical stability of the alveoli is highly dependent upon pulmonary surfactant, which consists predominantly (~ 90%) of lipids and about 10% of proteins (1). Phosphatidylcholine (PC) accounts for about 70% of surfactant lipids (2-4). About 50-60% of this PC consists of disaturated phosphatidylcholine (DSPC), almost entirely the dipalmitoyl (16:0/16:0) species, which is the main surface-tension-lowering component (5). Therefore adequate amounts of this surfactant component are essential for proper lung functioning immediately after birth. Since 1959 it has been known that neonatal respiratory distress syndrome is caused by lung immaturity with concomitant surfactant deficiency (6).

Abundant evidence indicates that the synthesis of surfactant PC increases during late gestation (1-3,7,8). The CDPcholine pathway is the primary pathway for de novo PC synthesis in the developing lung (reviewed in 2,3,9,10) (Fig. 1). Choline is brought into the cell by a facilitated transport system, and is phosphorylated by the choline kinase. The synthesis of the activated intermediate, CDPcholine, is catalyzed by the CTP:phosphocholine cytidylyltransferase (CT) (EC 2.7.7.15). Finally, the phosphocholine moiety is transferred to diacylglycerol by the CDPcholine:1,2-diacylglycerol phosphocholinetransferase (Fig. 1). Studies with whole lung have shown an increased activity of the CDPcholine pathway during late gestation (11). Pool size studies have demonstrated that the reaction catalyzed by the CT is a rate-limiting step in the CDPcholine pathway in fetal lung and isolated fetal type II cells (12,13). In addition, many studies indicate that CT is an important target for developmental (7,14-17) and hormonal regulation in

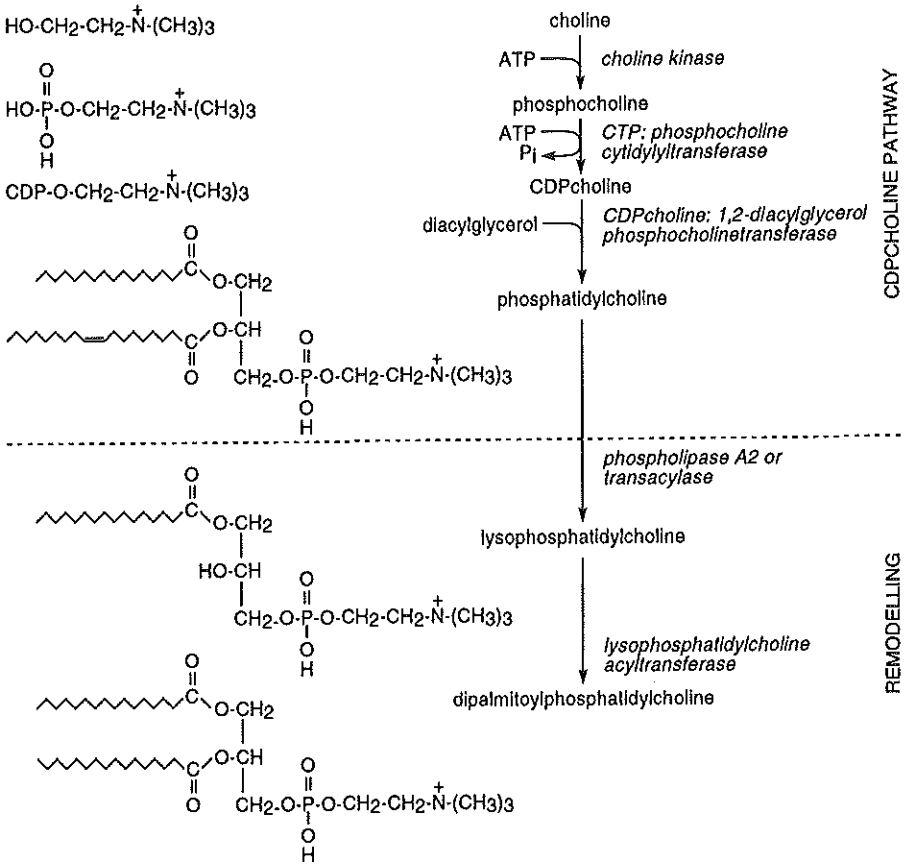

Figure 1. **CDPcholine pathway and remodelling of phosphatidylcholine.** The CDPcholine pathway for de novo phosphatidylcholine synthesis is shown in the upper part of the figure. About half of the newly synthesized phosphatidylcholine is disaturated, the other half contains an unsaturated fatty acid in the 2-position. Further remodelling of unsaturated phosphatidylcholine to dipalmitoylphosphatidylcholine is shown in the lower part of the figure.

alveolar type II cells of the developing lung (18-24). Although under most circumstances CT has indeed been shown to be the regulating step, we have to realize that an entire pathway is never regulated by the activity of one single enzyme under all circumstances. Recently, important progress has been made in the understanding of the regulation of CT activity. This was mainly made possible by the purification of the enzyme (25,26), followed by the availability of antibodies against the enzyme (27-29) and the cloning of the cDNA for CT from rat liver (30) and lung (31). These recent advancements have important implications for the understanding of the regulation of CT in developing fetal lung, which is the topic of this review.

As the lung consists of many different cell types and surfactant is produced by the alveolar type II cells, the regulation of CT activity in fetal type II cells will be the main focus of this review. The data are usually obtained by the study of isolated type II cells in primary culture (2,32). Although the cuboidal epithelium which lines the acinar tubules during the late pseudo-glandular and early canalicular stages of lung development does not contain lamellar bodies, the phenotypic marker for type II cells, it was shown that these cells do express other phenotypic features of type II cells and possess antigenic determinants of mature type II cells (33,34). Therefore these cells are usually called protodifferentiated type II cells or pre-type II cells (3). In this review the term 'type II cells' will be used for type II cells and pre-type II cells. Although the focus will be on type II cells, data obtained from whole lung studies will also be described, for two reasons. Firstly, the interaction of type II cells with other pulmonary cells is important in the regulation of surfactant synthesis and more specific in the regulation of CT activity (18,19). Secondly, many aspects of the regulation of CT activity in the developing lung have not been studied in isolated fetal type II

cells. I will also summarize mechanisms of CT regulation studied with purified CT and studies in other cell types or organs than the lung. Indeed, PC synthesis is necessary in all living cells as it is an essential component of cell membranes. In hepatocytes, CT activity is extensively studied because of the importance of PC in lipoproteins. The studies with these cell types can provide a better understanding of the mechanisms of regulation of CT in the developing lung, in which some of these mechanisms have not yet been studied.

It has been reported that in type II cells only about half of the DSPC, the main surface tension lowering component of surfactant, is being synthesized directly by the de novo pathway by using saturated diacylglycerols as precursors (7,35). A considerable part of the disaturated species of PC is formed by remodelling of de novo synthesized unsaturated (in the 2-position) phosphatidylcholine (Fig. 1). The most important mechanism for this remodelling is a deacylation of PC at the 2-position by a phospholipase A_2 or by a transacylation with another phospholipid, followed by a reacylation step of the resulting lysophosphatidylcholine by the lysophosphatidylcholine acyltransferase (reviewed in 2,3,9) (Fig. 1). The activity of the latter enzyme has been shown to be higher in type II cells than in whole lung, to increase during development and to exhibit specificity towards palmitoyl-CoA as a substrate in fetal type II cells (3,9,36). The remodelling of the de novo synthesized PC to a high proportion of DSPC will not be discussed further in this review.

2.2 The structure and characteristics of cytidylyltransferase

The enzyme CT has been purified to homogeneity (26) and the cDNA has been cloned (30) from rat liver and expressed in Chinese Hamster Ovary Cells (37,38) and insect cells using a baculovirus vector (39,40). We recently purified CT from adult rat lung (41) by the same protocol as used for rat liver and cloned the CT cDNA from fetal rat type II cells (31). This CT consists of 367 amino acids and has a molecular weight of 41 720 (41). The coding region demonstrated 99% sequence similarity between rat liver and rat type II cell CT cDNA. The putative amino acid sequence was different at four positions (31). Very recently, a human CT cDNA was cloned from a erythroleukemic K562 cell library and also showed close homology to other mammalian CTs (42).

The central domain of the CT protein has a close sequence homology to yeast CT (30,31,42). This region is thought to be the catalytic region (Fig. 2). There is one potential site for phosphorylation by cAMP-dependent protein kinase and six potential sites for phosphorylation by protein kinase C in the rat liver enzyme (30). The slightly different putative amino acid sequence of CT in the fetal type II cells resulted in an additional potential site for phosphorylation by protein kinase C (31). There are several potential sites for phosphorylation by other protein kinases (30,43). Purified rat liver CT has been shown to be a substrate for phosphorylation by cAMP-dependent kinase (44). The study of the phosphorylation of CT in insect cells using a recombinant baculovirus clone showed that only the carboxy-terminal region was phosphorylated and that phosphorylation was confined to serine residues (43)(Fig. 2). The potential role for the phosphorylation and dephos-

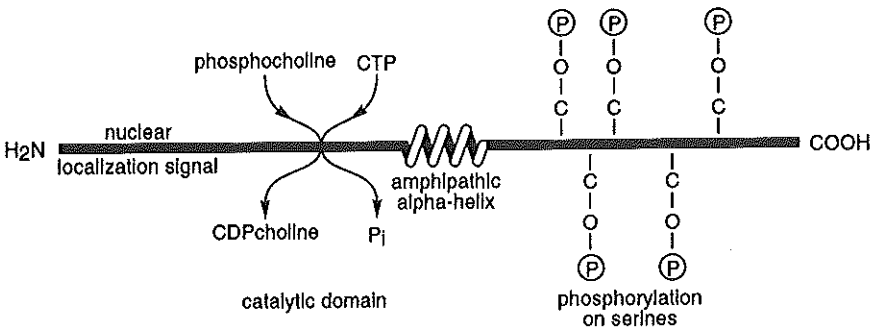

Figure 2. **The structure of CTP:phosphocholine cytidylyltransferase.** Details are described in the text.

phorylation of CT in the regulation of its activity will be discussed below. CT has an amphipathic α-helix at its C-terminal part (30) (Fig. 2). Recent studies using peptide-specific antibodies, limited chymotrypsin proteolysis and synthesis of a peptide corresponding to the amphipathic region, have identified this α-helix as the membrane-interacting domain of CT (45-47). This α-helix binds selectively to anionic membranes, and the binding involves intercalation of the hydrophobic face of the helix into the membrane core (47,48). The importance of the binding of CT to membranes for the regulation of CT activity will be discussed in the following sections. Finally, the N-terminal part contains a sequence which has been shown to play a role as a nuclear localization signal (49) (Fig. 2). Further studies are required to define the precise significance of this finding.

The purified CT catalytic subunit is inactive and requires phospholipids for activity (26,48,50). When bound to membrane vesicles CT exists as a dimer, but these dimers self-aggregate in the absence of lipids or detergents (51). In cytosol, CT exists in two forms (Fig. 3), a low molecular

weight L-form, which is inactive in the absence of added phospholipids and a high molecular weight H-form, which is active in the absence of added phospholipids (52-54). In the cytosol of most tissues, including fetal lung, the L-form is the predominant form, but in the adult lung and isolated type II cells from adult lung, the H-form is predominant (52-55). Many studies have demonstrated that CT becomes activated when bound to membranes (reviewed in 56-58)(Fig. 3). Recently, a 112-kDa CT binding protein has been described which is present in different organs including rat lung (59,60). The binding of CT to this binding protein is promoted by fatty acids (60). The precise structure and functional role of this binding protein remains unclear at present.

CT catalyses a reversible reaction. The pH optimum for the enzyme is 7.0. The equilibrium of this reaction in vitro, slightly favors the formation of CTP and phosphocholine ("reverse reaction"). The K_{eq} for the forward reaction was indeed found to be 0.2 (61). However, it is thought that in intact cells the reverse reaction is insignificant, because the levels of CDP-choline and inorganic pyrophosphate are far below their Km's. Purified CT has a Km for CDPcholine of 0.64 mM (62) while the concentration of CDPcholine in fetal and adult lung is only 46.2 and 31.8 nmol/g wet weight respectively (13,63). The Km for inorganic pyrophosphate is 0.007 mM (62) while inorganic pyrophosphate was undetectable (<2 pmol/μg DNA) in either whole adult lung or type II cells (63). This latter finding implies the presence of very active pyrophosphatases, which were suggested by Infante and Kinsella (61) to promote the forward reaction catalyzed by CT. Pure CT, assayed in the presence of saturating amounts of PC/oleic acid (OA) vesicles, has an apparent Km of 0.22 mM for CTP and 0.24 mM for phosphocholine, the substrates for the forward reaction (26). The Km's for

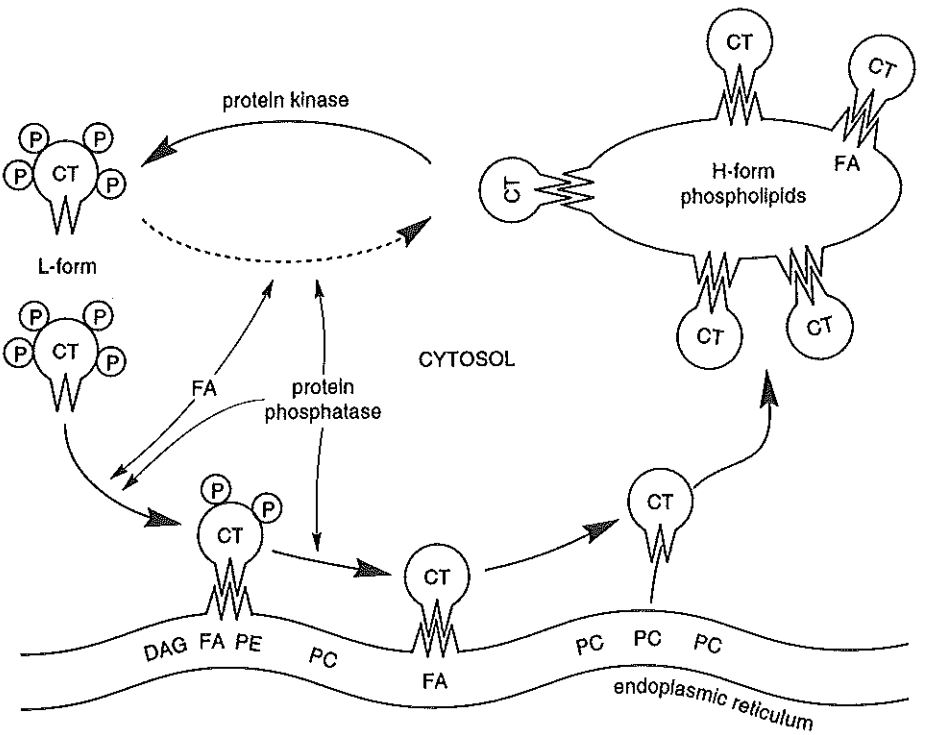

Figure 3. **A model for the regulation of CTP:phosphocholine cytidylyltransferase (CT) activity.** Details are described in the text. DAG: diacylglycerol; FA: fatty acids; PE:phosphatidylethanolamine; PC:phosphatidylcholine; P:phosphate group.

CTP and phosphocholine were found to be slightly higher but of the same order of magnitude in whole lung cytosol and microsomes when assayed in the presence of fatty acids (64,65). A recent study shows that the CT is highly specific for phosphocholine as a substrate, as the Km values for phosphodimethylethanolamine (Km=4 mM), phosphomonoethanolamine (6.9 mM) and phosphoethanolamine (68.4 mM) were much higher (66). Under normal circumstances, the concentrations of CTP and phosphocholine are not

limiting. The phosphocholine concentration was found to be 922 and 247 nmol/g wet weight in fetal (13) resp. adult rat lung, and the CTP concentration 53.5 nmol/g wet weight in adult rat lung (63). In isolated fetal or adult type II cells these concentrations were 18 to 60 times higher (13,63). Only under extreme circumstances, such as hypoxia or starvation, the supply of CTP or phosphocholine can be limited (reviewed in 57). The concentrations of substrates measured in tissues, cells or cell fractions do not necessarily reflect local concentrations in a particular cellular compartment. There is indeed good evidence for the channelling of intermediates during PC biosynthesis (reviewed in 57,58,67). This evidence was obtained by experiments with glioma cells permeabilized with electroporation (68). Such channelling would make the transfer of intermediates from one enzyme to the next much more efficient. This would certainly make sense in a specialized cell like the type II cell, which produces large amounts of surfactant PC in addition to the PC as part of membrane structure and function. The cytoskeleton may play a functional role in this channelling process (58,67). Hunt et al. (69) demonstrated the in vitro association between rat and human lung CT and the cytoskeletal actin, but so far, there is no further evidence for the channelling of substrates in type II cells. If such compartmentalization exists, the locally higher substrate concentrations would make it necessary to also consider the reversed reaction of CT.

2.3 Induction of cytidylyltransferase and regulation of gene expression

Overall CT activity in the cell can be altered by a change in CT enzyme protein or by a change in the activity of existing protein. Until recently, it was not possible to study CT protein mass in a reliable way. Relative CT enzyme mass was usually compared between two conditions by measuring CT activity in the presence of lipid vesicles that are known to maximally activate the enzyme. Since the availability of antibodies against CT (27-29), it is possible to study CT enzyme protein mass and CT localization in the cell in a more direct way. In addition, the cloning of the cDNA for CT from rat liver and lung (30,31) makes it possible to investigate CT gene expression.

That increased expression of CT mRNA is possible, was shown in a colony-stimulating factor 1 (CSF-1)-dependent murine macrophage cell-line BAC1.2F5, which was stimulated with CSF-1 (70). Houweling et al. (71,72) demonstrated increased activities of CT in liver cytosol and microsomal fractions after partial hepatectomy. This 1.4-fold increase in cytosolic activity was accompanied by a 1.5-fold increase in the amount of immunoreactive CT protein as well as by a 1.7-fold increase in [^{35}S]methionine incorporation into CT protein (72). Furthermore, Northern blot analysis showed a 2-3 fold increase in CT mRNA's at 12 hours after surgery (72).

Studies with fetal lung (8,73) and fetal type II cells (7) showed increased activities of both cytosolic and microsomal CT activities during maturation when CT activity was assayed in the absence of added lipids. Although specific activities of the enzyme were higher, similar developmental profiles were also found when the activities were assayed in the

presence of saturating concentrations of lipids, which are known to maximally activate the enzyme (7,8,73). These data suggest that CT is not only activated during lung development but that the amount of CT enzyme increases as well. The regulation of the amount of CT enzyme and also mRNA levels was confirmed in several experiments involving fetal lung or fetal type II cells. In day-18 fetal rat lung explant cultures (74) and, even more pronounced, in day-17 fetal rat lung type II cell cultures (75), a spontaneous increase in CT mRNA was seen over a few days in culture in the absence of hormones. Also in vivo, Mallampalli and Hunninghake (76) found an increased expression of immunoreactive CT protein mass in fetal lung, a decreased amount in neonatal lung and an even lower amount in adult lung. However, in the adult lung, a larger part of the cytosolic CT was found in a more active form (76). In fetal type II cells, we found a marked increase in the amount of CT in microsomes during development at late gestation, as measured by Western blot and densitometry (31). No change in the amount of cytosolic enzyme was found (31). This correlated with CT activities in these cell fractions (7,31). By reverse-transcriptase polymerase chain reaction (RT-PCR) analysis, CT mRNA content was shown to increase three-fold in fetal type II cells with advancing gestation, whereas CT mRNA levels in fetal lung fibroblasts remained constant (31). This finding demonstrates the need for studies with isolated type II cells, besides whole lung studies, as type II cells are only a small fraction of total lung cells.

The effect of corticosteroids on CT enzyme mass and mRNA content has also been investigated in fetal lung and fetal type II cells. Dexamethasone has been reported to increase the activity but not the amount of CT in fetal rat lung explants (28). Also, in vivo no increased amount of CT was found by Western blot in fetal rat lungs after maternal administration of

betamethasone (23). Although in several studies the effect of glucocorticoid stimulation on CT activity was markedly diminished when the activity was assayed in the presence of activating lipids (10,23,28,77,78), the effect was not completely abolished (23,28,77). In a study with human fetal lung explants (24), the stimulating effect was even more pronounced when CT was assayed in the presence of activating lipids. In contrast to the two studies which only found activation of existing CT by corticosteroids, this suggests an increase in enzyme mass in addition to enzyme activation (10). Indeed, Batenburg and Elfring (79) reported a small (around 30%) increase in CT mRNA content in fetal rat type II cells exposed to cortisol-containing fibroblast-conditioned medium. Such medium is known to activate CT in fetal type II cells and transmits the corticosteroid signal from fibroblasts to type II cells via a so called fibroblast-pneumocyte factor (FPF) (19,80). In contrast to these fetal type II cell studies, dexamethasone did not increase CT mRNA content in fetal rat lung explants (74). Therefore it remains an unresolved question whether the small increase in CT mRNA levels in fetal type II cells after corticosteroid stimulation is meaningful. It is possible that the increase is indeed meaningful but not seen with fetal lung explants because the effect is masked by the analysis of mixed cells.

In conclusion, the amount of CT enzyme can be regulated at a pre-translational level in developing lung or type II cells, and this regulation may be important together with enzyme activation. Whether corticosteroids increase the amount of CT mRNA and enzyme protein is not completely resolved.

2.4 The regulation of cytosolic cytidylyltransferase activity by (phospho)-lipids

The rest of this review will focus on the mechanisms of CT activation (Fig. 3) as opposed to CT enzyme induction. It was already shown several years ago that phospholipids are able to stimulate cytosolic CT activity in lung cytosol (8,52-54,81-84). The best stimulation was obtained with acidic phospholipids, especially phosphatidylglycerol (53,54,81-83) or by mixed PC/OA (in a 1/1 molar ratio) vesicles (54,83). This was also demonstrated for CT in fetal type II cell cytosol (85). Adult lung cytosolic CT is stimulated less by phosphatidylglycerol than fetal lung cytosolic CT activity (16,81). During the last few years, the studies using purified CT have confirmed the activation of the enzyme by acidic phospholipids and PC/OA vesicles (25,26,48,50). Cornell (50) showed that the negative surface potential is a major factor in the activation of purified CT by anionic lipids (50) and described the physical chemistry of the lipid activation of CT in detail (48,50).

It is now accepted by most investigators, that the cytosolic CT exists in two forms (Fig. 3), a low molecular weight L-form, which is inactive in the absence of added phospholipids and a high molecular weight H-form, which is active in the absence of added phospholipids (52-54). In the cytosol of most tissues, including fetal lung, the L-form is the predominant form, but in the adult lung and isolated type II cells from adult lung the H-form is predominant (52-55,76). The L-form can be converted into the H-form by the addition of phospholipids (52,54,76) or fatty acids (55,65,86-88). The H-form thus appears to be a lipoprotein consisting of L-form CTs complexed

with lipids (54,89). An unexplained observation is that the H-form was stable at 4°C, but dissociated during incubation at 37°C for 15 min (54).

Numerous studies have observed a developmental increase in fetal and neonatal CT activity in the cytosolic fraction of the lung (8,14,16,17,73,90-92). At the same time an increase in phospholipid concentration has been demonstrated in fetal lung cytosol (16,52). Chu and Rooney (16) demonstrated a strong correlation between CT activities in the developing rabbit lung cytosol and the ability of cytosolic phospholipids of the same lungs to activate delipidated cytosolic CT. The delipidated enzyme had very low activities without the re-addition of lipids (16). These data strongly suggest that phospholipids regulate cytosolic CT in the developing lung. Recently, we also demonstrated the importance of cytosolic lipids in the regulation of CT in isolated fetal type II cells (85,93). The stimulation of the activity of cytosolic CT by PC/OA vesicles decreased with advancing gestation, suggesting that the enzyme is in a more active form at the end of gestation. Moreover, lipids extracted from cytosol of fetal rat type II cells of various gestational ages differed in their ability to activate CT: cytosolic lipids from type II cells at the end of gestation were the most stimulatory (85,93). Which lipid is responsible for the developmental activation is not yet resolved. In the study of Chu and Rooney (16) the activation of CT could not be attributed to any individual (phospho)lipid species. In contrast to fetal lung, adult lung required not only the extracted phospholipids but also the extracted neutral lipids to fully re-activate the delipidated CT (16). The most important factor in these neutral lipids was the fatty acid fraction (16,86). Thus, adult lung displays a different lipid activation pattern, which may be related to the higher proportion of CT in the H-form in adult lung. The precise relation between the existence of L- and H-forms and the role of

phospholipids in the developmental activation of cytosolic CT is not completely resolved. In the study by Chu and Rooney (16), the developmental increase in cytosolic phospholipids in fetal lung (16,52) did not correlate with increased cytosolic CT activity. In addition, a developmental increase in H-form with an associated decrease in L-form has not been clearly demonstrated. However, an increase in H-form following birth has been demonstrated (15). The transition between fetal and adult type CT activity patterns (16,52,53,76) is not well described.

The activation of fetal lung cytosolic CT has also been demonstrated in response to several hormones such as estrogen, glucocorticoids and thyroid hormone (reviewed in 2,18). These experiments were performed in vivo, by maternal injection of the hormone, and in lung explants in culture. The effect of estrogen on CT activation has been shown to be mediated by phospholipids (22,94). The stimulatory effect of estrogens on fetal lung CT was not observed when the enzyme was assayed in the presence of phosphatidylglycerol, suggesting that the hormone increased the activation state of the enzyme rather than the amount of enzyme (82). The stimulatory effect of maternal injection of estrogen on fetal lung cytosolic CT activity was abolished by delipidation of the enzyme prior to the assay of CT activity (22). The enzyme activity and the effect of estrogen were restored by re-addition of cytosolic phospholipids obtained from lungs of estrogen treated fetuses or controls (22). Although these data demonstrate that the effect of estrogen on CT activity is mediated by phospholipids, estrogen had no effect on the phospholipid content or composition of fetal lung subfractions (82), and the stimulatory effect of estrogen could not be attributed to any individual phospholipid species (22). The relation between these findings of phospholipid-mediated activation of CT after estrogen treatment and the

conversion from L- to H-form still remains to be established. Mallampalli et al. (65) found a decreased H-form and an increased L-form in fetal lung cytosol after maternal administration of estrogen, the opposite of what would be expected. It is not clear whether species (rabbit versus rat) or timing differences (in hormonal treatment or delivery etc..) play a role in these dissimilar findings.

The stimulatory effect of corticosteroids on CT activity was also considerably reduced or completely abolished when the enzyme activity was assayed in the presence of a saturating concentration of activating lipids (10,23,28,77,78) or after delipidation (23). Further, Mallampalli et al. (23) showed that the addition of cytosolic lipid extracts from lungs of fetuses treated with betamethasone increased CT activity to a greater extent than cytosolic lipid extracts from control lungs (23). This lipid-mediated stimulatory effect of corticosteroids on cytosolic CT activity could be attributed to an increase in cytosolic H-forms associated with a decrease in L-forms (23,65). Several recent studies suggest that an increase in fatty acids, either in free form or after incorporation into (phospho)lipids, is responsible for the corticosteroid effect on CT activity (see reviews 10,95) via a conversion of L- to H-form (65).

2.5 Activation of cytidylyltransferase by translocation from cytosol to membranes

The mechanism of CT activation by translocation from cytosol to the membranes of the endoplasmic reticulum (Fig. 3) was suggested by Vance and Pelech in the early 1980's and was based on experiments with HeLa

cells and hepatocytes (reviewed in 56). The cytosolic CT was considered an inactive reservoir, while the microsomal fraction contained the active enzyme, which is activated by the lipidic microsomal environment, and which activity correlated with PC synthesis (reviews 56-58). The reversible translocation of CT between cytosol and endoplasmic reticulum has been shown in Krebs II cells to occur within minutes (96). Several mechanisms have been reported to be involved in the regulation of this subcellular translocation (Fig. 3). Fatty acids, diacylglycerols, phosphorylation/dephosphorylation of the enzyme and feedback inhibition by increasing PC levels have been studied in tissues and cells, especially in HeLa cells and hepatocytes (reviewed in 57,58,67). The location of the active CT on the endoplasmic reticulum has the advantage of producing CDPcholine on the site of its utilization by the phosphocholinetransferase, the next enzyme in the CDPcholine pathway for de novo PC synthesis. However, other membrane locations of CT have been described. In rat liver cells CT was found in the Golgi apparatus (97). In Chinese Hamster Ovary Cells and HeLa cells a nuclear localization was found or could be induced (98-100). In lung no such localizations have been described. However, two recent studies make it necessary to consider this possibility in the lung. A nuclear localization of CT by indirect immunofluorescence microscopy in several cells, including HepG2, and in rat liver (101) has recently been described. In addition, Wang et al. (49%) found that a N-terminal domain of CT functions as a nuclear localization signal (Fig. 3). Further studies are necessary to confirm these findings and to define the role of this possible nuclear localization.

In lung, only the study by Weinhold et al.(84) showed an increase in microsomal CT activity with an associated decrease in cytosolic CT activity, which is considered strong evidence for a translocation of CT from cytosol

to microsomes. The translocation of CT in the rat lung following birth was mediated by fatty acids, which was further substantiated by in vitro evidence (84). Several studies, however, showed an increased microsomal CT activity in association with increased PC synthesis in fetal lung (15,21,102), or fetal (7,103) and adult type II cells (104-106). In these and other studies a proportional redistribution was found (with an increased percentage of total activity found in microsomes and a decreased percentage in cytosol), but in absolute terms, cytosolic activity did not decrease in association with the increased microsomal activity (7,17,20,73,102-107). A developmental increase in microsomal CT activity of fetal lung has been demonstrated to occur with advancing gestation (8,73) and following premature birth (15,84). A similar increase in microsomal activity and shift in subcellular distribution has been found in fetal type II cells with advancing gestation (7,17,31). Hormones such as corticosteroids, alone (20,21) or in combination with thyroid hormone (21), increase microsomal CT activity in fetal lung or fetal type II cells without causing a subcellular redistribution of CT activity.

2.6 The regulation of cytosolic and microsomal cytidylyltransferase activity: a unifying hypothesis

In the current literature some controversy exists around the question whether developmental and hormonal activation of fetal lung CT takes place in cytosol or in microsomes, via a translocation of CT from cytosol to the endoplasmic reticulum. Our hypothesis is that both cytosolic and microsomal activation of CT occurs during lung development and during hormonal stimulation. CT activity of both cytosolic and microsomal fractions increases

due to the conversion of cytosolic L-form to microsomal CT and cytosolic H-form, which are similar forms of the enzyme.

As discussed in the previous sections, several studies with fetal lung and fetal type II cells demonstrated a developmental or hormone-induced increase in cytosolic CT activity while other studies showed an increase in microsomal CT activity. However, a large number of studies that measured both cytosolic and microsomal CT activity found an increased enzyme activity in both fractions (7,8,15,17,21,73,78). This was frequently, although certainly not always (20,22,78), associated with a relative redistribution of CT activity from cytosol to microsomes, without showing good evidence for a translocation mechanism because the absolute cytosolic CT activity did not decrease (7,15,17,31,73). One study in lung did show an increased microsomal activity together with a decreased cytosolic CT activity after birth (84). Taken together, it is reasonable to conclude that the increased activity of CT found in fetal lung or isolated fetal type II cells during late gestation is caused by an increase in both cytosolic and microsomal enzyme activity.

A few recent studies directly support this view that both cytosolic and microsomal activities can be activated in a coordinated way (Fig. 3). The study by Feldman et al. (55), although performed with adult instead of fetal lung, describes the relationship between the cytosolic and membrane forms of CT. It turns out that microsomal CT existed in a form similar if not identical to the cytosolic H-form. The microsomal form would be formed after binding of the L-form to microsomal membranes, which can be induced by fatty acids, and the H-form is released from the membrane (Fig. 3). The same group of researchers already showed some evidence for such interpretation in the perinatal lung several years ago (84). Firstly, the type of

response to lipid stimulators (fatty acids and phosphatidylglycerol) and to albumin treatment (which removes fatty acids by binding) was similar for the H-form and the microsomal form of CT. Secondly, the phospholipid composition of the H-form was similar to that of microsomes (81). These data support the idea that the H-form of the cytosolic CT appears to be CT bound to small membrane fragments (84). Another study with HepG2 cells showed evidence for activation of both cytosolic and microsomal CT activity by OA (89). The cytosolic activation was accompanied by an increase in H-form. The unique finding was that the immunoreactive CT protein increased in both the cytosolic and membrane fractions in these HepG2 cells treated with OA (89). The short response time and the inability of cycloheximide to prevent the increase, argue against the synthesis of new enzyme. These results suggested that fatty acids promoted the formation of active CT (H-form and microsomal enzyme) from a pre-existing inactive form, which was not (completely) detected by antibodies. The increased activity was distributed between membranes and H-form in cytosol (89).

It is not yet clear in which state the inactive, immunologically less detectable, form exists. It is possible that the large and enzymatically inactive aggregates, that are formed when purified CT is Triton- and lipid-depleted (51), constitute such an inactive state. The possible role of the recently discovered CT binding protein (59,60) and of post-translational modifications of the enzyme should also be considered. Whether a similar process exists in the developing lung remains to be demonstrated. In any case, with the above findings in mind, it is easy to understand that fatty acids may increase both cytosolic H-form and microsomal enzyme activity, as they are essentially the same form of the enzyme (55,89). This may provide a possible explanation for the paradoxical finding in fetal lung that enzyme

translocation may take place and yet cytosolic activity increases at the same time. As will be described in the next section, fatty acids may indeed play a very important role in the regulation of both the activity of the cytosolic CT (by increasing the H-form) and the microsomal enzyme activity (possibly via a translocation mechanism) in the lung or fetal type II cells during development or hormone-induced stimulation. The precise role of fatty acids as opposed to phospholipids in the activation of cytosolic CT is not yet completely clear. There are however two obvious relations between phospholipid and fatty acid activation of CT. Firstly, fatty acids can be incorporated into phospholipids, which then activate CT (95). Secondly, fatty acids promote the conversion of L-form to H-form (65,87,88), which consists of aggregated CT, complexed with mainly phospholipids (Fig. 3).

2.7 Regulation of cytidylyltransferase activity by fatty acids

In vitro, fatty acids promote the binding of CT to lipid vesicles (108) and membranes (84). Recent studies with the purified CT or a synthetic peptide sequence have shown that this membrane-binding part of the enzyme consists of the amphipathic α-helical domain (45-47). Thus, fatty acids do not activate CT on their own but need a vesicle or membrane structure to activate CT (25,48,83,109). In intact cells these lipid structures are the cytosolic H-form and the membranes of the endoplasmic reticulum (Fig. 3).

Fatty acids have been shown to activate CT by translocation from cytosol to microsomes in different cell types or organs (84,96,109-112), including perinatal lung (84). Several studies showed a fatty-acid-induced increase of CT activity in the microsomal fraction of adult (104,105) and

fetal type II cells (103) in primary culture. Fatty acids also have been shown to increase cytosolic CT activity in adult type II cells (105) and fetal lung in culture (88). Also in vitro, CT activity in fetal type II cell cytosol (85) or fetal lung cytosol (86,87) was activated by fatty acids. Several studies by Mallampalli et al. (65,87,88) demonstrated that this cytosolic CT activation in fetal lung is due to a conversion of L-form to H-form induced by fatty acids.

During normal lung development, fatty acids play at least two important roles in the de novo surfactant PC synthesis. Besides their role in regulating CT activity (and thus PC synthesis), fatty acids are a substrate for PC synthesis and they are synthesized de novo by perinatal fetal type II cells at a high rate (113,114) from a variety of substrates (115-118). The following observations point to the importance of *de novo* fatty acid synthesis for surfactant phospholipid formation in the perinatal period (reviews 4,95): [1] in fetal rat lung the rate of fatty acid synthesis (116,119) and the specific activities of acetyl-CoA carboxylase (116,120) and fatty acid synthase (120,121) are increased in the period when surfactant production is accelerated; [2] even in the presence of exogenous palmitate, inhibitors of fatty acid synthesis depress the rate of saturated phosphatidylcholine formation in explants of fetal rat lung and isolated lung cells (122,123); [3] in lungs of newborn rabbits fatty acids synthesized from acetate are preferentially incorporated into surfactant PC (saturated and total) compared to exogenous palmitate (124). All these findings suggest the importance of fatty acid synthesis in developing type II cells. These newly synthesized fatty acids play a role in the activation of cytosolic and microsomal CT. Weinhold et al.(84) found an increased amount of CT activity in lung microsomes shortly after birth in association with an increase in free fatty acid content in

microsomes. Viscardi and McKenna (17) studied the fatty acid content of microsomal phospholipids in fetal and neonatal lung and also showed an increase in these fatty acids together with increased microsomal CT activity. Free fatty acids were however not measured. The increase of cytosolic H-form has been demonstrated in fetal lung following premature birth (15) and the role of free fatty acids has been shown in the transition of fetal to adult lung forms of cytosolic CT (16,86). A progressive increase in cytosolic free fatty acids together with an increase in H-form during lung development has however not yet been demonstrated. Although free fatty acids can activate CT and possibly do so in the developing lung in vivo (84), it is very well possible that the activation of CT takes place after fatty acids have been incorporated into phospholipids or into diacylglycerols. The fact that free fatty acids can directly activate CT in different cells and do so in a reversible way (as shown by the addition of albumin (84,100,111)) within minutes (96,100), would suggest a role for free fatty acids themselves. Furthermore the role of free fatty acids in the activation of CT during hormonal stimulation, together with the importance of these hormones for lung development, would suggest a similar role for fatty acids during type II cell maturation.

The importance of fatty acids in the *activation of lung CT by corticosteroids* is better defined. Firstly, fatty acid synthesis is regulated by corticosteroids. Dexamethasone has been shown to accelerate the normal developmental increase in fetal lung fatty acid synthesis *in vivo* and *in vitro* (119,125,126) and to enhance the activity of fatty acid synthase in fetal lung tissue (121,125,127,128). Batenburg and collaborators have also demonstrated this in isolated fetal type II cells (79,120) and have stressed again that type II cell-fibroblast interactions are important in modulating the effect of hormones, not only on PC synthesis (18-20,80), but also on fatty acid

synthesis (79,120). The effect of dexamethasone on fatty acid synthase activity in cultured fetal lung is due to enzyme induction (127) and is regulated at a pre-translational level (74,79,129,130). Secondly, quantitation of CT protein in fetal lung by immunotitration (28) and Western blotting (23) confirmed that CT enzyme mass is not increased by glucocorticoids. Earlier studies had already suggested an activation of the enzyme instead of an increased enzyme mass, because the stimulatory effects of glucocorticoids could be severely diminished or abolished by assaying CT in the presence of lipid activators (10,23,28,77,78). Thirdly and most importantly, inhibitors of de novo fatty acid synthesis, which act at steps in the pathway prior to those catalyzed by fatty acid synthase, abolished the stimulatory effect of dexamethasone on CT activity in fetal rat lung explants (77). The similar time course of fatty acid synthase and CT activity in these fetal rat lung explants cultured in the presence of dexamethasone further supports the importance of increased fatty acid synthesis through increased fatty acid synthase activity in the regulation of CT activity (77). The role of a fatty acid mediated activation of CT by corticosteroids was further confirmed by the in vivo experiments performed by Mallampalli et al.(65). Maternal adminstration of betamethasone increased the total amount of free fatty acids associated with the cytosolic H-form by 62% (65) in association with increased CT activity caused by a conversion of L-form to H-form (23,65). This supports a direct role for free fatty acids but an effect of fatty acids after incorporation into (phospho)lipids or diacylglycerols cannot be excluded.

Taken together, the studies available to date support the following sequence (Fig. 4): corticosteroids induce the production of fibroblast-pneumocyte factor (FPF) in lung fibroblasts adjacent to the alveolar epithelial cells (34) at a pre-translational level (review 80); this FPF induces fatty acid

synthase and other enzymes involved in fatty acid synthesis in fetal type II cells at a pre-translational level (79); this leads to an increase in fatty acid biosynthesis, and fatty acids, their metabolites, or lipids into which they become incorporated ultimately activate CT (10) by increasing cytosolic H-form and possibly translocation of CT from cytosol to microsomes. A similar sequence could take place during type II cell maturation at late gestation or around birth.

Some recent studies start to give some idea which fatty acids may be important for CT activation in vivo. In general, these in vivo studies show that an increase in total saturated and unsaturated fatty acids may be the most important mechanism (17,65). This confirms earlier studies which only measured total fatty acids (16,84,86). From in vitro and culture studies, using hepatocytes (109), HeLa cells (110) but also fetal type II cells (85, 103), fetal (87) and adult lung (86), it was known however that long chain (mono-)unsaturated fatty acids (OA was used most frequently) were the best activators of CT. This was confirmed with purified enzyme, but free fatty acids were only able to activate CT in the presence of lipid vesicles (48). Mallampalli et al.(88) suggested from in vitro evidence and fetal lung explants that polyunsaturated n-3 fatty acids were the best stimulators. Their in vivo study with maternal administration of betamethasone suggested a selective increase in myristic, oleic and linoleic acids in H-form lipids, the latter two fatty acids being very effective in the conversion of L-form to H-form (65). If exogenous fatty acids such as linoleic or n-3 poly-unsaturated fatty acids, which are not synthesized de novo by human cells, are indeed important for CT activation, the role of the endogenous fatty acid synthesis should be re-evaluated.

Further studies are necessary to confirm the role of the described

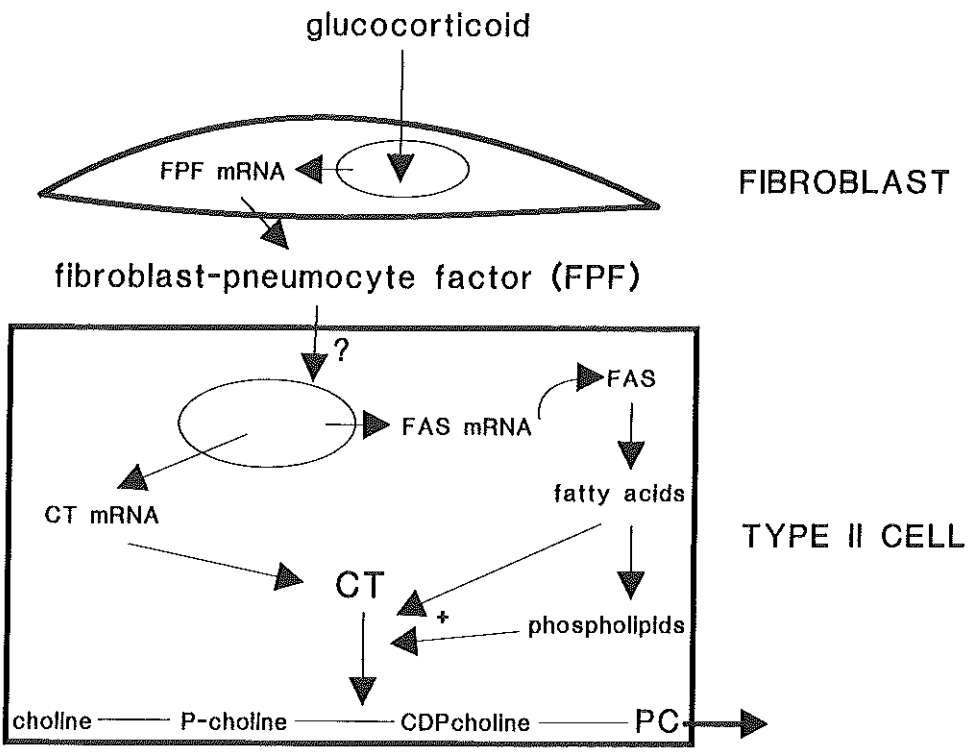

Figure 4. **A model for the regulation of CTP:phosphocholine cytidylyltransferase (CT) in fetal lung after glucocorticoid administration.** Fetal type II cell-fibroblast interactions have been shown to play a very important role in this regulation. FPF: fibroblast-pneumocyte factor; P-choline: phosphocholine; PC: phosphatidylcholine; FAS: fatty acid synthase

sequence of events in vivo and to elucidate the role of the various fatty acids. The role of fatty acids in the promotion of CT binding to a CT binding protein, as recently described (60), also needs further evaluation.

2.8 Regulation of cytidylyltransferase activity by diacylglycerols

Studies in different cell types, other than type II cells, stimulated with various agents suggest that diacylglycerols may play an important role in CT activation (review 58). All of the following observations were shown to be related to increased diacylglycerol levels: the translocation of CT induced by the phorbolester 12-O-tetradecanoylphorbol-13-acetate (TPA) in HeLa cells (131); the increase in H-form of CT in rat liver cytosol induced by phospholipase C or by feeding rats a high cholesterol diet (132); the reversal of CT inhibition of okadaic acid by fatty acids through enzyme translocation in rat hepatocytes (133); the translocation of CT in GH3 pituitary cells (134) and phospholipase C treated chick-embryonic muscle cells (135). Thus, the activation of CT by a conversion of L- to H-form or by a translocation of CT from cytosol to microsomes, can be mediated by diacylglycerols in response to various stimuli.

The molecular mechanism of the activation of CT by diacylglycerols is suggested to be an increased degree of curvature of the membrane (48) or, more in general, a disruption of the packing of the membrane lipid bilayer (136) with increased intercalation of the α-helical domain of CT into the lipid membrane (48,136) (Fig. 3). This can activate CT by translocation (131,133-135) or by binding to cytosolic lipoprotein aggregates as H-form (132).

Until now, evidence is lacking that diacylglycerols play a major role in the regulation of CT in the developing lung. In fetal type II cell cytosol we did not find any activation of CT by 1,3-diolein and 1-oleyl-2-acetylglycerol (85). A study with adult type II cells in primary culture showed an activation

of CT when 1-oleyl-2-acetylglycerol, diolein or mixed diacylglycerols were included in the medium (137). Two studies with adult lung suggested that the major CT activating component of neutral cytosolic lipids was free fatty acid and not diacylglycerol (16,86). However, the possible effect on CT translocation was not examined in these studies. It is clear that the physiologic significance of CT activation by diacylglycerols in the developing lung is far from proven but the data from other cell types make it worth to investigate.

2.9 Feedback regulation of cytidylyltransferase activity by phosphatidylcholine

Two models have provided good evidence for the existence of a feedback regulation of CT, the rate-limiting step of the CDPcholine pathway, by PC, the end product of the pathway.

The first and most convincing evidence is the model of choline depletion. In this model, choline depletion of cells, in vitro or in vivo, leads to a decrease of PC in all cellular membranes with a resulting activation of CT by translocation from cytosol to microsomes. Resupplementation of choline has the reverse effect. Choline starvation (138) or supplementary feeding with choline-analogues (139) in Chinese Hamster Ovary Cells translocates CT to the PC-deficient membranes (139). A more physiologically relevant system using the same idea is the choline-deficient liver caused by feeding rats a choline-deficient diet. (27). In choline-deficient hepatocytes, a translocation of CT from cytosol to endoplasmic reticulum was found which was reversed by choline supplementation (140). There was

a highly significant correlation between the concentration of PC in the membranes and the increased activity of CT in cytosol and decreased activity in the membranes (140). Methionine or lysophosphatidylcholine supplementation, which also increase membrane PC, also reversed the translocation with a decrease of CT activity in membranes (140). This feedback mechanism of CT activity is quite specific for the PC head group, which is a trimethyl-aminogroup (141,142). Tijburg et al.(143) demonstrated that fasting and then re-feeding of rats had similar effects on PC levels and binding of CT to membranes as described for the choline deficiency.

A second, less convincing model to study PC deficiency of membranes and the subsequent translocation of CT to those membranes, is the treatment of cells with phospholipase C. The suggested mechanism by phospholipase C is a PC degradation in the cell membrane. The subsequent movement of PC from the endoplasmic membrane to the cell membrane makes the membranes of the endoplasmic reticulum relatively PC depleted (57,136). These relatively PC depleted microsomal membranes then activate CT by increased binding, thus promoting translocation from cytosol to microsomes. The translocation was demonstrated in Chinese Hamster Ovary Cells (144,145), Krebs II cells (146), chick-embryonic muscle cells (135) and hepatocytes (136).

Jamil et al.(136) suggested a general molecular mechanism to explain the feedback inhibition by PC, but also most other effects of lipids on CT activity. The ratio of bilayer- to non-bilayer-forming lipids may be the overriding common factor in the regulation of CT binding to membranes (136). PC, for example, is a bilayer-forming lipid and inhibits the intercalation of the α-helical domain of CT into the lipids, while diacylglycerol, fatty acids and phosphatidylethanolamine are non-bilayer-forming lipids and thus

stimulate binding of CT to the membranes by promoting the intercalation of the α-helical domain of CT into the lipids (136) (Fig. 3).

A few studies suggest that a negative PC feedback on CT activity may also play a role in the lung. When rats were fed a choline and methionine deficient diet, lung PC production was relatively maintained compared to liver PC production (147). A possible mechanism was suggested to be an increased activity of lung CT, because also CDPcholine levels were maintained (148). More convincing evidence that similar mechanisms, as described in this section, may play a role in lung, was given by depleting adult rat type II cells of choline (107). This caused subcellular redistribution of CT together with activation of the enzyme (107). Aeberhard et al.(149) treated fetal rabbit type II cells with phospholipase C and found marked increased CT activity. The role of such mechanisms during fetal lung development remains to be established.

2.10 Regulation of cytidylyltransferase activity by phosphorylation and dephosphorylation, protein kinases and protein phosphatases

From the cloning and sequencing of rat liver and lung CT cDNA, it was deducted that CT has one potential site for phosphorylation by cAMP-dependent protein kinase, several potential sites for phosphorylation by protein kinase C, and also for other protein kinases (30,31,43). In the last few years, it has become clear that CT is phosphorylated and dephosphorylated in intact cells (43,100,133,150-153). The study of the phosphorylation of CT in insect cells using a recombinant baculovirus clone showed that only the carboxy-terminal region was phosphorylated and that

phosphorylation was confined to serine residues (43), which was also found in HeLa cells (150). The phosphorylation state of CT was shown to correlate with the location of CT: increased phosphorylation in cytosol and decreased phosphorylation when bound to membranes (100,133,151-153). The interaction with other mechanisms of CT regulation and the timing of phosphorylation/dephosphorylation with respect to translocation has recently been investigated (Fig. 3). Several studies suggest that a dephosphorylation of CT is required for CT translocation from cytosol to membranes (100, 151,152). These studies were performed in Chinese Hamster Ovary Cells after phospholipase C treatment (151), in HeLa cells after oleate treatment (100) and in HepG2 cells after choline depletion and repletion (152). On the other hand, the study by Houweling et al.(153) demonstrated that a prior dephosphorylation was not required for binding of CT to membranes in rat hepatocytes treated with OA or phospholipase C. In this study, CT becomes dephosphorylated after translocation to membranes (153). The very rapid translocation of CT to endoplasmic reticulum in Krebs II cells (96) also supports the view that translocation to membranes happens before dephosphorylation of CT. For the reversed translocation from membranes to cytosol, studies agree on the fact that the release of CT from membranes occurs before the enzyme is phosphorylated, and that subsequent phosphorylation occurs (100,152) with further loss of activity (100). It is interesting to speculate that the membrane bound CT is first converted to cytosolic H-form and then gradually phosphorylated to become L-form (100) (Fig. 3). This is consistent with earlier studies of Pelech (154,155), who showed, the other way around, that the conversion from L-form to H-form in liver cytosol was much faster under dephosphorylating conditions and reduced by sodium fluoride, a phosphatase inhibitor.

All these studies taken into account, it seems reasonable to conclude that CT activity can be regulated by phosphorylation and dephosphorylation and that all mechanisms of regulation of CT discussed so far are interrelated. In particular, the reversible phosphorylation and dephosphorylation of CT are closely related to interconversion between cytosolic L- and H-form and to the reversible translocation of CT between cytosol and microsomes.

If CT is phosphorylated and dephosphorylated, then, which enzymes are responsible for the dephosphorylation and which for the phosphorylation of CT?

Dephosphorylation of CT by protein phosphatases and an associated increase in CT activity by translocation has been shown in hepatocytes (133,156). These studies with intact cells incubated with okadaic acid, a protein phosphatase inhibitor, confirmed the previous in vitro evidence for translocation of CT to membranes induced by phosphatases (157) obtained with rat liver cytosol incubated with okadaic acid or sodium fluoride. In the developing lung no similar studies with intact cells have been performed so far. In vitro evidence with fetal rabbit lung cytosol shows that under phosphorylating conditions with Magnesium/ATP, cytosolic CT activity is reduced (158). We showed in fetal rat type II cell cytosol that dephosphorylation with alkaline phosphatases increased CT activity in a concentration-dependent manner. This was also found with purified lung CT. Alkaline phosphatase had no effect on the activity of purified CT in the presence of PC/OA vesicles, which are known to maximally activate the enzyme. Sodium fluoride decreased CT activity in cytosol (159). Further studies in the developing lung are necessary.

In vitro, purified rat liver and lung CT have been shown to be a substrate for *phosphorylation by cAMP-dependent kinase* (41,44). The binding of the purified liver CT to membranes was inhibited by phosphorylation by cAMP-dependent protein kinase. Studies with hepatocytes in culture suggested that cAMP analogues decreased PC synthesis with a decrease in CT activity in microsomes (160). More recent studies with hepatocytes however, did not show any change in CT activity, phosphorylation state or distribution between microsomes and cytosol in response to cAMP-analogues nor in response to an elevation of cAMP levels by cholera toxin (29,161). The inhibition of PC synthesis by cAMP-analogues in these experiments, could be explained by decreased diacylglycerol levels, which then become limiting for the last enzyme in the CDPcholine pathway, the 1,2 diacylglycerol phosphocholinetransferase (29), but CT activity was not affected. We recently demonstrated that cAMP analogues or cAMP-dependent protein kinase inhibitors did not alter CT activity in fetal type II cell cytosol in vitro, nor in intact fetal type II cells in primary culture (41). The activity of cAMP-dependent protein kinase was stimulated by cAMP analogues under similar conditions (41). Thus, it is unlikely that a phosphorylation by cAMP-dependent protein kinase regulates CT activity.

However, there is still a possible role for the long-term regulation of CT activity by cAMP and cAMP-dependent protein kinases. In fetal lung (162) and fetal type II cells (41) changes in cAMP-dependent protein kinase during development have been demonstrated, which correlated with a change in the phosphorylation of several proteins (162). Long-term stimulation (one to several days) by cAMP-analogues or agents that increase cellular cAMP content, increased PC synthesis in fetal (rat and human) lung explants, in an alveolar type II cell line (A549) and in fetal rabbit type II cells (163-167).

Interestingly, in the human fetal lung explants fatty acid synthase gene expression (165) and fatty acid synthase activity were increased by increasing levels of intracellular cAMP (164). As increased fatty acid synthesis is known to activate CT, the *indirect effect* of cAMP-dependent protein kinase activity on CT activity mediated by fatty acids, and its consequences for developmental regulation of CT activity need to be examined.

Protein kinase C was another possible candidate for phosphorylating CT. Phorbol esters, such as TPA, which are well known to activate protein kinase C, stimulated PC synthesis in a variety of cells (87,131,150,168-172) including fetal rabbit type II cells (173). Some studies with HeLa cells treated with TPA, support the activation of CT by translocation to membranes (131, 169, 170), while one study does not (150). Studies with other cells are also controversial regarding TPA induction of CT translocation to membranes (170,172,174,175). In any case, it appears that direct phosphorylation of CT by protein kinase C does not account for the possible increase in CT activity in HeLa cells (131,150,168). Taking into account that phosphorylation of CT usually decreases its activity, it would indeed be quite surprising if CT phosphorylation would increase its activity. The possible increased activity of CT in HeLa cells stimulated with TPA has recently been shown to be related to an increased diacylglycerol production instead of to an increased phosphorylation of CT (131). Moreover pure CT is reported not to be phosphorylated by protein kinase C (58). In adult type II cells, Warburton et al.(176) demonstrated protein kinase C-dependent phosphorylation of several proteins, suggesting key roles for these proteins in type II cell functions. A change of protein kinase C (and protein phosphatase) activities during fetal type II cell development (177) which was similar to that of growth related genes, c-myc and histone (178) also sug-

gests a major role for protein kinase C in fetal type II cell function. However, no effects of TPA and protein kinase C inhibitors were found on CT activity, neither in cytosol in vitro, nor in intact fetal type II cells in primary culture (177).

I conclude that CT activity is regulated by a phosphorylation/dephosphorylation mechanism and that this mechanism is closely interrelated to other mechanisms of CT regulation. Dephosphorylation of CT by protein phosphatases has been demonstrated. There is also some evidence for CT dephosphorylation by phosphatases in fetal type II cells. However, it is unclear which protein kinase phosphorylates CT in intact cells and in vivo. Considering recent studies, protein kinase C and cAMP-dependent protein kinase are very unlikely candidates to play that role. This was also demonstrated in fetal type II cells (41,177).

2.11 Summary and conclusions

Increased production of surfactant at the end of gestation is essential for the stability of alveoli during air breathing after birth. PC is the most abundant component of surfactant. CT has been shown to be the rate-regulatory step in de novo PC synthesis in type II cells, which are the producers of surfactant. CT activity increases with advancing gestation in association with the increased production of PC. In addition, many studies indicate that CT is an important target for developmental and hormonal regulation in alveolar type II cells of the developing lung. Since the purification of CT in 1986 and the cloning of CT cDNA in rat liver, the progress in the understanding of

the regulation of CT activity has accelerated. Not all new insights into the mechanisms of CT regulation have been examined yet in the developing lung.

CT is essentially inactive without lipids. The amphipathic α-helical domain of the CT protein is involved in the binding of the enzyme to lipid membranes. In the type II cell, the most important binding sites involved in the activation of CT are the microsomal membranes and the lipids of the cytosolic H-form (Fig. 3). The currently available data are indeed most compatible with an activation of both cytosolic and microsomal CT activity during fetal lung development and after corticosteroid administration. Cytosolic CT is regulated by phospholipids. The activation of cytosolic CT is accompanied by a conversion of a low molecular weight L-form to a high molecular weight H-form, which is a lipoprotein complex consisting of aggregated CT complexed with (phospho)lipids. The H-form is the predominant form in the adult lung. Fatty acids, either in free form or possibly after incorporation into (phospho)lipids, induce the conversion from L-form to H-form and are shown to be a very important regulator of CT activity after corticosteroid administration and, most likely, also during normal lung development. Translocation of CT from cytosol to the membranes of the endoplasmic reticulum activates the enzyme. This mechanism is also regulated by fatty acids (Fig. 3) and plays an important role during lung development. Recent evidence demonstrates that the cytosolic interconversion from L-form to H-form and the translocation of CT from cytosol to endoplasmic reticulum are closely related.

Taken together, recent studies support the following sequence in the fetal lung after exogenous corticosteroid administration (Fig. 4): corticosteroids induce the production of fibroblast-pneumocyte factor (FPF)

in lung fibroblasts adjacent to the type II cells at a pre-translational level; this FPF induces fatty acid synthase and other enzymes involved in fatty acid synthesis in fetal type II cells at a pre-translational level; this leads to an increase in fatty acid biosynthesis, and fatty acids, their metabolites, or lipids into which they become incorporated, ultimately activate CT by increasing cytosolic H-form and translocation of CT from cytosol to microsomes. A similar sequence could take place during normal type II cell maturation at late gestation or around birth, caused by endogenous corticosteroid production.

CT contains several potential sites for phosphorylation by protein kinases. There is now convincing evidence that CT is phosphorylated and dephosphorylated in intact cells and that the phosphorylation state of the enzyme regulates its activity. The phosphorylation state of the enzyme is correlated with its location (Fig. 3): when the enzyme translocates from membranes to cytosol it becomes subsequently phosphorylated, when it translocates from cytosol to membranes it becomes dephosphorylated and active but the order of events and the precise significance is not yet clear. Protein phosphatases 1 and/or 2A have been shown to dephosphorylate the enzyme, but which protein kinase is involved in the phosphorylation is still unclear. Protein kinase C and cAMP-dependent protein kinase are very unlikely candidates as is demonstrated by recent studies, also involving fetal type II cells. The number of studies about the phosphorylation and dephosphorylation mechanism in developing lung are still very limited, and clearly further studies are required to elucidate the precise role of this mechanism in the regulation of CT activity.

In several cell types the regulation of CT activity by diacylglycerols was demonstrated but no convincing evidence exists for the fetal lung. The

feedback inhibition of CT activity by increased PC in membranes or in general, the regulation of CT activity by altered membrane composition, may be a very important mechanism. Some evidence for this exists in type II cells.

From recent experiments, it has become very clear that all mechanisms in the regulation of CT activity are very closely interrelated (Fig. 3). The precise role of these mechanisms and its interdependence has to be further investigated in the developing lung and isolated type II cells. Recent studies show that not only the activity of CT is regulated, but also that CT protein expression is regulated at a pre-translational level in the II cells of the developing lung. The relative importance and the regulation of these mechanisms will surely be investigated in the near future. It is expected that the gene sequence for CT will be known soon, which will make it much easier to study the regulation of gene expression. Structure-function relationships will be further investigated with the help of site-directed mutagenesis and transgenic animals. New laboratory techniques will help to resolve the question of the recently suggested and intriguing possibility of the channelling of intermediates of the CDPcholine pathway from one enzyme to the next. The role of the cytoskeleton in this channelling will be further examined.

The regulation of CT has frequently been used as an example for the mechanisms of enzyme regulation in general. It is evident that the study of the regulation of CT has not yet come to an end.

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CHAPTER 3

REGULATION OF PHOSPHATIDYLCHOLINE SYNTHESIS IN FETAL TYPE II CELLS BY CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE

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3.1 Abstract

Phosphatidylcholine (PC) synthesis increases in fetal rat type II cells during late gestation, as demonstrated by an increased incorporation of radiolabeled palmitate, glycerol, acetate and choline into PC. However, the percentage of PC present in the saturated form remains essentially constant. The developmental profile of the enzymes of the CDPcholine pathway suggests that CTP:phosphocholine cytidylyltransferase (CT) catalyses a rate-regulatory step in *de novo* PC synthesis by fetal type II cells. When CT activity is assayed in different subcellular fractions the greatest increase, as a function of development, is found in microsomes. This developmental increase is accompanied by a shift in subcellular distribution of CT activity from cytosol to microsomes in fetal type II cells during late gestation. This shift is evident even when CT activity is assayed in the presence of 0.5 mM PC/ oleic acid (OA) (1/1 molar ratio) vesicles. We speculate that either a subcellular translocation of CT from cytosol to microsomes or an increase in CT gene expression are responsible for the developmental increase of *de novo* PC synthesis by fetal type II cells.

3.2 Introduction

Adequate amounts of pulmonary surfactant are essential for proper lung functioning immediately after birth. Pulmonary surfactant prevents alveolar collapse at end-expiration by reducing surface tension at air-liquid interfaces. Phospholipids are quantitatively the most important constituents of

surfactant. Phosphatidylcholine (PC) makes up 70-85% of the phospholipids (1,2). Abundant evidence indicates that the synthesis of PC increases during late gestation (1-3). The CDPcholine pathway is the primary pathway for *de novo* PC synthesis in the developing lung (reviewed in 1). Studies with whole lung have shown an increased activity of the CDPcholine pathway during late gestation (4). Pool size studies have demonstrated that the reaction catalysed by CTP:phosphocholine cytidylyltransferase (CT) is the rate-limiting step in the *de novo* synthesis of PC in fetal lung (5,6). However, when assayed under optimal conditions *in vitro*, only an increased activity of CT just before or immediately after birth has been demonstrated (7-10), but no good evidence exists for an activation of the enzyme during fetal development. The increased activity around birth has mainly been observed in cytosol (3,7). However, there is evidence from a number of systems, including whole lung (1), that the endoplasmic-associated enzyme is the more active form, and that the enzyme can be activated by subcellular translocation from cytosol to endoplasmic reticulum (reviewed in 11). These studies showed an activation by translocation of CT in response to different agents, but the physiologic importance of this mechanism during development has not been demonstrated. Furthermore, no data are available that demonstrate the importance of this mechanism in fetal type II cells, the producers of surfactant. Herein, we report that increased PC formation from radioactive precursors during fetal type II cell development is associated with an increase in the activity of CT. This increase in CT activity with advancing gestation correlates with a shift in subcellular distribution of total CT activity in type II cells from cytosol to endoplasmic reticulum.

3.3 Materials and methods

Materials

Female (200-250g) and male (250-300g) Wistar rats were purchased from Charles River (St. Constant, Quebec) and bred in our animal facilities. Cell culture media, antibiotics, and trypsin were obtained from Gibco Canada (Burlington, Ontario). Fetal calf serum (FCS) was from Flow Laboratories (McLean, VA), collagenase and DNase from Worthington Biochemical Corporation (Freehold, NJ). Cell culture flasks were purchased from Falcon (Becton Dickinson, Lincoln Park, NJ). All radioactive chemicals were from New England Nuclear Research (Dupont Canada, Mississauga, Ontario). All remaining unlabeled biochemicals were obtained from Sigma (St. Louis, MO).

Cell cultures

Timed pregnant rats were killed by diethylether excess on days 18 to 22 of gestation (term = day 22) and the fetuses were aseptically removed from the dams.

The epithelial cells were isolated as described in detail elsewhere (12,13). Although the term "type II cells" is used in this paper, the cuboidal epithelium which lines the acinar tubules during the late pseudoglandular and early canalicular stages of lung development, does not contain lamellar bodies, the phenotypic marker for type II cells. In previous studies, we have shown that these cells do express other phenotypic features of type II cells and possess antigenic determinants of mature type II cells (14).

Radioactive precursor incorporation into diacylglycerol and PC

Fetal epithelial cells were plated into plastic 24-well plates. At confluency (next day), the cells were rinsed with serum-free Eagle's minimal essential medium (MEM) and incubated with serum-free MEM containing either 1 μCi/ml [*methyl*-^{3}H]choline, 5 μCi/ml [1(3)-^{3}H]glycerol, 5 μCi/ml [1-^{14}C]acetate, or 10 μCi/ml [9,10 (n)-^{3}H]palmitate (complexed to bovine serum albumin in a molar ratio of 5.3:1). After 24 h of incubation, the medium was removed and the cells were washed with serum-free MEM. Following trypsinization to remove the cells from the plate, cellular lipids were extracted according to the method of Bligh and Dyer (15). In experiments with radioactive glycerol, palmitate, and acetate, half of the lipid extract was used for diacylglycerol and the other half for phospholipid determination. Incorporation of radiolabeled glycerol, acetate, and palmitate into diacylglycerols was measured. Neutral lipids were separated from the lipid extracts by thin-layer chromatography on precoated silica G plates using diethylether/petroleum ether/acetic acid (20:80:2, vol/vol/vol) as developing solution. The neutral lipid spots were visualized with iodine vapour.

PC was isolated from the lipid extract by thin-layer chromatography on silica H plates with chloroform/methanol/water (65:25:4, vol/vol/vol) as developing solution. To isolate disaturated phosphatidylcholine (DSPC), lipid extracts were treated with osmium tetroxide after which DSPC was isolated by thin-layer chromatography on precoated silica gel H plates with chloroform/methanol/ water (65:25:4) as eluent. PC spots were visualized with a bromothymol blue solution. The PC and diacylglycerol spots were transferred to scintillation vials and radioactivity was measured in a scintillation counter.

Cell fractionation

After overnight incubation in 75-cm^2 culture flasks, attached cells were collected by scraping in homogenization buffer of 145 mM NaCl, 50 mM Tris-HCl (pH 7.4), 50 mM NaF, and 2.5 mM EDTA (hereafter referred to as Tris saline). For each experiment, cells from two culture flasks were combined. After homogenization with 50 strokes of a Dounce homogenizer and sonication with a probe sonicator (3 x 20 sec at maximal output), the homogenate was centrifuged at 300x*g* for 10 min. The resulting supernatant was centrifuged at 13,000x*g* for 10 min to obtain a postmitochondrial supernatant. After an aliquot of this supernatant was stored, microsomal and cytosolic fractions were obtained by centrifugation at 313,000x*g* for 15 min. Microsomes were resuspended in a volume of Tris saline equal to that of the cytosol. All steps were carried out at 4°C. Cell homogenates and cell fractions were stored at -70°C until enzyme activities were measured.

Enzyme assays

Choline kinase activity was assayed by measuring the rate of incorporation of radiolabeled choline into phosphocholine. The incubation medium (0.1 ml) contained 100 mM Tris-HCl (pH 8.0), 30 mM $MgCl_2$, 10 mM ATP, 0.25 mM [*methyl*-^{14}C]choline (specific activity: 50 μCi/μmol), and up to 100 μg of protein. After a 15-min incubation period at 37°C, the reaction was terminated by the addition of 30 μl of glacial acetic acid. The reaction product, phosphocholine, was separated from radioactive choline by paper chromatography as described previously (16).

CT activity was assayed in the forward direction by measuring the rate of incorporation of [*methyl*-^{14}C]phosphocholine into CDPcholine. The incubation medium (0.2 ml) contained 20 mM Tris-succinate (pH 7.8), 6

mM $MgCl_2$, 4 mM CTP, 1.6 mM [*methyl*-^{14}C]phosphocholine (specific activity: 0.625 μCi/μmol) and up to 150 μg protein. Some assays were performed in the presence of 0.5 mM PC-OA (1:1 molar ratio) vesicles. These vesicles were prepared by sonication as described previously (17). After 40 minutes of incubation at 37°C, the reaction was stopped by addition of 0.1 ml of 25% (wt/vol) trichloroacetic acid and 0.5 ml of charcoal suspension (6% charcoal in 50 mM phosphocholine). The samples were placed on ice and [*methyl*-^{14}C]CDPcholine was isolated as described previously (18). The recovery (69-74%) of CDPcholine was determined in each set of assays by adding a known amount of [*methyl*-^{14}C]CDPcholine to a complete assay mixture. All assays were corrected for background and recovery.

Cholinephosphotransferase activity was determined by measuring the rate of incorporation of radiolabeled CDPcholine into PC using either endogenous or exogenous (15 mM) diacylglycerols as substrates. The cell homogenates (50-100 μg of protein) were incubated for 15 minutes at 37°C in 0.15 ml of incubation medium containing 100 mM Tris-maleate (pH 8.0), 25 mM $MgCl_2$, 10 mM ß-mercaptoethanol, 1 mM EDTA, and 0.5 mM [*methyl*-^{14}C]-CDPcholine (specific activity: 42 μCi/μmol). A 50-μl aliquot was taken for the measurement of the formation of radioactive PC by the filter disc method (19). The remaining 100 μl of the reaction mixture was extracted with chloroform/methanol (1:2, vol/vol) and the lipid extract was used for the determination of saturated phosphatidylcholine as described above. In preliminary experiments we established that, under the conditions used, product formation in all assays was directly proportional to incubation time and amount of protein.

Measurement of choline containing metabolites

The aqueous phase remaining after lipid extraction from cells incubated with radioactive choline was used to determine the choline intermediates. An aliquot (250 μl) of the aqueous layer (2.5 ml) was subjected to high performance liquid chromatography on an ion exchange column (Aminex A-27) in an isocratic mode (20). The phosphocholine and CDPcholine fractions were recovered from the column in scintillation vials and assayed for radioactivity.

Protein and DNA measurements

The protein concentrations were determined by the method of Bradford (21), using bovine serum albumin as standard. DNA was measured by the modified method of Burton (22).

Statistical analysis

Data are presented as means $\pm$ SE. The trend of enzyme activities and protein-to-DNA ratio with advancing gestation were analyzed with the Pearson product moment correlation coefficient (r). Statistical significance was accepted at the $p<0.05$ level (two-tailed).

3.4 Results

Incorporation of radioactive precursors into diacylglycerol and PC

In order to investigate whether surfactant PC production increases with type II cell development, the incorporation of several radioactive precursors into PC by type II cells isolated at different gestational ages was assessed.

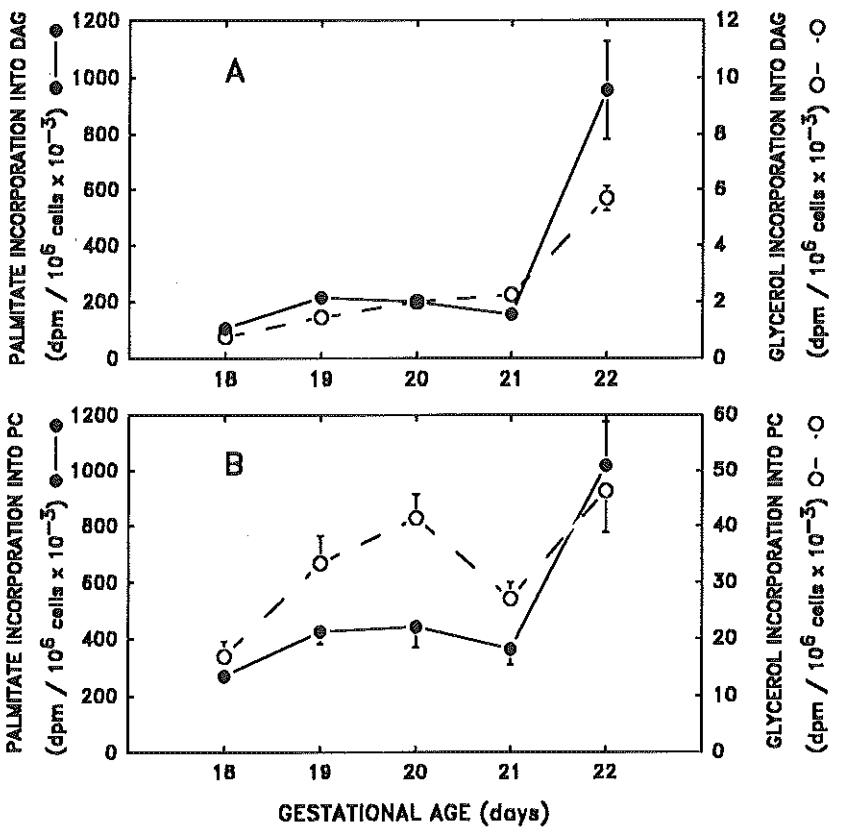

Figure 1. **Developmental profile of diacylglycerol (DAG) and phosphatidylcholine (PC) synthesis from radiolabeled palmitate and glycerol by fetal type II cells.** Type II cells in primary culture were incubated with MEM supplemented with 10 μCi/ml [9,10 (n)-^{3}H]palmitate or 5 μCi/ml [1(3)-^{3}H]glycerol. After 24 hours of incubation, cellular lipids were extracted and incorporation of radiolabeled precursors into DAG (A) and PC (B) was measured as described under "Materials and Methods". The data represent the means $\pm$ SE of at least three independent experiments carried out in quadruplicate. Where no error bars are drawn, SE are small and within the size of the data symbols.

As diacylglycerols provide a substrate for PC formation, the incorporation of labeled precursors into this important neutral lipid was also measured. As gestation advanced, incorporation of palmitate into diacylglycerols showed an initial small increase, followed by a small decrease on day 21, then a sixfold increase on day 22 (Fig. 1A). A similar pattern was observed for acetate incorporation into diacylglycerols (data not shown). Glycerol incorporation into diacylglycerols (Fig. 1A) increased progressively with advancing

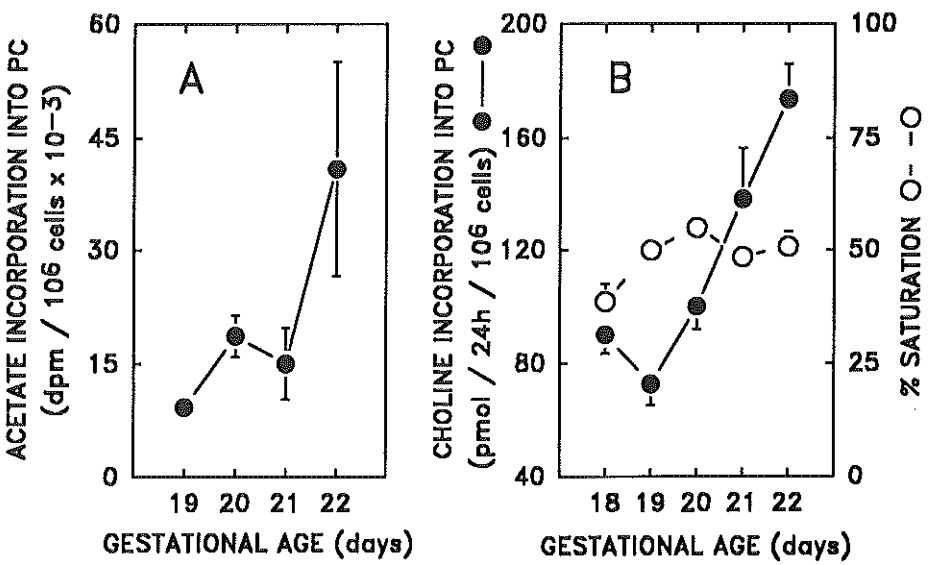

Figure 2. **Developmental profile of phosphatidylcholine (PC) synthesis from radiolabeled acetate and choline by fetal type II cells.** Type II cells in primary culture were incubated with MEM supplemented with 5 μCi/ml [1-^{14}C]acetate (A) or 1 μCi/ml [*methyl*-^{14}C]choline (B). After 24 hours of incubation, cellular lipids were extracted and incorporation of radiolabeled precursors into PC, as well as radiolabeled choline into disaturated PC, were measured as described under "Materials and Methods". Percentage of disaturated PC was calculated from disaturated and total PC (B). The data represent the means ± SE of at least three independent experiments carried out in quadruplicate. Where no error bars are drawn, SE are small and within the size of the data symbols.

gestation, again with the largest increase on day 22.

Glycerol and palmitate incorporation into total phospholipids increased 2.5-fold and >4-fold, respectively between day 18 and 22. Glycerol incorporation into PC increased more than twofold between day 18 and 20. A 35% decrease in incorporation was observed on day 21 followed by an increase on day 22 (Fig. 1B). Although incorporation of label into PC generally showed an increase during development, incorporation of glycerol into PC, as a percentage of total phospholipids, remained essentially constant

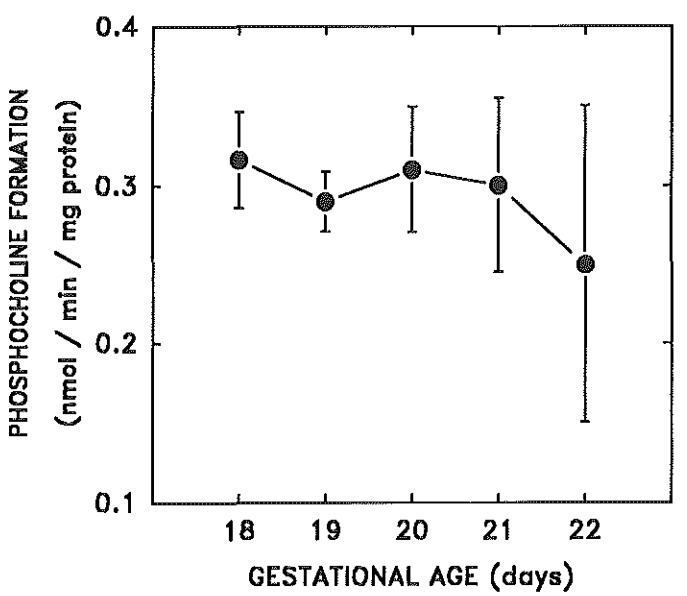

Figure 3. **Choline kinase activity in fetal type II cells during development.** Type II cells in primary culture, isolated from fetal rats at 18 to 22 days gestation, were homogenized and choline kinase activity was assayed by measuring the rate of incorporation of radiolabeled choline into phosphocholine. The data represent the means ± SE of three independent experiments carried out in duplicate (r=-0.21, p= N.S).

throughout late gestation (not shown).

Palmitate incorporation into PC exhibited a developmental profile similar to that observed with glycerol incorporation (Fig. 1B) and it resembles the pattern for palmitate incorporation into diacylglycerols (Fig. 1A). Again, the incorporation of palmitate into PC as a percentage of total phospholipids remained virtually unchanged (not shown). The developmental profile for acetate incorporation into PC was almost identical to that described for glycerol and palmitate incorporation into PC (Fig. 2A). As can be seen in Fig. 2B, the choline incorporation into PC by fetal type II cells increased with advancing gestation. Interestingly, the percentage of PC present in the saturated form remained essentially unchanged after day 19. Taken together, these data clearly indicate that PC synthesis increases in maturing type II cells.

Developmental profiles of enzymes involved in the CDPcholine pathway

As the CDPcholine pathway is the primary pathway for *de novo* PC synthesis in type II cells (1), the activities of choline kinase, CT and cholinephosphotransferase were measured in maturing type II cells. The specific activity of choline kinase exhibited no significant change with advancing gestation (Fig. 3). An increase in the specific activity of CT (Fig. 4) was noted during fetal development. The ratio of phosphocholine to CDPcholine also decreased (Fig. 4) and was mainly due to a decrease in incorporation of radiolabel into phosphocholine. This observation also supports the idea for an enhanced flux through the reaction catalysed by CT. Figure 5 illustrates the developmental profile for the specific activity of cholinephosphotransferase using endogenous diacylglycerols as substrate. No significant change was observed from day 18 to 22. Approximately 20% of the PC formed in

this reaction was present in the saturated form. This percentage did not change significantly with advancing gestation (Fig. 5). Although diacyl-

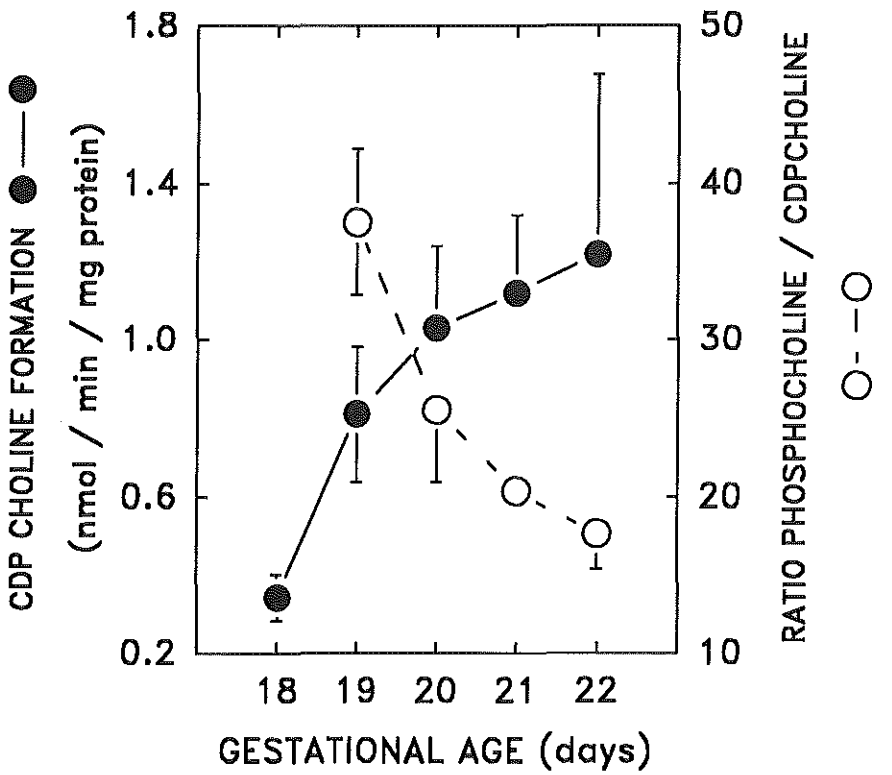

Figure 4. **CT activity and phosphocholine / CDPcholine ratio in fetal type II cells during development.** Type II cells in primary culture, isolated from fetal rats at 18 to 22 days gestation, were homogenized and CT activity was assayed by measuring the rate of incorporation of radiolabeled phosphocholine into CDP-choline (in the absence of lipid vesicles) ($r=0.61$, $p<0.02$). High-performance liquid chromatography was used for determinations of choline containing metabolites in the aqueous phase remaining after lipid extraction from type II cells from the choline incorporation studies ($r=-0.80$, $p<0.01$). The data represent the means ± SE of three independent experiments carried out in duplicate and quadruplicate. SE bars for ratio phosphocholine/CDPcholine of day 21 data point are small and within size of data symbol.

glycerol formation increased slightly during fetal development, it is possible that diacylglycerol availability influenced the measurements of the enzyme. Therefore, we measured the cholinephosphotransferase activity in the presence of 15 mM exogenous diolein (Fig. 6). The cholinephosphotransferase activity using exogenous diacylglycerols as substrate remained constant from day 18 to 20 but increased significantly at day 21. The increase in cholinephosphotransferase activity occurred, however, after the increase in CT activity. To exclude the possibility that the observed enzyme changes

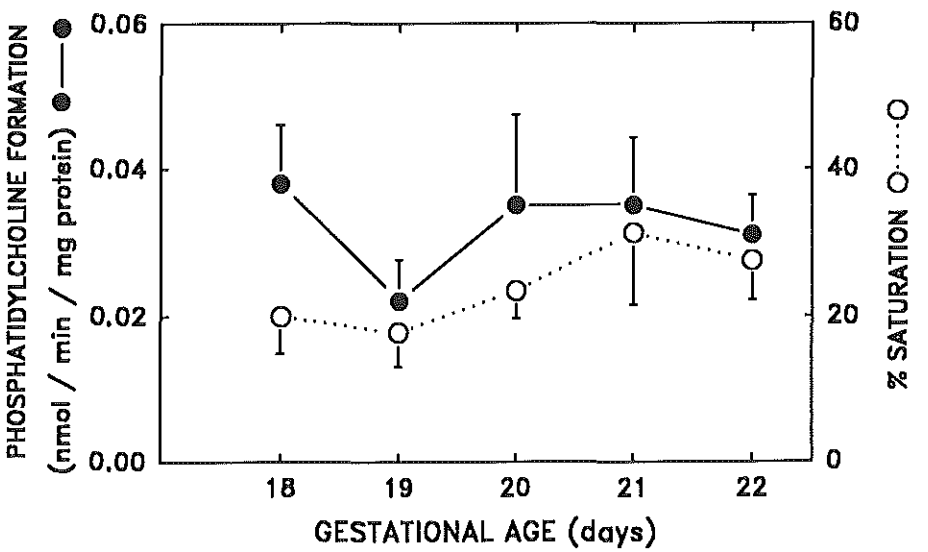

Figure 5. **Cholinephosphotransferase activity in fetal type II cells during development and percentage disaturated phosphatidylcholine formed from endogenous diacylglycerols.** Type II cells in primary culture, isolated from fetal rats at 18 to 22 days gestation, were homogenized and cholinephosphotransferase activity was assayed by measuring the rate of incorporation of radiolabeled CDPcholine into phosphatidylcholine (r=-0.01, p=N.S.).
Disaturated phosphatidylcholine formed in this reaction was measured and is presented as the percentage of total phosphatidylcholine synthesized (r=0.41, p=N.S.). The data represent the means ± SE of three independent experiments carried out in duplicate.

were due to increased overall protein synthesis during development, the protein and DNA levels of the cells were measured. The protein-to-DNA ratio ($\mu g/\mu g$) in the cells did not significantly change with advancing gestation (20.92 $\pm$ 6.60 on day 18 to 13.79 $\pm$ 4.47 on day 22; mean $\pm$ SE; r=-0.27 and p=NS). Although cholinephosphotransferase may play a regulatory role in PC synthesis at term, the findings suggest that CT controls PC synthesis in type II cells during fetal development.

Distribution of CT in subcellular fractions

Several studies with other systems, particularly HeLa cells and hepatocytes (reviewed in 11) have suggested that CT can be activated by subcellular translocation from cytosol to endoplasmic reticulum. To investigate whether the observed increases in PC synthesis (Figs. 1B and 2) and CT activity (Fig. 4) in type II cells during fetal development is accompanied by a shift in distribution of enzyme activity from cytosol to endoplasmic reticulum, the CT activity in the subcellular fractions of fetal type II cells was measured. As shown in figure 7A, the increase in specific activity of CT during development was greater in the microsomal fraction than in the cytosolic fraction. As expected, the specific activities in the post-mitochondrial supernatant were found to be intermediate of the activities of the fractions (not shown). During fetal development, the percentage of total activity of CT in cytosol of type II cells decreased (r=-0.52, p=0.023) while a corresponding increase in microsomal CT activity was noted (r=0.52, p=0.023) (Fig. 7B). CT activity was also determined in the presence of 0.5 mM PC/OA vesicles (1:1 molar ratio) (19). The developmental shift in subcellular distribution of CT from cytosol to microsomes was similar

when the enzyme activity was measured in the presence of the mixed lipid vesicles (in cytosol from 59.73±4.55% on day 18 to 42.29±3.08% on day 21; in microsomes from 40.27±2.41% on day 18 to 57.71±3.08% on day 21: r=±0.56, p=0.013). Also when unstimulated microsomes were compared with lipid stimulated cytosol, an almost identical shift in distribution of enzyme activity during fetal development was observed (r=±0.56, p=0.012) (not shown).

The stimulation of the cytosolic CT activity by lipids decreased from 262% at day 18 to 181% of control at day 21, while the lipid stimulation of microsomal CT activity (approx. 150% of control) remained constant during fetal development.

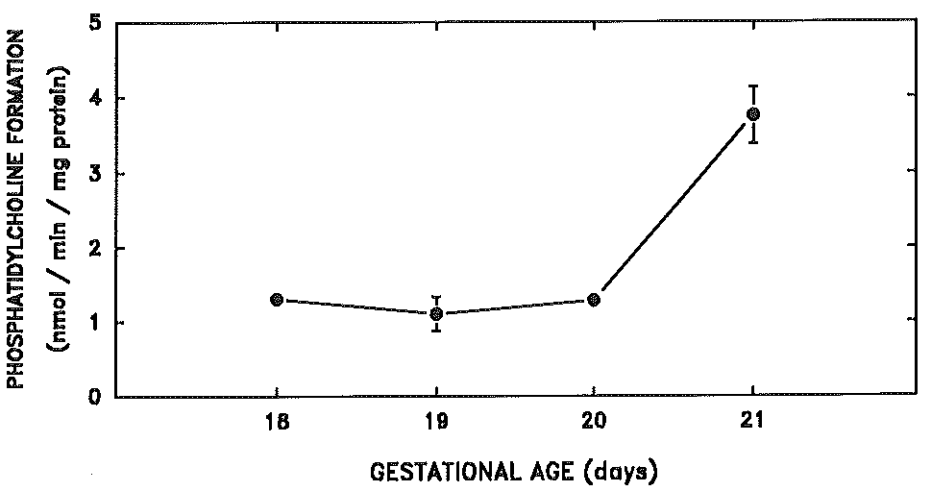

Figure 6. **Cholinephosphotransferase activity in fetal type II cells using exogenous diacylglycerols.** Type II cells in primary culture, isolated from fetal rats at 18 to 21 days gestation, were homogenized and cholinephosphotransferase activity was assayed by measuring the rate of incorporation of radiolabeled CDPcholine into phosphatidylcholine using 15 mM diolein. The data represent the means ± SE of a representative experiment carried out in triplicate.

3.5 Discussion

The present study shows that the developmental increase in PC synthesis in fetal type II pneumocytes during late gestation is associated with an increase in CT activity. The choline kinase and cholinephosphotransferase activities remain constant or increase just before birth. This increase in specific activity of CT during fetal development is more pronounced in microsomes than in cytosol. The developmental increase in CT activity is accompanied by a developmental shift of enzyme activity from cytosol to endoplasmic reticulum.

Taken together, these data provide further evidence that CT catalyses a regulatory step in the CDPcholine pathway for *de novo* PC synthesis in fetal type II cells. The finding that a shift in subcellular distribution of enzyme activity from cytosol to microsomes coincides with increased CT activity led us to conclude that the enzyme may be activated during development by a subcellular translocation of enzyme from cytosol to endoplasmic reticulum (11). Another explanation for the observed shift might be that gene expression of CT increases with advancing gestation and that new CT protein is mainly bound to microsomes. Both mechanisms may be involved in controlling surfactant PC synthesis in maturing type II cells. It is evident, however, that both translocation of CT molecules from cytosol to microsomes and CT gene expression need further investigation to elucidate their role in PC synthesis by fetal type II cells.

The present study also focuses on whether direct *de novo* synthesis may contribute to the formation of DSPC in fetal type II cells. Earlier

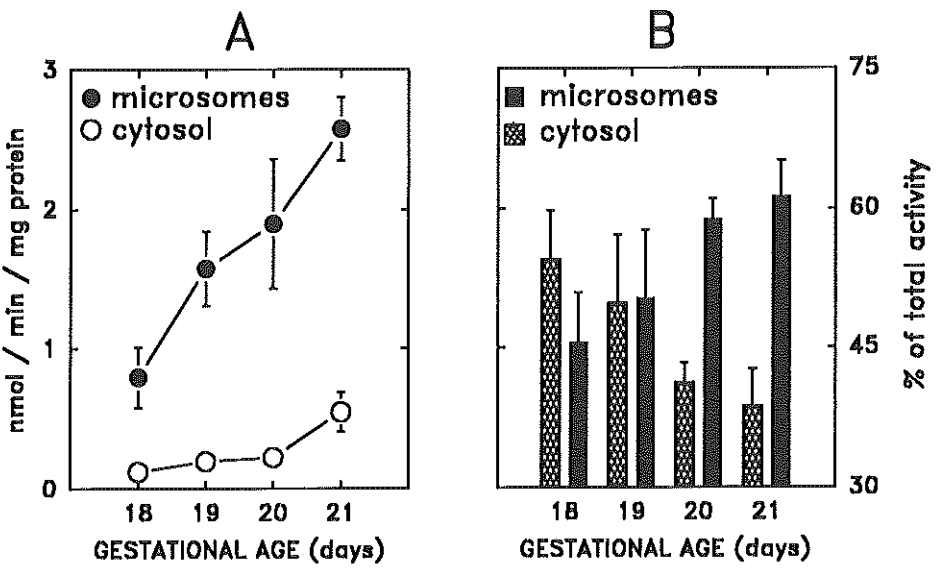

Figure 7. **Specific activity and subcellular distribution of CT in cytosol and microsomes of fetal type II cells as function of development.** Cytosolic and microsomal fractions were prepared from type II cells in primary culture, isolated from fetal rats at 18 to 21 days gestation. CT activity was assayed in the different fractions. In figure A specific activities are expressed as nmol/min/mg protein. In figure B the percentage of total activity in each fraction is calculated as the total activity in that fraction (specific activity x total mg protein) x 100, over the sum of the total activities in cytosol and microsomes. The data represent the means ± SE of four or five independent experiments carried out in duplicate. Where no error bars are drawn for cytosolic activities in *A*, SE are small and within the size of the data symbols.

studies with rat lung microsomes, homogenates and also adult type II cells have suggested that cholinephosphotransferase does utilize endogenous disaturated diacylglycerols for the formation of DSPC (23-26). In the present study, we found that incubation of fetal type II cell homogenates with CDP[*methyl*-^{14}C]choline resulted in the formation of labeled PC that was 20-25% saturated. This finding suggests a direct conversion of endogenous

disaturated diacylglycerols into DSPC. However, other explanations cannot be excluded. Unsaturated PCs synthesized by cholinephosphotransferase could be rapidly remodelled, or labeled CDPcholine could be hydrolysed to choline or phosphocholine with subsequent introduction of these substrates into existing DSPCs by a base-exchange mechanism. Previous studies (27) with type II cells isolated from adult rat lung have shown that the cells have the capability of hydrolysing CDPcholine. However type II cell homogenates incubated under the same conditions as with CDPcholine, did not incorporate the hydrolysis products choline and phosphocholine into PC, even under conditions which promote base exchange. If we assume that direct *de novo* synthesis of DSPCs occurs from disaturated diacylglycerols, the results of the present study suggest that approximately half of the total DSPCs are synthesized from this source. Similar contributions of *de novo* synthesis to total synthesis of saturated PC have been reported in previous studies using adult type II cells (25) or lung microsomes (23). Lower figures have been published by Mason and Nellenbogen (24), who estimated from pulse-chase experiments that 25% of the DSPC in type II cells was made via synthesis de novo.

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CHAPTER 4

INCREASED EXPRESSION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE IN MATURING TYPE II CELLS

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4.1 Abstract

We previously reported that phosphatidylcholine (PC) synthesis increased in fetal rat lung type II cells with advancing gestation. This increase was accompanied by an increase in CTP:phosphocholine cytidylyltransferase (CT) activity, which catalyses a rate-regulatory step in *de novo* PC synthesis by fetal type II cells. To determine whether this increase in CT activity is due to an increase in CT protein levels, the gene and protein expression of CT was investigated in maturing type II cells. The CT cDNA was cloned from fetal rat type II cells and showed 99% sequence homology with rat liver cDNA. The cDNA detected two mRNA transcripts (1.8 and 7.5 kb) in fetal rat lung. By reverse-transcriptase polymerase chain reaction (RT-PCR) analysis, CT mRNA content increased threefold in fetal type II cells with advancing gestation, whereas CT mRNA levels in fibroblasts remained constant. An antibody against rat liver CT was used to assess CT protein. Western blotting revealed that CT protein content increased threefold in the microsomal fraction of type II cells with advancing gestation. The enzyme protein levels in the cytosolic fraction did not significantly change with development. Enzyme activity studies confirmed these latter observations. We conclude that the increase in surfactant PC synthesis by type II cells at late fetal gestation is due in part to an increase in the amount of CT protein.

4.2 Introduction

Adequate amounts of pulmonary surfactant are essential for proper lung functioning immediately after birth. Pulmonary surfactant stabilizes the lung at end expiration by reducing the surface tension at the air-liquid interface of the alveoli. Pulmonary surfactant is composed of lipids and proteins. Phospholipids are quantitatively the most important constituents of surfactant. Phosphatidylcholine (PC) makes up 70-85% of the phospholipids (1,2). Ample evidence has accumulated indicating that the synthesis of PC increases during late gestation (1-3). The CDPcholine or Kennedy pathway is the primary pathway for *de novo* PC synthesis in the developing lung (reviewed in 1). Studies with whole lung have shown an increased activity of the CDPcholine pathway during late gestation (4). Pool size studies have demonstrated that the reaction catalysed by CTP:phosphocholine cytidylyl-transferase (CT) is the rate-limiting step in the *de novo* synthesis of PC in fetal lung (5,6). Recently, we reported that increased PC formation during fetal type II cell development is associated with an increase in the activity of CT (7). This increase in CT activity with advancing gestation was mainly observed in the microsomal membrane fraction of type II cells (7). In addition, we noted a developmental shift in the distribution of CT activity from cytosol to microsomes. This increase in CT activity in maturing type II cells might be due to a subcellular translocation of CT from cytosol to endoplasmic reticulum. Another possibility is that the developmental increase in CT activity is caused by an increase in CT protein. In this report, we cloned CT from fetal type II cells and further investigated CT gene and protein expression in fetal type II cells during late gestation.

Evidence is presented that the increased synthesis of surfactant PC by fetal type II cells during late gestation is due to an increase in CT expression.

4.3 Materials and methods

Materials

Female (200-250g) and male (250-300g) Wistar rats were purchased from Charles River (St. Constant, Quebec) and bred in our animal facilities. The sources of all cell culture material have been described elsewhere (8). A rabbit polyclonal antibody to a synthetic peptide (D-F-V-A-H-D-D-I-P-Y-S-S-A) corresponding to residues 164-176 of rat liver CT was a generous gift of Dr. D.E. Vance [University of Alberta, Edmonton, Canada] (9). [*Methyl*-^{14}C]phosphocholine was from New England Nuclear (Dupont, Mississauga, Ontario). Biotinylated goat anti-rabbit immunoglobulin G, streptavidin-biotinylated peroxidase complex and a chemiluminescence detection system were from Amersham (Oakville, Ontario). Phosphodiester oligodeoxy-nucleotides were synthesized on a 391 DNA synthesizer from Applied Biosystems (Foster City, CA). All remaining biochemicals were obtained from Sigma Chemical (St. Louis, MO).

Cell culture

Rats were killed by diethylether inhalation on days 18 to 21 of gestation (term = 22 days). The fetuses were aseptically removed from the mothers, the fetal lungs dissected out in cold Hanks' balanced salt solution (HBSS) without calcium or magnesium [HBSS (-)] and cleared of major airways and vessels. The lungs were washed twice in HBSS (-), minced and

suspended in HBSS (-). Fibroblasts and epithelial cells were isolated from the fetal lungs as previously described in detail (8). Briefly, the lung tissue was digested for 20 min in an enzymatic solution of 0.125% trypsin and 0.4 mg/ml DNase. After filtering through 100-μm mesh nylon bolting cloth, Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS) was added and the mixture centrifuged. The pellet was resuspended in MEM containing 0.1% collagenase. After a 15-min incubation, the collagenase activity was neutralized by adding MEM+10% FBS. Two 1-h differential adhesion periods in tissue culture flasks allowed removal of fibroblasts. The nonadherent cells were removed, transferred to new culture flasks, and incubated overnight for attachment of epithelial cells. Non-adherent cells were removed from all cell cultures after overnight incubation. All experiments were performed 24-36 h after the start of isolation. Viability and purity of the cultures were not affected by gestational age and were comparable to previously published data (8,10). Although the term "type II cells" is used in this paper, the cuboidal epithelium which lines the acinar tubules during the late pseudoglandular and early canalicular stages of lung development does not contain lamellar bodies, the phenotypic marker for type II cells. In previous studies , we have shown that these cells do express other phenotypic features of type II cells and possess antigenic determinants of mature type II cells (11).

Cell fractionation

After overnight incubation in 75-cm^2 culture flasks, attached cells were collected by scraping in a homogenization buffer of 145 mM NaCl, 50 mM Tris-HCl (pH 7.4), 50 mM NaF and 2.5 mM EDTA (hereafter referred to as Tris-saline). For each experiment, cells from two culture flasks were

combined. After homogenization with 50 strokes of a Dounce homogenizer and sonication with a probe sonicator (3 x 20 sec at maximal output), the homogenate was centrifuged at 300 x *g* for 10 min. The resulting supernatant was centrifuged at 13,000 x *g* for 10 min to obtain a post-mitochondrial supernatant. After an aliquot of this supernatant was stored, microsomal and cytosolic fractions were obtained by centrifugation at 313,000 x *g* for 15 min. Microsomes were resuspended in a volume of Tris-saline equal to that of the cytosol. All steps were carried out at 4°C. Protein content of samples was determined according to Bradford (12). Cell homogenates and cell fractions were stored at -70°C until CT activities and protein levels were measured.

CT activity assay

CT activity was assayed in the forward direction by measuring the rate of incorporation of [*methyl*-^{14}C]phosphocholine into CDPcholine. The incubation medium (0.2 ml) contained 20 mM Tris-succinate (pH 7.8), 6 mM $MgCl_2$, 4 mM CTP, 1.6 mM [*methyl*-^{14}C]phosphocholine (specific activity: 0.625 μCi/μmol) and up to 150 μg protein. All assays were performed in the presence of 0.5 mM PC-oleic acid (OA) (1:1 molar ratio) vesicles. These vesicles were prepared by sonication as described previously (13). After 40-minutes of incubation at 37°C, the reaction was stopped by addition of 0.1 ml of 25% (wt:vol) trichloroacetic acid and 0.5 ml of charcoal suspension (6% charcoal in 50 mM phosphocholine). The samples were placed on ice and [*methyl*-^{14}C]CDPcholine was isolated as described previously (14). The recovery (69-74%) of CDPcholine was determined in each set of assays by adding a known amount of [*methyl*-^{14}C]CDPcholine to a complete assay mixture. All assays were corrected for background and

recovery.

Reverse-transcribed polymerase chain reaction (RT-PCR) cloning of rat lung type II cell CT

The protocol was modeled after the rapid amplification of cDNA ends (RACE) procedure (15). Briefly RNA was isolated by lysing the tissue in 4 M guanidinium thiocyanate followed by centrifugation on a 5.7 M cesium chloride cushion to pellet RNA (16). After sequential extraction with phenol:chloroform (1:1, vol:vol) the RNA was ethanol precipitated and collected by centrifugation. This RNA was lyophilized and dissolved in sterile water. For 3-RACE, the RNA was reverse transcribed with avian myeloblastosis virus (AMV) reverse transcriptase and a dT_{17}-adaptor primer, T17-CATGATCAGCTGCGCACCGG. The cDNA was then amplified for two rounds of 35 cycles with amplitaq DNA polymerase (Perkin-Elmer/ Cetus, Norwalk, CT). The primers for the first round of amplification were CT-specific sense primer 1: 5'-GATTTCGTCGCCCATGACGATAT-3' complementary to nucleotide 591-614 of the conserved region of rat liver CT (17) and antisense primer: 5'-GGCCACGCGTCGACTAGTAC-3' complementary to the adaptor sequence of the dT_{17}-adaptor primer. The 3'-RACE products were then reamplified in a second round of amplification using the nested sense CT-specific primer: 5'-CCCGCATTGTCCGTGACTATGATG-3' complementary to nucleotides 721-745 of the conserved region of rat liver CT (17) and the 3'-primer complementary to the adaptor sequence of the dT_{17}-adaptor primer. For 5'-RACE, the RNA was reverse transcribed using a 3'-RACE sequence specific CT primer:
5'-CTTTTCGTTGATAAAGCTGACATT-3'. Terminal deoxynucleotidyl-transferase was then used to attach oligo(dC) tails to the 3'ends of the first-

strand cDNA. Tailed cDNA was amplified with 40 cycles of PCR using a nested antisense CT-specific primer:
5'-CACATCGTCGCTCCCTGCCGA-3' complementary to nucleotides 625-646 of the conserved region of rat liver CT (17) and a sense anchor primer 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3' which was specific for the oligo(DC) tail. After PCR amplification, RACE-products were analyzed by 2% agarose gel electrophoresis. DNA of positive PCR reactions were then directly ligated into pCR vector (Invitrogen, San Diego, CA) with T4 DNA ligase. After transformation of competent *Escherichia Coli*, positive colonies were picked for sequence analysis using the dideoxy chain termination method (Pharmacia, Baie D'Urfe, Quebec) according to the manufacturer's instructions.

Northern analysis

Total RNA was isolated from day 21 fetal rat lung and liver essentially as described by Chirgwin *et al.* (16). This RNA was used to prepare poly(A)$^+$ RNA and poly(A)$^-$ RNA using one cycle of chromatography on oligo(dT)-cellulose. Total RNA and poly(A)$^+$ and poly(A)$^-$ selected RNA were dissolved in 25 mM EDTA/0.1% (wt:vol) sodium dodecyl sulfate (SDS), denatured for 2 min at 100°C, fractionated on 1 % (wt:vol) agarose gels containing 6% (vol:vol) formaldehyde and transferred to a nylon membrane, which was fixed by baking at 80°C for 2 h. CT cDNA was labeled with deoxycytidine 5'-[α-^{32}P] triphosphate by a random primed labeling system. Prehybridization (overnight), and hybridization, was performed in hybridization solution of 50% (vol:vol) formamide, 750 mM NaCl, 75 mM sodium citrate, 5xDenhardt's solution, 10% (wt:vol) dextran sulfate, and 100 μg/ml denatured salmon sperm DNA for 24 h at 42°C. The

blot was washed twice with 2xSSC; 0.2% (wt:vol) SDS at 42°C for 10 min. The blot was exposed for 24-48 h to Kodak XAR-5 film using Dupont Cronex Intensifying screens.

RT-PCR assay for CT message

Total RNA was isolated essentially as described by Chirgwin *et al.* (16). This RNA was used to prepare cDNA using AMV reverse transcriptase. The cDNAs from the reverse transcription reaction were then amplified using PCR. The primers chosen for amplification of CT were primers 1 and 4 (Fig. 1A). The predicted product size with these primers was 225 bp. The primers chosen for amplification of ß-actin, based on the mouse ß-actin mRNA sequence reported by Alonso *et al.* (18), predict a 540-nucleotide product. These were: 5'-primer: 5'-GTGGGCCGCTCTAGGCACCAA-3', complementary to nucleotides 25-46; and 3'-primer:
5'- CTCTTTGATGTCACGCAGGATTTC-3', corresponding to nucleotides 540-564. The PCR products were separated by electrophoresis on a 2% (wt:vol) agarose gel and then visualized by ethidium bromide staining. If required, amplified DNA was transferred to a nylon membrane by Southern blotting. The blots were hybridized with radiolabeled CT probe and results obtained by autoradiography.

Immunoprecipitation and Western analysis of CT

Cytosol and microsomes were prepared from fetal type II cells as described in *Cell fractionation*. To remove all cytosolic CT, microsomal fractions were incubated for 8 min with cold digitonin buffer containing phosphate-buffered saline (PBS), 250 mM sucrose, 0.5 mg/ml digitonin, and 0.5 mM phenylmethylsulfonyl fluoride. Digitonin-released proteins and

membranes were separated by centrifugation at 100,000 x *g* for 60 min at 4°C. Cytosolic and membrane samples (400 μg of protein) were precleared by incubation with 3 μl of nonimmune rabbit IgG for 30 min at 4°C, followed by the addition of 50 μl of 10% (vol:vol) Formalin-fixed *Staphylococcus aureus* Cowan strain A (Zysorbin) in PBS. The incubation was continued for 30 min at 4°C before centrifugation for 5 min at 12,000 x *g*. Rabbit anti-CT (10 μl) was added to the samples and incubated overnight on an end to end rotator at 4°C. Zysorbin (50 μl) was then added to precipitate the immune complexes. After a 60-min incubation at 4°C, immune complexes were washed three times with lysis buffer, boiled for 3 min in sample buffer [10% (vol:vol) glycerol, 2% (wt:vol) SDS, 5% (vol:vol) β-mercaptoethanol, 0.0025% (wt:vol) bromophenol blue, 0.06 M Tris-HCl, pH 8.0] to dissociate immune complexes, and the immunoprecipitated proteins were separated on 10% (wt:vol) SDS-polyacrylamide gel (SDS-PAGE) as described by Laemmli
(19). The separated proteins were electrophoretically transferred to nitrocellulose membrane (20). Nonspecific binding was blocked by incubating the nitrocellulose membrane with 3% (wt:vol) dry skim milk in PBS at 4°C for 60 min, then rabbit anti-CT (1:100) was added to detect the immunoprecipitated CT. After overnight incubation at 4°C, the nitrocellulose membrane was washed three times with PBS, followed by incubation with biotin-conjugated goat anti-rabbit IgG (1:1,000) for 60 min at 4°C. The membrane was washed again three times with PBS, and then incubated with streptavidin-biotinylated horseradish peroxidase complex (1:700 dilution) for 60 min at 4°C. The membranes were then thoroughly washed with cold PBS (2 x 10 min), immersed in chemiluminescence detection reagents for 1 min and exposed to Amersham Hyperfilm-ECL for

10 s in an X-ray cassette.

4.4 Results and discussion

Cloning of CT

We used RT-PCR to clone CT cDNA from fetal rat lung type II cell RNA in several fragments as shown in figure 1. We first amplified a 225-bp fragment using two oligonucleotide primers (1 and 4) based on the rat liver CT cDNA sequence (17). The region between the primers covered part of the central region of the rat liver CT with 100 % sequence homology to yeast CT. The nucleotide sequence of the 225-bp fragment (Fig. 1) exhibited a 100 % homology with the corresponding region of the rat liver CT cDNA. We used this sequence information to design nested primers 2 and 3 for RACE-PCR. CT sequence-specific primers 1 and 2 with nonspecific primer 5, which annealed to a sequence element incorporated during the reverse transcription, were then used to amplify the 3' half of the coding region and the 3' untranslated region of the mRNA out to the poly(A) tract (Fig. 1). This amplification yielded one major and three minor bands which were cloned and sequenced. Clones of the major band showed 100% sequence identity with rat liver cDNA. The other three clones displayed 100% sequence homology for the conserved region of rat liver cDNA but showed no further similarity with 3' end of the coding region of rat liver CT. After cloning and sequencing of 3' RACE products, we used CT sequence-specific antisense primer 6 to start first strand cDNA synthesis for 5' RACE. Following homopolymeric tailing of 3' ends of cDNA, tailed cDNA was then used to amplify the 5' end of the coding region and the 5'

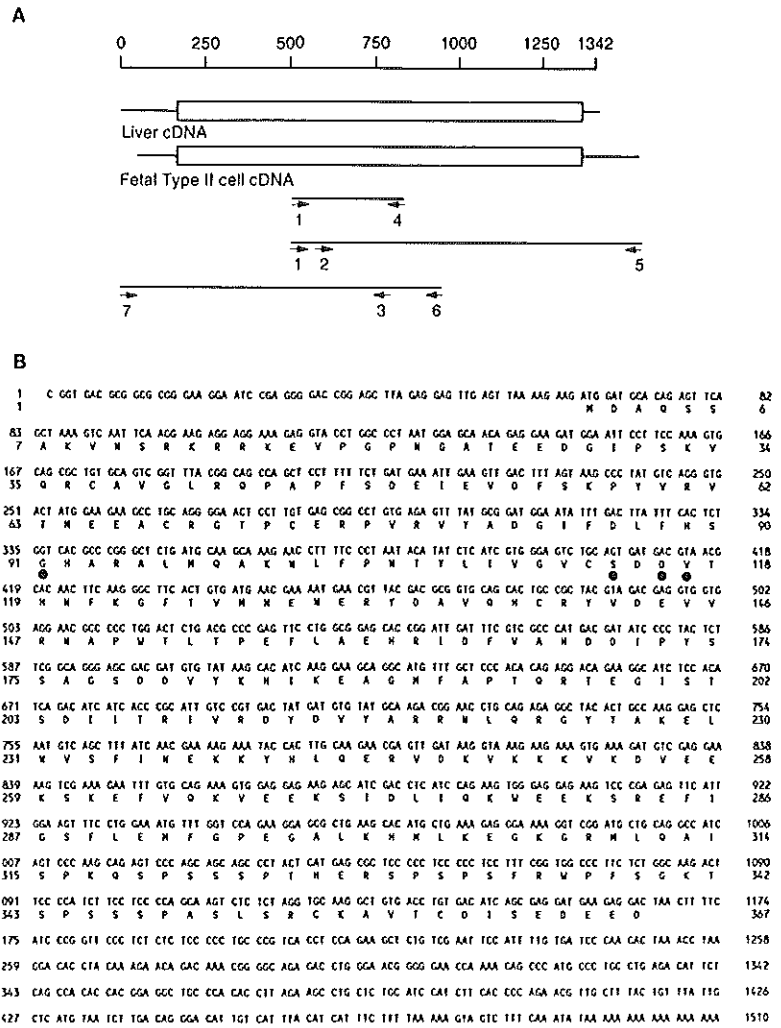

Figure 1. **Cloning and sequence of fetal rat type II cell CTP:phosphocholine cytidylyltransferase (CT).** A: The cloning strategy used for isolation of fetal type II cell CT cDNA. Three fragments were polymerase chain reaction (PCR) amplified from fetal rat type II cell cDNA. Small numbered arrows denote primers referenced in text. B: Sequence of fetal type II cell CT 3 clones. Nucleotide sequence is shown with presumed reading frame. (●) amino acids in presumed reading frame which are different from liver CT (17).

untranslated region of message (Fig. 1). CT sequence specific primer 3 and nonspecific primer 7 , which anneals to a sequence element incorporated during 3' homopolymeric tailing, were used for amplification. One major 5'RACE product was obtained, cloned, and sequenced. The rat lung type II cell CT cDNA sequence is shown in figure 1B. The coding region demonstrated 99% sequence similarity with rat liver cDNA. The putative amino acid sequence was altered at four positions when compared to that of rat liver CT (17). All changes were observed at the 5' end of the coding region. The changes resulted in an additional potential site for phosphorylation by protein kinase C compared with rat liver CT (17). Evidence suggests that reversible phosphorylation might be one of the mechanisms by which CT activity is regulated (1,9). However, it is also possible that the nucleotide changes are due to misreading of DNA during the PCR reaction.

By using the 5'RACE cDNA product (Fig.1) as a CT probe, we found that the probe hybridized to two different sizes of RNA on blots of poly(A)$^+$ selected RNA isolated from day 21 fetal rat lung and liver (Fig. 2). In both tissues, there was a major band of $\approx$ 7.5 kb and a second, less abundant, RNA of $\approx$ 1.8 kb. The RNA sizes are consistent with published data for rat liver (17) and fetal rat lung (21).

CT gene and protein expression in maturing type II cells

In previous experiments with fetal type II cells, we found that CT activity increased with advancing gestation (7). To investigate whether the increase in CT activity was accompanied by an increase in CT gene expression, we measured the steady-state CT mRNA levels. Our Northern analysis suggested that CT mRNA is present in very small quantities. Therefore, we

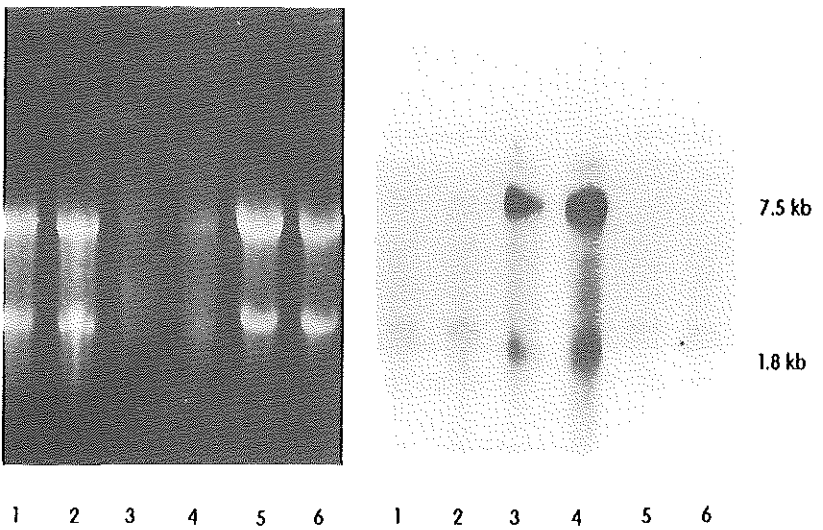

Figure. 2. **Northern blot analysis of RNA from day 21 fetal rat lung and liver for cytidylyltransferase.** *Left:* a photograph of the ethidium-bromide stained gel. *Right:* a Northern blot of mRNA hybridized with ^{32}P-labeled CT cDNA after 48h of autoradiography at -70°C. *Lanes* 1, 3 and 5, fetal liver; *lanes* 2, 4 and 6, fetal lung. Lanes 1 and 2: 15 μg total RNA; *lanes* 3 and 4: 8 μg poly(A)$^+$ RNA; *lanes* 5 and 6: 15 μg poly (A)$^-$ RNA. The sizes of the hybridizing transcripts are indicated in kilobases.

measured CT mRNA by RT-PCR using the oligonucleotide primers 1 and 4 (Fig. 1). RT-PCR of 1 μg of total RNA of fetal type II cells yielded the predicted single 225-bp cDNA fragment (Fig. 3). No bands were observed when PCR was performed without first transcribing mRNA with reverse transcriptase, suggesting that the amplified RT-PCR products are not due to DNA contamination of the RNA samples. To test RNA integrity and the efficiency of the reverse-transcriptase reaction of each sample, RT-PCR for ß-actin was carried out. Message of ß-actin in type II cells did not change with advancing gestation (Fig. 3). Southern analysis of the RT-PCR prod-

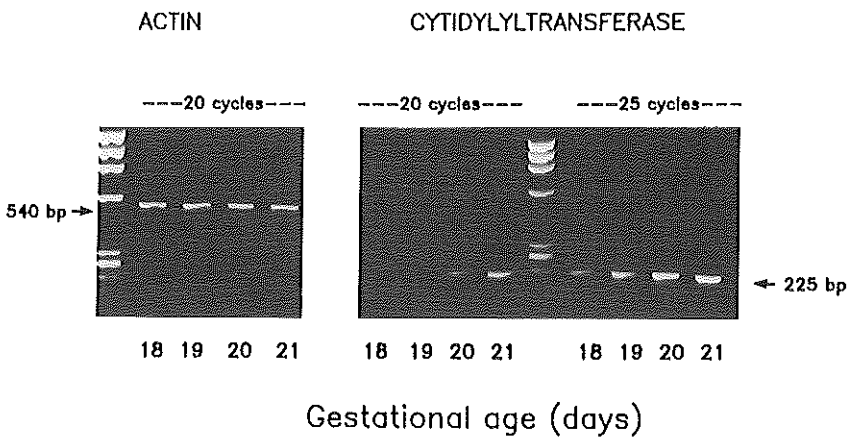

Figure 3. **Expression of cytidylyltransferase (CT) and ß-actin mRNAs in fetal type II cells.** Total RNA was extracted from fetal type II cells in primary culture. Reverse transcriptase (RT)-PCR analysis was carried out on 1 μg total RNA as described in "Materials and Methods". Ethidium bromide-stained agarose gel showing amplified CT (225 bp) and ß-actin (540 bp) products after either 20 or 25 cycles of PCR is representative of 4 experiments.

ucts, using a [^{32}P]-labeled CT cDNA corresponding to positions -65 to 708 in figure 1B as probe, revealed that the amplified 225-bp CT cDNA fragment was detectable with 20 cycles of PCR in fetal type II cells (Fig. 4). The CT mRNA content increased with advancing gestation (Figs. 3 and 4). We observed a threefold increase in the relative amount of CT mRNA of fetal type II cells between day 18 and day 21 of gestation (Fig. 5). In contrast, CT mRNA content of fetal lung fibroblasts did not change with development (Fig. 5). This increase in message for CT in maturing type II cells correlates with the previously observed increase in CT activity (7).

We then investigated whether the increase in CT gene expression was accompanied by an increase in enzyme mass. To measure the content of CT

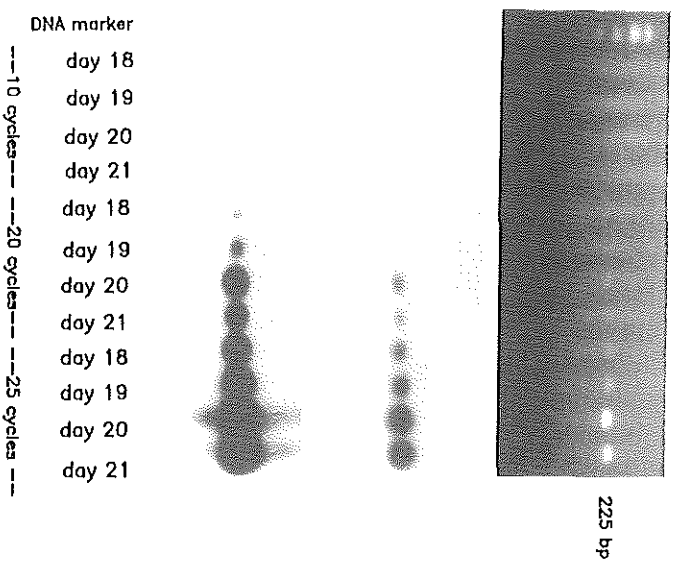

Figure 4. **Southern analysis of cytidylyltransferase (CT) RT-PCR products of fetal type II cell mRNA.** *Top:* Ethidium bromide-stained agarose gel showing amplified CT product (225 bp) after either 10, 20 or 25 cycles of PCR on 1 μg RNA of fetal type II cells. *Middle:* Southern blot of amplified (10, 20 and 30 cycles) CT PCR product hybridized with ^{32}P-labeled CT cDNA after 2h autoradiography at room temperature. *Lower:* Southern blot of amplified (10, 20 and 30 cycles) CT PCR product after 2h autoradiography at -70°C.

in the soluble and membrane fractions of fetal type II cells, we first immunoprecipitated CT with CT antiserum and then determined CT in the immunoprecipitates by enhanced chemiluminescence Western blot immunoanalysis. Figure 6 shows a representative Western blot immunostained with CT antiserum. The rabbit antibodies raised against the CT peptide (9) immunoprecipitated and recognized a major 42-kDa protein in both cytosolic and microsomal samples of fetal type II cells, consistent with the previously reported molecular mass for the M_r 42,000 catalytic subunit from purified rat liver CT (9,14,22). This observation suggests that CT of fetal rat type II

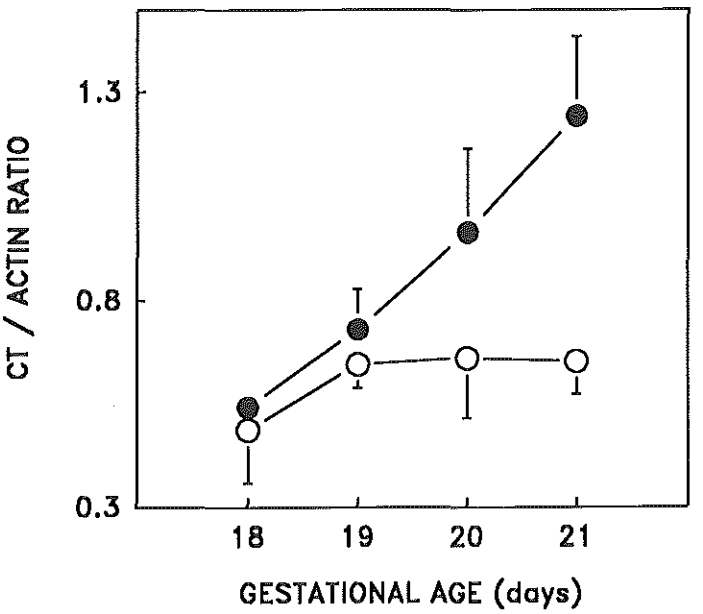

Figure 5. **Determination of message for cytidylyltransferase (CT) in fetal type II cells and fibroblasts as a function of development.** Total RNA was extracted from type II cells and fibroblasts isolated from fetal rat lung at 18 to 21 days' gestation. CT and ß-actin gene expression was assessed by RT-PCR as described in "Materials and Methods. The gels were photographed and the negatives were used to quantify messages for CT and ß-actin by laser densitometry. Ontogeny of CT mRNA is expressed as a ratio of the arbitrary units of CT over ß-actin. Values are mean ± S.E. of 4 separate experiments. (O) fetal lung fibroblasts; (●) fetal lung type II cells.

cells is immunologically similar to that of adult rat liver. As assessed by laser densitometry the immunoreactive content of microsomal CT increased approximately threefold between day 18 and 21 of fetal gestation, whereas the CT content in the cytosol remained constant (Fig. 7). These results are in agreement with our previous finding that microsomal CT activity in type II cells increased with advancing gestation (7). The observation that the cytosolic content of immunoreactive CT did not change with advancing

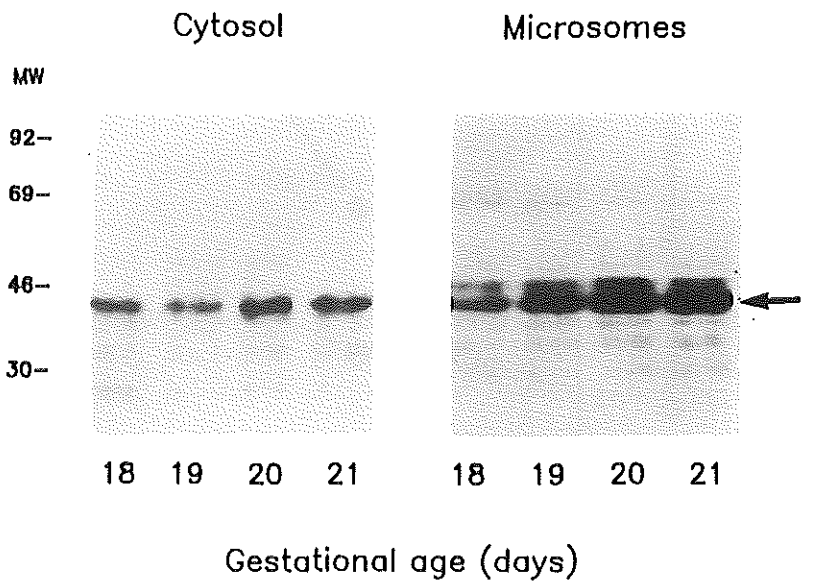

Figure 6. **Immunoprecipitation and Western blot immunoanalysis of cytidylyltransferase (CT) in cytosol and microsomes of fetal type II cells as a function of development.** Cytosolic and microsomal fractions were prepared from type II cells in primary culture, isolated from fetal rats at 18 to 21 days of gestation. Aliquots (400 μg of protein) of both fractions were incubated with 10 μl of rabbit anti-cytidylyltransferase as described in "Materials and Methods". Immunoprecipitated CT was loaded and separated on a 10% (wt:vol) polyacrylamide gel under reducing conditions. Protein was transferred to a nitrocellulose membrane and immunostained with cytidylyltransferase antibodies. Western blot is representative of 2 separate experiments.

gestation suggests that the increase in microsomal CT mass is due to the observed increase in CT gene expression. However, we have to assume that the peptide antibody recognizes both cytosolic forms of CT, the inactive L-form and the active H-form (23,24). The cytosolic H-form, which is a lipoprotein complex consisting of L-form and lipids, seems to be similar to the active enzyme associated with microsomal membranes (24). If the

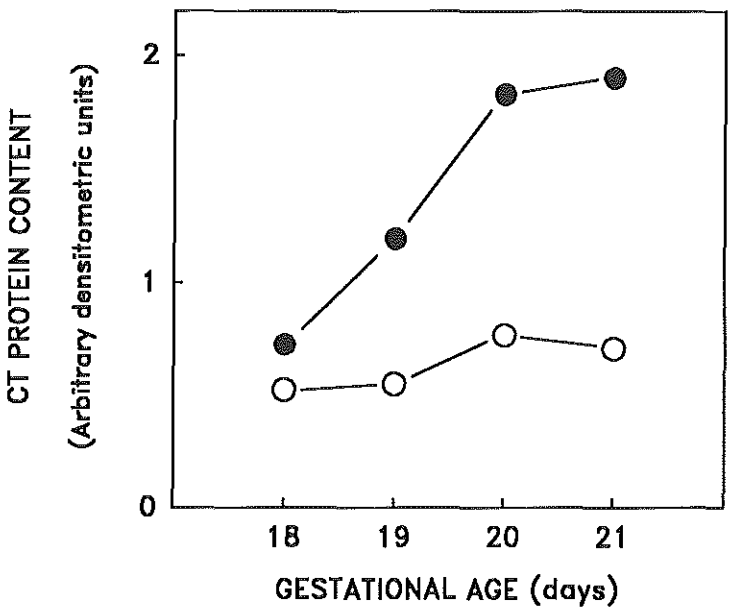

Figure 7. **Ontogeny of cytidylyltransferase (CT) protein in cytosol and microsomes of fetal type II cells as a function of development.** Cytosolic and microsomal CT was determined by immunoprecipitation and Western immunoanalysis as described in "Materials and Methods". The enhanced chemiluminescence Western blot was scanned on a laser densitometer. (O) cytosol; (●) microsomes. Figure is representative of 2 separate experiments.

antibody does not recognize the predominantly cytosolic L-form, it is possible that the increase in microsomal CT protein is in part due to a translocation of the cytosolic L-form to the microsomal membrane. To investigate this possibility, we determined the ontogeny of CT activity in the presence of optimal amounts of lipid activators (13). Although the specific activities of CT measured in the presence of lipids (Fig. 8) were significantly higher than CT measured in the absence of the lipids (7), the developmental profiles of both cytosolic and microsomal CT activities measured in the presence of lipids were similar to those measured in the absence of lipid

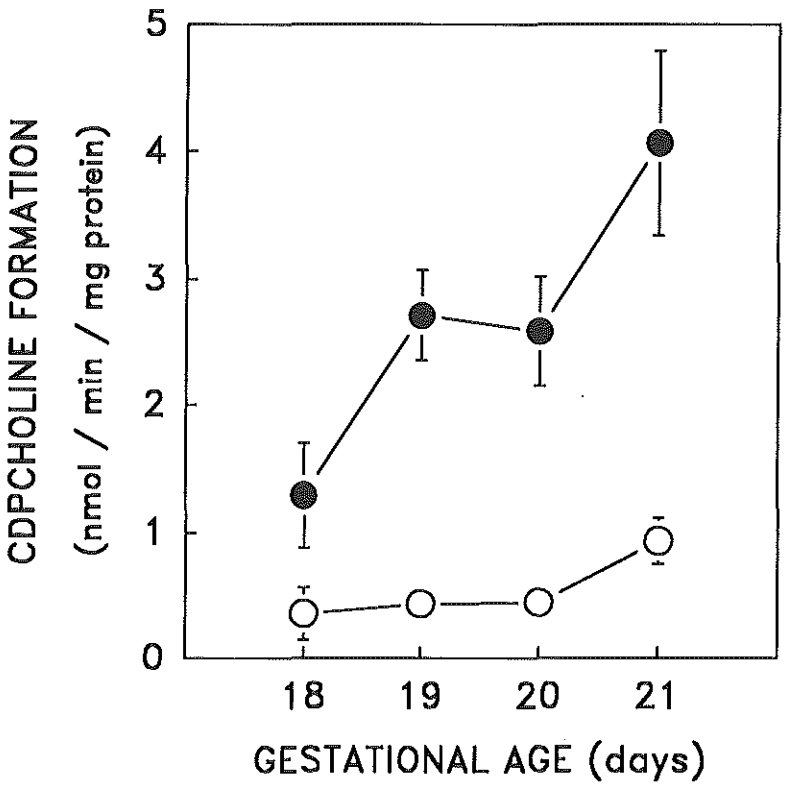

Figure 8. **Specific activity of cytidylyltransferase (CT) in cytosol and microsomes of fetal type II cells as a function of development.** Cytosolic (O) and microsomal (●) fractions were prepared from type II cells in primary culture, isolated from fetal rats at 18 to 21 days of gestation. CT activity was assayed in different fractions in presence of 0.5 M phosphatidylcholine/oleic acid vesicles. Data represent the means ± S.E. of 4 independent experiments carried out in duplicate.

activators (7). Independent of the assay condition, microsomal CT activity increased threefold with advancing gestation whereas cytosolic CT remained constant (7). This suggests that the increase in microsomal CT activity in maturing type II cells is not due to an activation of preexisting inactive enzyme by translocation from cytosol to microsomes. We have reported

earlier (7) that the protein-to-DNA ratio did not change with advancing gestation. The percentage of total protein in cytosol and microsomes remained also constant during fetal development (in cytosol from 56.1 ± 3.8% on day 18 to 63.3 ± 3.7% on day 21; in microsomes from 12.5 ± 1.8% on day 18 to 14.5 ± 0.5% on day 21). Based upon these findings, we calculated that total CT protein increased approximately twofold with advancing gestation and that the percentage of total CT protein in the microsomal fraction increased from approximately 20% on day 18 to 45% on day 21.

In conclusion, our results indicate that the increased synthesis of surfactant PC by maturing type II cells is due to an increase in CT gene and protein expression. The observation by Fraslon and Batenburg (21) that CT mRNA increases in hormone-free explant cultures of 18-day fetal rat lung supports this idea. It is of interest that the developmental profiles of surfactant proteins A, B and C mRNAs are similar to that of CT (25-28). When the need to produce large amounts of surfactant becomes important, expression of both CT and surfactant proteins increases in maturing type II cells. This suggests that the expression of both surfactant proteins and lipids in fetal type II cells is well coordinated. Although it is tempting to speculate that CT expression in type II cells is regulated in a similar fashion as surfactant proteins, the developmental regulation of CT expression remains to be elucidated. The differential gene expression of CT in fetal type II cells and fibroblasts suggests that CT gene expression in fetal type II cells is regulated at the transcriptional level.

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CHAPTER 5

REGULATION OF CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE BY CYTOSOLIC LIPIDS IN RAT TYPE II PNEUMOCYTES DURING DEVELOPMENT

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5.1 Abstract

CTP:phosphocholine cytidylyltransferase (CT) catalyses a rate regulatory step in the *de novo* synthesis of surfactant phosphatidylcholine (PC). We have previously shown that CT activity increases during late gestation in alveolar type II cells, and that this increase is most pronounced in microsomes. As it is known that CT is activated by lipids, we investigated the lipid activation of CT in fetal type II cells during late gestation. The degree of activation of cytosolic CT by PC/oleic acid (OA) (1:1 molar ratio) vesicles was gestation dependent (a 3-fold stimulation on day 18 and a 1.5-fold stimulation on day 21). In contrast, microsomal CT activation by PC/oleic acid (OA) vesicles (1.5-fold) remained constant with advancing gestation. Lipids extracted from microsomes of fetal type II cells of different gestational ages (day 18 to 21) did not differ in their ability to activate either cytosolic CT of day 18 or day 21 fetal type II cells, purified CT from adult lung or delipidated purified CT. In contrast, lipids extracted from cytosol of fetal type II cells of different gestational ages (day 18 and 21) differed in their ability to activate either delipidated cytosolic CT of fetal type II cells, or delipidated purified CT from adult lung. Day 21 cytosolic lipids activated CT more than day 18 cytosolic lipids. Both cytosolic and purified CT, when delipidated by acetone/butanol extraction, showed reduced activities. Several lipids were tested for their ability to activate cytosolic CT. Acidic phospholipids and the mixture of PC/OA (1:1) were the strongest stimulators of cytosolic CT activity. We conclude that cytosolic but not microsomal lipids are involved in the developmental activation of cytosolic CT in fetal type II cells at late gestation.

5.2 Introduction

The production of pulmonary surfactant, which is produced by the alveolar type II cells, is initiated during the latter part of gestation. The subsequent accumulation of surfactant in the airways coincides with the ability of the newborn to establish regular air breathing. Surfactant deficiency due to lung immaturity is the main factor responsible for the occurrence of respiratory distress syndrome in premature neonates. Phosphatidylcholine (PC) is a major component of surfactant (1,2). CTP:phosphocholine cytidylyltransferase (CT) (E.C. 2.7.7.15) has been demonstrated to catalyse a rate-limiting step in the de novo synthesis of PC in the developing lung (3,4). We have previously shown that CT activity increases in fetal type II cells at late gestation (5). The increase in enzyme activity coincides with an increase in PC synthesis (5). The developmental regulation of CT in fetal type II cells remains to be elucidated. Recently, we have reported that the increased PC synthesis by fetal type II cells at late gestation is due, in part, to an increase in CT protein (6). However, there is overwhelming evidence that CT may also be regulated by enzyme-membrane interactions (reviews 7,8). The enzyme exists in an inactive soluble form and an active membrane-bound form (9,10). Recent studies have identified the lipid-binding domain of CT as an α-helical domain of the protein (11,12). Our previous studies with fetal type II cells demonstrated that CT protein content and activity increased in the microsomal fraction with advancing gestation with no developmental change in the cytosolic fraction (5,6). In whole fetal lung, fatty acids (13-15) and phospholipids (16,17) may play an important physiologic role in the activation of CT during development, possibly through CT-membrane

interactions (13). However, results from studies with whole lung cannot be directly extrapolated to developmental changes in type II cells, the producers of surfactant, as pointed out in several reviews (2,18,19). In the present study, therefore, we investigated CT activation by microsomal and cytosolic lipids of maturing type II pneumocytes. An abstract of these studies has been published previously (20).

5.3 Materials and methods

Materials

Female (200-250g) and male (250-300g) Wistar rats were purchased from Charles River (St. Constant, Quebec) and bred in our animal facilities. Cell culture media, antibiotics and trypsin were obtained from Gibco Canada (Burlington, Ontario). Fetal calf serum was from Flow Laboratories (McLean, VA), collagenase and DNAse from Worthington Biochemical (Freehold, NJ). Cell culture flasks were purchased from Falcon (Becton Dickinson, Lincoln Park, NJ). [*Methyl*-^{14}C]phosphocholine and [*Methyl*-^{14}C]CDPcholine were from New England Nuclear Research (Dupont Canada, Mississauga, Ontario). All remaining unlabeled biochemicals were obtained from Sigma (St. Louis, MO).

Cell cultures

Timed pregnant rats were killed by diethylether excess on days 18 to 21 of gestation (term = day 22) and the fetuses were aseptically removed from the dams. The epithelial cells were isolated from the fetal lungs as described in detail elsewhere (21-23). Although the term 'type II cells' is used in this paper, the cuboidal epithelium which lines the acinar tubules

during the late pseudo-glandular and early canalicular stages of lung development does not contain lamellar bodies, the phenotypic marker for type II cells. In previous studies, we have shown that these cells do express other phenotypic features of type II cells and possess antigenic determinants of mature type II cells (22,24).

Cell fractionation

After overnight incubation in tissue culture flasks, attached cells were collected by scraping in homogenization buffer of 145 mM NaCl, 50 mM Tris-HCl (pH 7.4), 50 mM NaF and 2.5 mM EDTA (hereafter referred to as Tris-saline). Postmitochondrial supernatant, microsomal and cytosolic fractions were obtained as previously described (5) and stored at -70°C until processed further.

Enzyme assay

CT activity was assayed in the forward direction by measuring the rate of incorporation of [*methyl*-^{14}C]phosphocholine into CDPcholine as previously described (5,21).

Purification of CT

Rats were killed by diethylether excess and immediately afterwards a tracheostomy was performed and lungs were inflated with a pressure of 15 cm H_2O. The thorax was opened and the lungs were thoroughly perfused with normal saline via injection in the right ventricle and opening the left atrium. Lungs were immediately frozen in liquid nitrogen and kept at -70°C till the purification was started. CT was purified from 100 g lung as described by Weinhold *et al.* for liver (25). A 2278-fold purification was

obtained and a yield of 9.34% of total cytosolic activity (21).

Delipidation of purified CT and cytosol

Purified CT and cytosolic CT were delipidated by acetone/butanol extraction according to the method of Fiscus and Schneider (26) as modified by Chu and Rooney (17). The final pellet was resuspended in Tris-saline (see above) by sonication for 2 x 20 s at 0°C and foam was removed under vacuum.

Lipid extraction and preparation of (phospho)lipid vesicles

Total lipids were extracted from cell fractions with chloroform/methanol by the method of Bligh and Dyer (27). Phospholipid phosphorus was measured according to Bartlett (28). All microsomal and cytosolic lipid concentrations are therefore given as μM of lipid phosphorus. Lipids to be tested were dried under a stream of N_2 at 40°C. After traces of solvent were removed under high vacuum, lipids were resuspended in Tris-saline (see above) by sonication (29) for 3x30 s, or longer when the turbid suspension had not clarified.

Protein measurements

Protein concentrations were determined by the method of Bradford (30), using bovine serum albumin as standard.

Statistical analysis

Statistical differences between various groups were analyzed by paired or unpaired t-test (for two groups) or by analysis of variance with the Neumann-Keuls test (for more than two groups). Statistical significance was

accepted at the $p < 0.05$ level (two-tailed).

5.4 Results

Stimulation of CT activity of fetal type II cells by phospholipid vesicles

We first investigated whether activation of CT activity in cytosol and microsomes by phospholipids varied as a function of development. As PC/OA (1:1 molar ratio) vesicles at a (combined) concentration of 0.5 mM (PC and OA each 0.25 mM) have been shown to stimulate CT activity maximally (29), we assayed CT activity in both cell fractions in the absence and presence of 0.5 mM PC/OA (1:1) vesicles. The CT activities in cytosol and microsomes measured in the absence of PC/OA were in the same range as published previously (5). Independent of gestational age, CT activity in the microsomal fraction of fetal type II cells was stimulated approximately 1.5-fold by PC/OA vesicles (Fig. 1). In contrast, activation of cytosolic CT by PC/OA vesicles was gestation dependent. It was activated 3-fold on day 18 but 1.5-fold on day 21 (Fig. 1). The greater lipid stimulation of cytosolic CT activity in day 18 compared to that of day 21 fetal type II cells suggests that fetal type II cell cytosol contains a higher proportion of inactive CT at 18 days than at 21 days of gestation.

Activation of CT by microsomal lipids of maturing type II cells

We then determined whether microsomal lipids of maturing type II cells differed in their ability to activate CT. Microsomal lipids of day 18 and day 21 fetal type II cells were extracted and their effect on cytosolic CT activity of day 18 and day 21 fetal type II cells was assessed. In addition,

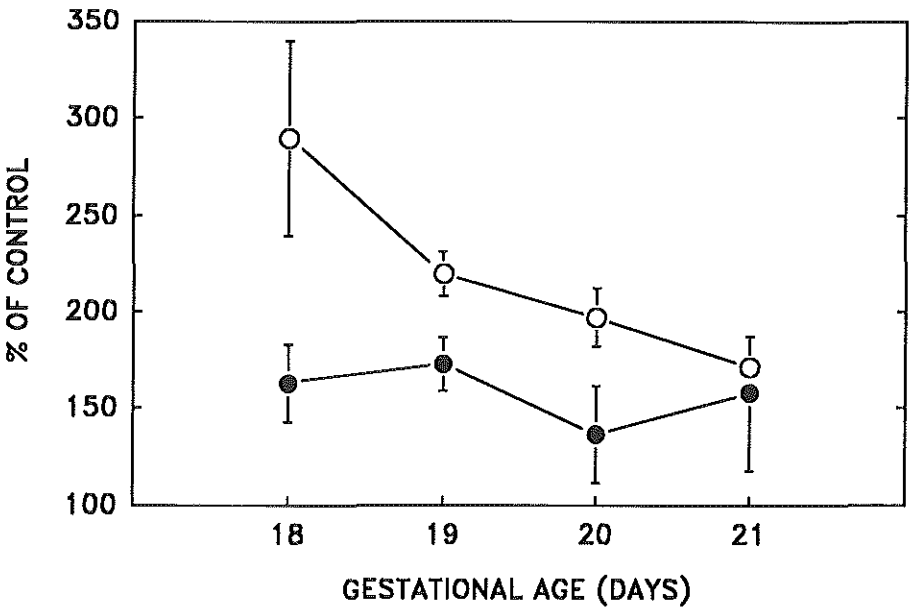

Figure 1. **Gestation-dependent activation of cytidylyltransferase in subcellular fractions by phosphatidylcholine/oleic acid (PC/OA) vesicles.** Cytosolic (◯) and microsomal (●) fractions were prepared from type II cells in primary culture, isolated from fetal rats at 18 to 21 days of gestation. CT activity was assayed in both fractions in the absence (control) and presence of 0.5 mM PC/OA vesicles (1/1 M ratio, 0.25 mM each), which are known to activate the enzyme. Data are presented as percentage of control (activity in the presence of PC/OA x 100 / activity in the absence of PC/OA). Each data point represents mean ± S.E. of at least 4 separate experiments, performed in duplicate.

this effect was compared with those of microsomal lipids from whole adult lung and PC/OA (1:1) vesicles. Figure 2 shows that day 18 and day 21 microsomal lipids stimulated the cytosolic CT activity in a concentration-dependent manner and to a similar degree as PC/OA vesicles. Stimulation of cytosolic CT activity by microsomal lipids of adult lung was less pronounced than the stimulation by microsomal lipids of fetal type II cells. Day 18 and day 21 cytosolic CT activities were different when assayed without lipids

(0.29 versus 0.56 nmol/min/mg protein) but CT activity in both cytosols was stimulated maximally to the same value (1.82 and 1.86 nmol/min/mg protein) by 100 μM lipids (assayed by phospholipid phosphorus) of either day 18 or day 21 fetal type II cell microsomes. The amount of microsomal lipids of type II cells increased slightly from day 18 (0.016 μg phosphorus/μg protein) to day 21 (0.020 μg phosphorus/μg protein). Thus, although the amount of microsomal lipids increases during development, there is no qualitative developmental difference in the ability of these microsomal lipids to activate CT in fetal type II cells. This was confirmed by testing the ability of 50 μM microsomal lipids of fetal type II cells of 18-21 days' gestation to stimulate purified lung CT (Table 1). Microsomal lipids from day 18 to day 21 fetal type II cells stimulated CT activity. However, no significant gestation-dependent differences in CT activation were observed. Since the presence of residual lipids may mask the effect of microsomal lipids on purified CT, we delipidated purified CT by acetone/butanol extraction. The extraction drastically reduced the CT activity (1310 nmol/min/mg protein to 46 nmol/min/mg protein measured with 25 μl of the purified enzyme solution). The activity of the delipidated CT was partially restored by the addition of 50 μM PC/OA (1:1, 25 μM each) vesicles (817 nmol/min/mg protein), but not to the same level as the unextracted purified CT activated by 50 μM PC/OA (1:1) vesicles (3184 nmol/min/mg protein). Microsomal lipids (50 μM lipid phosphorus) of fetal type II cells also increased the activity of the delipidated enzyme but less than the PC/OA vesicles (Table 1). Again, there was no difference in the ability of day 18 and day 21 microsomal lipids of fetal type II cells to stimulate delipidated purified CT (Table 1).

Table 1. **Activation of purified lung cytidylyltransferase by microsomal and cytosolic lipids.**

Addition	cytidylyltransferase activity nmol/min/mg protein (fold increase)			
	purified CT		delipidated purified CT	
no lipids added	1310±127	(1)	46±14	(1)
PC/OA (1/1 M)	3184±275	(2.43)	817±308	(17.78)
Microsomal lipids				
- day 18	2358±131	(1.80)	268±70	(5.82)
- day 19	2397±197	(1.83)		
- day 20	2083±118	(1.59)		
- day 21	1900±223	(1.45)	234±38	(5.09)
Cytosolic lipids				
- day 18	3013±26	(2.30)	386±75	(8.39)*
- day 19	3263±157	(2.49)		
- day 20	3236±170	(2.47)		
- day 21	3275±13	(2.50)	682±172	(14.82)*

CT was purified from whole adult lung and the activation of CT was studied in the presence of 50 μM mixed PC/OA (1/1 M ratio, 25 μM each) vesicles or lipids (50 μM lipid phosphorus) extracted from microsomes or cytosol from type II cells isolated at different days of gestation. In separate experiments, CT was first delipidated by acetone/butanol extraction before studying lipid activation (protein recovery during extraction was 60%). The CT activities are expressed nmol/min/mg protein (and fold change compared to controls without the addition of lipids). Mean ± S.D. are shown for 4 separate experiments.
* significantly different ($p<0.02$) by t-test.

Activation of CT by cytosolic lipids of maturing type II cells

As previous studies with whole fetal lung have suggested that cytosolic CT can be activated by cytosolic lipids (16,17), we also examined the effect of cytosolic lipid extracts of fetal type II cells on CT activation. In preliminary experiments it was found that the activation of CT in delipidated cytosol by cytosolic lipids was concentration dependent. Saturation (maximal stimulation) of CT activity was obtained with 5 times the amount of lipids present in the original cytosol (approximately 95 μM of lipid phosphorus)

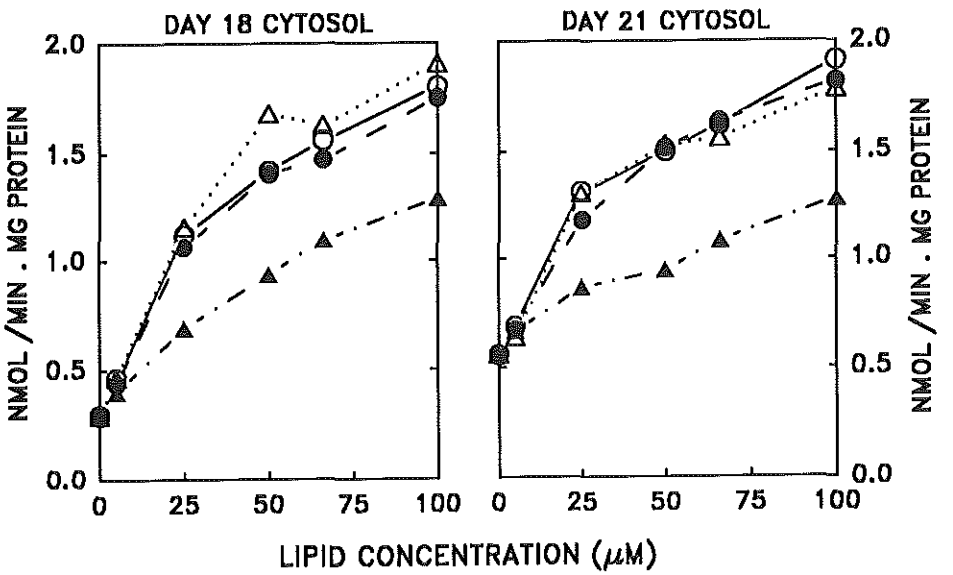

Figure 2. **Activation of cytosolic cytidylyltransferase by microsomal lipids.** CT activity was assayed in day 18 (left) and day 21 (right) cytosol in the presence of different concentrations of PC/OA (1/1 M ratio) vesicles (△) (expressed as sum of [PC]+[OA]) or in the presence of different concentrations of lipids (expressed as μM of lipid phosphorus) extracted from microsomes of day 18 fetal type II cells (○), day 21 fetal type II cells (●) or adult whole lung (▲). Assays were performed in duplicate. The experiment was repeated with almost identical results.

(not shown). Using the same saturating conditions, we then compared the ability of cytosolic lipids from day 18 and day 21 fetal type II cells to stimulate delipidated cytosolic CT of day 18 and day 21 fetal type II cells (Fig. 3). Delipidation of cytosol of day 18 and day 21 fetal type II cells resulted in a drastic reduction of CT activity. The CT activities of both day 18 and day 21 delipidated cytosol were restored on re-addition of cytosolic lipids (approx. 0.5 nmol/min/mg protein). However, this activity is still lower than that of unextracted cytosolic CT activated by lipids (approx. 1.85

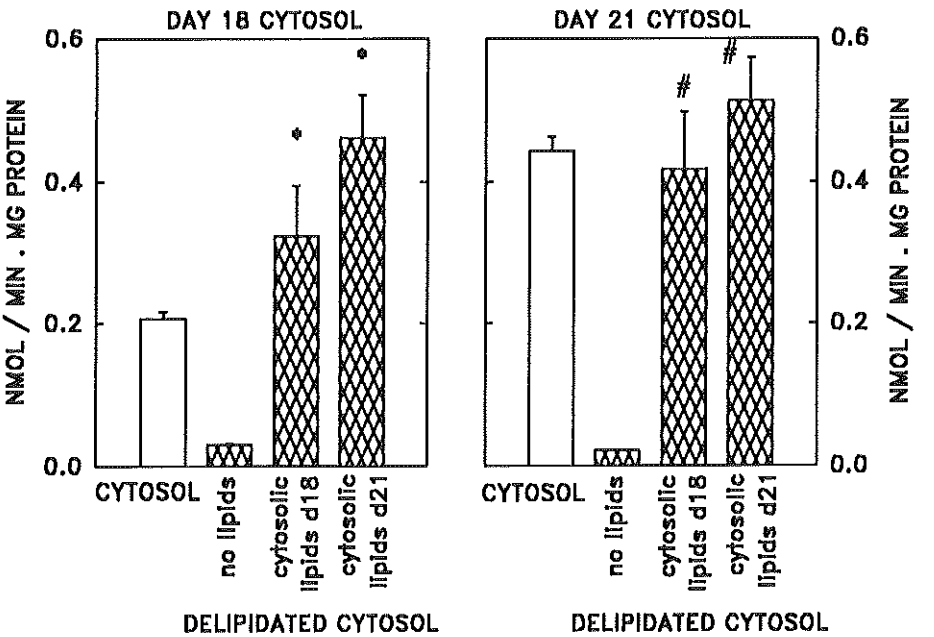

Figure 3. **Activation of delipidated cytosolic cytidylyltransferase by cytosolic lipids from day 18 and 21 fetal type II cells.**
CT activity was assayed in normal cytosol (open bars) or in cytosol delipidated by acetone/butanol (cross-hatched bars) of day 18 (graph on the left) and day 21 (graph on the right) fetal type II cells. CT activity in delipidated cytosols was assayed in the absence and presence of cytosolic lipids from day 18 and 21 fetal type II cells. The amount of lipids added to the delipidated cytosol was 5 times the amount of lipid present in the original cytosol (the average final concentration in the assay was 96 μM for day 18 cytosolic lipids and 92.4 μM for day 21 cytosolic lipids). Data are presented as mean $\pm$ S.E. of 4 separate experiments, all performed in duplicate. Comparison between the activities in the presence of either d18 or d21 cytosolic lipids by paired t-test (two-sided): * p=0.075 (n=4, all in duplicate) and # p=0.064 (n=4, all in duplicate); all 8 comparisons (day 18 and day 21 CT data) taken together p=0.019 (n=8, all in duplicate).

nmol/min/mg protein). It appeared that cytosolic lipids of day 21 had a greater stimulatory effect on CT activity than day 18 cytosolic lipids [significantly different when all 8 comparisons (day 18 and 21 enzyme data) were

taken together (n=8), p=0.019 by paired t-test]. As these measurements were performed under saturating conditions of lipids, these data suggest that there is a difference in cytosolic lipid composition between day 18 and day 21 cytosol of fetal type II cells. In order to confirm these cytosolic findings, we tested the ability of cytosolic lipids (50 μM of lipid phosphorus) to activate purified CT delipidated by acetone/butanol extraction (Table 1). Day 21 cytosolic lipids activated delipidated CT to a significantly greater extent than day 18 cytosolic lipids. When the same experiment was carried out with non-delipidated purified CT, no significant gestation-dependant differences were observed (Table 1), likely because the effect is masked by the presence of residual lipids in the purified CT preparation.

Comparison of activation of cytosolic CT by various lipids

Cytosolic lipids, microsomal lipids or combinations of both (all from day 20 fetal type II cells), stimulated CT activity of delipidated day 20 cytosol (0.04 nmol/min/mg protein) to the same level as 0.5 mM PC/OA (1:1, each 0.25 mM) vesicles (1.27 nmol/min/mg protein), when saturating conditions of lipids were used. However, CT in delipidated cytosol could not be activated to the level found in cytosol before acetone/butanol extraction (2.42 nmol/min/mg protein in presence of 0.5 mM PC/OA vesicles). To further investigate which lipid(s) may be responsible for the activation of cytosolic CT, we investigated the effect of two phospholipids, PC and phosphatidylglycerol, and OA on cytosolic CT activity. Figure 4 shows that the stimulatory effect of PC/OA (1:1) vesicles on cytosolic CT activity was dependent on concentration up to 50 μM (25 μM of each PC and OA) when maximum stimulation was reached. Stimulation of CT with PC/OA (1:1) vesicles was noted up to at least 1 mM. A similar concentration vs. CT

activity curve was obtained with phosphatidylglycerol vesicles. OA alone stimulated CT activity at low (<100 μM) but not at high concentrations (>200 μM). PC vesicles alone inhibited cytosolic CT activity. Based upon these results a variety of other lipids were tested at a concentration of 50 μM for their ability to activate cytosolic CT from day 20 fetal type II cells (not shown). Consistent with previous studies with CT obtained from other sources (29,31-33), vesicles of acidic phospholipids, phosphatidylglycerol

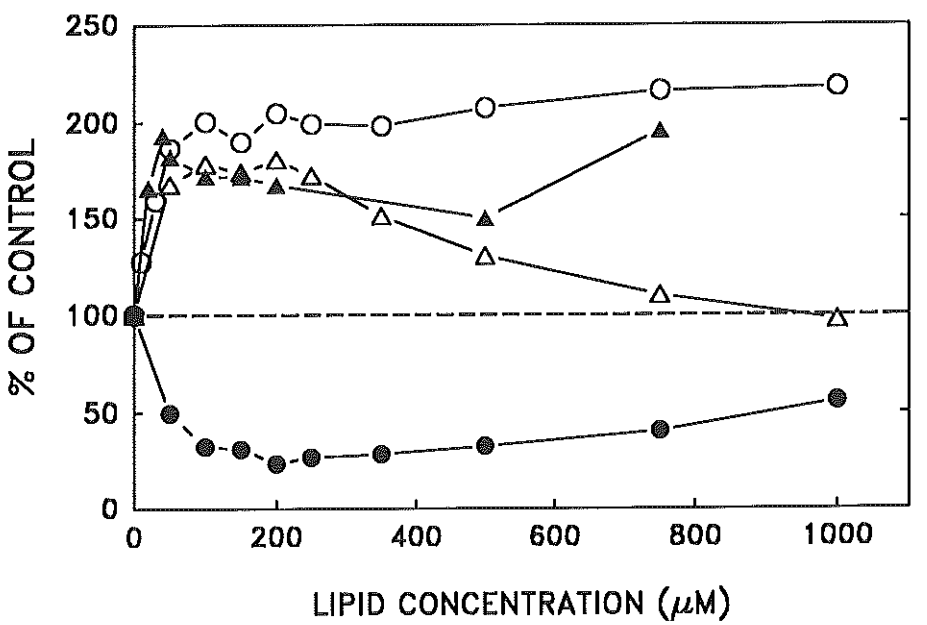

Figure 4. **Effect of different lipids on cytosolic cytidylyltransferase activity.** CT activity was assayed in cytosol prepared from day 20 fetal type II cells in the absence (control) or presence of different concentrations of vesicles consisting of phosphatidylcholine (●), oleic acid (△), phosphatidylglycerol (▲) or mixed phosphatidylcholine/oleic acid vesicles (1/1 M ratio) (○) (concentration expressed as sum of [PC]+[OA]). Values are expressed as percentage of control (0.35 nmol/min/mg protein). Assays were performed in duplicate. The experiment was repeated with almost identical results.

(6.44±0.08 fold stimulation), phosphatidylinositol (5.01±0.15 fold) and phosphatidylserine (4.04±0.02 fold) were the strongest stimulators of cytosolic CT activity in fetal type II cells. Phosphatidic acid (2.29±0.01 fold stimulation), phosphatidylethanolamine (2.92±0.24) and 1,2-dioctanoyl-glycerol vesicles (3.04±0.02) stimulated CT activity to some extent. Independent of fatty acid chain, PC vesicles did not stimulate CT activity. Addition of 1,3-diolein and 1-oleyl-2-acetylglycerol vesicles did also not activate CT. Oleic acid (5.13±0.34 fold) but not palmitic acid vesicles (1.38±0.05 fold) stimulated CT activity to the same maximal value as the acidic phospholipids. The effect of most stimulatory lipids was less when experiments were repeated at a concentration of 500 μM (not shown).

5.5 Discussion

Several mechanisms by which the activity of CT is regulated, and which are probably interrelated, have been studied in the fetal lung including sub-cellular translocation of the enzyme from cytosol to microsomes (13), activation by fatty acids (13,14) and phospholipids (16,17,31,34), and regulation by phosphorylation/dephosphorylation (35). While activation of cytosolic CT by lipids has been shown in a number of these studies, the role of this mechanism in activating CT in maturing type II cells has not been determined. In the present study, we found that the developmental increase in CT activity in fetal rat type II cells during late gestation (5) was in part mediated by an increased capacity of cytosolic lipids to activate CT with advancing gestation. Microsomal lipids did also stimulate CT activity, but no developmental difference was demonstrated. In previous studies with whole

lung it has been shown that activation of cytosolic CT by either estrogen (17) or betamethasone (36) is lipid dependent. Mallampalli et al. (36) demonstrated that the administration of betamethasone to pregnant rats increased CT activity in fetal rat lungs by increasing the proportion of cytosolic CT in the H-form. These hormonal findings cannot be extrapolated directly to CT activation during normal lung development. However, Chu et al. (16) have found that lipids also regulate cytosolic CT activity during whole rabbit lung development. Our present findings with isolated maturing type II cells of fetal rat lungs are in agreement with a developmental regulation of CT by cytosolic lipids. In addition, we showed the developmental activation of purified rat lung CT by cytosolic lipids of fetal type II cells. It was somewhat surprising that no developmental difference was found in the ability of microsomal lipids to activate CT, because we have previously observed that the specific and total activity of CT increased more in the microsomal fraction with advancing gestation than in the cytosolic fraction (5). This may be explained by two mechanisms, which may be complementary. It is possible that a developmental change in the cytosolic lipids promotes the translocation of CT from cytosol to microsomes, which are also more abundant at a later gestational age (37) and contain an increased amount of phospholipid fatty acids (38). A likely lipid candidate is free fatty acid. Fatty acid synthesis increases at late gestation (39,40) and translocation of CT to microsomes by free fatty acids has been demonstrated in whole lung (13). Another possibility is that newly synthesized CT remains associated with the microsomal membranes, thereby increasing microsomal CT activity with advancing gestation (6), while cytosolic CT activity remains a less active pool, which is regulated by cytosolic lipids. Our present data are compatible with developmental activation of pre-existing CT enzyme as opposed to

increased enzyme synthesis during late gestation. We have previously reported, however, that CT mRNA and protein levels increased in fetal type II cells with advancing gestation (6). Increased CT activity in fetal type II cells stimulated with conditioned medium from cortisol-treated fetal lung fibroblasts (41) also appears in part to be regulated at a pre-translational level (42). Therefore, both mechanisms of increased synthesis and activation may be important, and may complement each other during type II cell development.

In the present study, activities of both CT in fetal type II cell cytosol and purified lung CT were significantly reduced when lipids were removed. CT activity was partially restored by re-addition of lipids, confirming the lipid dependency of CT activity. However, re-addition of lipids to the delipidated cytosolic or purified CT was unable to activate CT to the same level as unextracted CT activated by lipids. The reason for this finding is not known, but it is possible that the extraction of lipids had a damaging effect on CT or that non-lipid factors necessary for optimal CT activity are lost during the extraction. With regard to the question of which lipids are able to activate CT in fetal type II cell cytosol, we demonstrated that vesicles of anionic phospholipids, oleic acid alone or mixed egg PC/OA (1:1 molar ratio) vesicles stimulated CT activity. This is consistent with studies using whole fetal lung cytosol (29,31) and purified rat liver CT (32,33). OA was enough to provide the required negative charge for activation of cytosolic CT. It is likely that OA incorporated in lipids present in the cytosol, thereby forming anionic membranes which bound and stimulated CT activity (29,32). We did not investigate the lipid composition of the type II cell cytosol and therefore are, at this moment, unable to answer the question which lipid is physiologically important during type II cell development. Chu *et al.*

determined the lipid composition of whole fetal lung cytosol during development (16) and after estrogen stimulation (17) but were unable to identify any individual lipid component that could explain the activation of CT.

Mallampalli's studies (15,36) suggest that phosphatidylglycerol and unsaturated fatty acids play an important role in cytosolic CT activation by the conversion of the L-form to the H-form either in vitro (15) or after betamethasone stimulation in vivo (36). The physiologic importance of these findings during normal development however remain to be established. In the present study we found that cytosolic CT activity of fetal type II cells was inhibited by PC vesicles. This may be due to a feedback inhibition of the end product of the pathway, PC, on the rate-limiting enzyme, CT, as was suggested by Jamil *et al.* (43,44) using rat hepatocytes.

In conclusion, cytosolic lipids play an important role in the developmental activation of CT in fetal rat type II cells during late gestation, but the precise mechanism of activation remains to be elucidated.

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CHAPTER 6

CYCLIC AMP-DEPENDENT PROTEIN KINASE DOES NOT REGULATE CTP:PHOSPHOCHOLINE CYTIDYLYL-TRANSFERASE ACTIVITY IN MATURING TYPE II CELLS

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6.1 Abstract

CTP:phosphocholine cytidylyltransferase (CT) catalyses a rate regulatory step in the *de novo* synthesis of surfactant phosphatidylcholine (PC) in alveolar type II cells. To investigate if CT can be regulated by cAMP-dependent protein kinase, we first studied the ontogeny of cAMP-dependent protein kinase activity in type II cells of fetal rat lung. Total cAMP-dependent protein kinase activity, measured in the presence of 10 μM cAMP, as well as endogenous activity, measured without cAMP, increased with advancing gestation. CT activity showed a similar developmental profile. This temporal relationship between cAMP-dependent protein kinase and CT supports a potential role for cAMP-dependent protein kinase in regulating CT phosphorylation. CT purified from adult rat lung was, indeed, phosphorylated *in vitro* by cAMP-dependent protein kinase. Despite the phosphorylation, however, no change in CT activity was noted. Pre-incubation of fetal type II cell cytosol with ATP and Mg^{++} did not affect CT activity. Addition of either cAMP, dibutyryl-cAMP or the catalytic subunit of cAMP-dependent protein kinase to the pre-incubation medium did also not alter CT activity. Furthermore, neither cAMP-dependent protein kinase inhibitor peptide, nor H8, a cyclic nucleo-dependent protein kinase inhibitor, affected CT activity in fetal type II cell cytosol. Treatment of intact fetal type II cells with either cAMP, dibutyryl-cAMP or 8-[4-chlorophenylthio]-cAMP activated cAMP-dependent protein kinase but did not alter CT activity. We conclude that the increase in CT activity in fetal type II cells at late gestation is not regulated by the developmental activation of cAMP-dependent protein kinase.

6.2 Introduction

Phosphatidylcholine (PC) is a major component of pulmonary surfactant (1,2). It is well known that the production of surfactant is initiated during the latter part of gestation. The subsequent accumulation of surfactant in the airways coincides with the ability of the newborn to establish regular air breathing. A surfactant deficiency due to insufficient lung maturity is, indeed, the prime factor responsible for the occurrence of respiratory distress syndrome (RDS) in premature neonates. CTP:phosphocholine cytidylyltransferase (CT) (EC 2.7.7.15) has been demonstrated to catalyse a rate-limiting step in the *de novo* PC synthesis in the developing lung (3,4). Its developmental regulation in lung remains to be elucidated. There is substantial evidence that CT is regulated by enzyme-membrane interaction (5-10). Studies with numerous cell types (5-12) have indicated that PC synthesis is regulated by translocation of inactive CT from the cytosol to the endoplasmic reticulum where it becomes activated by association with membrane lipids (12). A similar model for the regulation of pulmonary CT has been proposed by Weinhold *et al.* (13,14). Recently, we have reported that CT activity increases in fetal type II cells at late gestation (15). The increase in enzyme activity with advancing gestation was accompanied by a developmental shift in the distribution of CT activity from cytosol to endoplasmic reticulum.

The mechanism by which CT is activated is unknown, but some evidence suggests that reversible phosphorylation might be involved. In short term incubations, cyclic AMP (cAMP) and its analogues have been shown to inhibit PC synthesis in hepatocytes (16). The inhibition was

accompanied by partial inactivation of microsomal enzyme and redistribution of CT to the cytosolic compartment (16). In addition, it has been demonstrated that serine residue(s) of rat liver CT are substrates for cAMP-dependent protein kinase *in vitro* (17). Phosphorylation and dephosphorylation of proteins has also been demonstrated for adult type II cells (18). In contrast to the hepatocyte studies, investigations with adult type II cells showed no short-term effect of cAMP or its analogues on PC synthesis (19) and CT activity (20). Recent studies suggest, however, that protein phosphorylation by cAMP-dependent protein kinase may play an important role in regulating cellular functions in the developing lung (21). Furthermore, there is suggestive evidence for the involvement of cAMP-dependent protein kinase in regulating CT activity in whole fetal lung (22). As no data are currently available for isolated fetal type II cells, we investigated the ontogeny of cAMP-dependent protein kinase and the possible regulation of CT by cAMP-dependent protein kinase in fetal rat type II cells.

6.3 Materials and methods

Materials

Female (200-250g) and male (250-300g) Wistar rats were purchased from Charles River (St. Constant, Quebec) and bred in our animal facilities. Cell culture media, antibiotics, trypsin and a cAMP-dependent protein kinase enzyme assay system were obtained from Gibco Canada (Burlington, Ontario). Fetal bovine serum was from Flow Laboratories (McLean, VA), collagenase and DNase from Worthington Biochemical (Freehold, NJ). Cell culture flasks were purchased from Falcon (Becton Dickinson, Lincoln Park,

NJ). [*Methyl*-^{14}C]phosphocholine was from New England Nuclear Research (Dupont Canada, Mississauga, Ontario). [*Methyl*-^{14}C]CDPcholine and adenosine 5'-[γ-^{32}P]triphosphate were obtained from Amersham Canada (Oakville, Canada). *N*-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide dihydrochloride (H8) was from Seikagaku America (St. Petersburg, Florida). Catalytic subunit of cAMP-dependent protein kinase A (bovine heart) and cAMP-dependent protein kinase inhibitor peptide (rabbit sequence) and all remaining unlabelled biochemicals were obtained from Sigma (St. Louis, MO).

Cell cultures

Rats were sacrificed by diethylether inhalation on day 18 to day 21 of gestation (term = 22 days). The fetuses were aseptically removed from the mothers, the fetal lungs dissected out in cold Hank's balanced salt solution without calcium or magnesium [HBSS] and cleared of major airways and vessels. The lungs were washed twice in HBSS, minced and suspended in HBSS. Epithelial cells were isolated from the fetal lungs as described in detail previously (23). Briefly, the lung tissue was digested for 20 min in an enzymatic solution of 0.125% trypsin and 0.4 mg/ml DNase. After filtering through 100 μm mesh nylon bolting cloth, Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS) was added and the mixture centrifuged. The pellet was resuspended in MEM containing 0.1% collagenase. After 15 min of incubation, the collagenase activity was neutralized by adding MEM+10% FBS. Two differential adhesion periods of 1 h in tissue culture flasks allowed removal of fibroblasts. The non-adherent cells were removed, transferred to new culture flasks and incubated overnight for attachment of epithelial cells. Non-adherent cells were removed from all

epithelial cell cultures after overnight incubation. All experiments were performed 24 h after isolation. Although the term "type II cells" is used in this paper, the cuboidal epithelium which lines the acinar tubules during the late pseudoglandular and early canalicular stages of lung development does not contain lamellar bodies, the phenotypic marker for type II cells. In previous studies, we have shown that these cells do express other phenotypic features of type II cells and possess antigenic determinants of mature type II cells (24).

Cell fractionation

After overnight incubation in tissue culture flasks, attached epithelial cells were collected by scraping in homogenization buffer, the composition of which varied with each experiment. After homogenization with 50 strokes of a Dounce homogenizer, the homogenate was centrifuged at 300 x *g* for 10 min. The resulting supernatant was centrifuged at 13,000 x *g* for 10 min to obtain a postmitochondrial supernatant. Microsomal and cytosolic fractions were obtained by centrifugation at 313,000 x *g* for 15 min. Microsomes were resuspended in the same homogenization buffer as initially used to scrape the cells. All steps were carried out at 4°C. Cell homogenates and cell fractions were stored at -70°C until enzyme activities were measured.

Enzyme assays

CT activity was assayed in the forward direction by measuring the rate of incorporation of [*methyl*-^{14}C]phosphocholine into CDPcholine. The incubation medium (0.2 ml) contained 20 mM Tris-succinate (pH 7.8), 6 mM $MgCl_2$, 4 mM CTP, 1.6 mM [*methyl*-^{14}C]phosphocholine (specific

activity: 0.625 μCi/μmol) and up to 150 μg protein. After 30 minutes (20 min for purified adult lung CT) of incubation at 37°C, the reaction was stopped by addition of 0.1 ml of 25% (w/v) trichloroacetic acid and 0.5 ml of charcoal suspension (6% charcoal in 50 mM phosphocholine). The samples were placed on ice and [*methyl*-^{14}C]CDPcholine was isolated as described by Weinhold *et al.* (25). The recovery (69-74%) of CDPcholine was determined in each set of assays by adding a known amount of [*methyl*-^{14}C]CDPcholine to a complete assay mixture. All assays were corrected for background and recovery.

For cAMP-dependent protein kinase activity measurements, cells were scraped and homogenized in 0.75 ml of 50 mM Tris, 5 mM EDTA, pH 7.5. A 10 μl aliquot of the homogenate was incubated at 30°C in 50 mM Tris, 10 mM $MgCl_2$, 0.25 mg/ml BSA, 50 μM Kemptide, 100 μM [γ-^{32}P]ATP (specific activity 65 μCi/μmol), pH 7.5 (26). After 5 min of incubation, samples were spotted on phosphocellulose filters. The filters were washed with 1% (v/v) phosphoric acid and water, transferred to scintillation vials and radioactivity was measured. Total cAMP-dependent protein kinase activity was defined as the activity measured in the presence of 10 μM cAMP minus the activity determined in the presence of cAMP and cAMP-dependent protein kinase inhibitor peptide. Endogenous cAMP-dependent protein kinase activity was defined as the activity assayed in the absence of cAMP minus the activity measured in the presence of cAMP-dependent protein kinase inhibitor peptide. Percentage activated cAMP-dependent protein kinase was defined as (endogenous activity x 100) / total activity.

In preliminary experiments we established that, under the conditions used, product formation in all assays was directly proportional to incubation time and amount of protein.

Purification of CT

Adult rats were sacrificed by diethylether inhalation and immediately afterwards a tracheostomy was performed and lungs were inflated with a pressure of 15 cm H_2O. The thorax was opened and the lungs were thoroughly perfused with normal saline via injection in the right ventricle and opening the left atrium. Lungs were immediately frozen in liquid nitrogen and kept at -70°C till purification was started. CT was purified from 100 g of lung tissue as described by Weinhold *et al.* (25). A 2278-fold purification of CT was obtained with a yield of 9.3% of total cytosolic activity.

Pre-incubation of purified CT with the catalytic subunit of cAMP-dependent protein kinase A 25 μl aliquot of purified CT (0.45 nmol/min measured in the presence 0.5 mM [1:1 molar ratio] PC/oleic acid (OA) vesicles) was incubated at 37°C in a total volume of 155 μl of buffer A (50 mM Tris-HCl, 150 mM NaCl, 2 mM DTT, 1 mM EDTA and 0.025% (w/v) sodium azide, pH 7.4) with 0-60 units of pure catalytic subunit of cAMP-dependent protein kinase, 4 mM $MgCl_2$ and 0.1 mM ATP. After 30 min of incubation, CT activity was assayed. In some experiments purified CT was dephosphorylated prior to incubation with catalytic subunit of cAMP-dependent protein kinase. The dephosphorylation was performed by incubating 60 μl of purified CT (1.08 nmol/min measured in presence of 0.5 mM PC/OA vesicles) with 5 units of alkaline phosphatase bound to agarose at 4°C for 1 h in a volume of 250 μl of buffer A supplemented with 4 mM $MgCl_2$. The alkaline phosphatase was removed by centrifugation at 2500 x *g* for 5 min. Preliminary experiments revealed that 5 units of alkaline phosphatase was optimal for activating purified CT.

Phosphorylation of purified CT with the catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP

A 500 μl aliquot of purified lung CT (9.03 nmol/min measured in presence of 0.5 mM PC/OA vesicles or approx. 2.9 μg) was first dephosphorylated with 25 units of alkaline phosphatase bound to agarose in buffer A with 4 mM $MgCl_2$ (total volume 1 ml). After a 1 h incubation at 4°C, the alkaline phosphatase was removed by centrifugation (2 times 2500 x *g* for 5 min at 4°C). The supernatant was then incubated with 100 units of catalytic subunit of cAMP-dependent protein kinase with 0.1 mM [γ-^{32}P]-ATP (specific activity 108 μCi/μmol) in buffer A (total volume 1 ml). After 30 min of incubation at 37°C the reaction was stopped by adding 5 μg sodium deoxycholate and 400 μl of 25% trichloroacetic acid (17). The sample was kept on ice for 30 min and the precipitate was pelleted by centrifugation at 2500 x *g* for 20 min. The supernatant was discarded and the pellet washed twice with 1 ml of chilled acetone. The precipitate was then resuspended in sample buffer and separated on 12% (w/v) SDS-polyacrylamide gels under reducing conditions according to the method of Laemmli (27). The gel was dried and exposed for 48 h to Kodak XAR-5 film using Dupont Cronex intensifying screens.

Incubation of fetal type II cells in culture with cAMP or cAMP analogues

Fetal type II cells were grown to confluence in MEM plus 2% FBS and 5 μg/ml transferrin. The cells were washed three times with fresh serum-free MEM prior to incubation with serum-free MEM containing either cAMP, dibutyryl-cAMP or 8-[4-chlorophenylthio]-cAMP. After 1 or 3 h of incubation, cells were washed twice with phosphate buffered saline (PBS) and scraped in Tris/saline buffer (145 mM NaCl, 50 mM NaF, 2.5 mM

EDTA, 50 mM Tris-HCl, pH 7.4). Post-mitochondrial supernatant, cytosol and microsomes were prepared as described above and CT activity was assayed. When cAMP-dependent protein kinase activity was measured, solutions were used as described for cAMP-dependent protein kinase assay.

Protein measurements

Protein concentrations were determined by the method of Bradford (28), using bovine serum albumin as standard.

Statistical analysis

Data are presented as means ± S.E. Statistical differences between various groups were analysed by unpaired t-test (for two groups) or by analysis of variance with the Neumann-Keuls test (for more than two groups). Statistical significance was accepted at the $p < 0.05$ level (two-tailed).

6.4 Results

Ontogeny of cAMP-dependent protein kinase activity in fetal type II cells during late gestation

The developmental profiles of activities of cAMP-dependent protein kinase and CT were determined in postmitochondrial supernatants of fetal type II cells. Total cAMP-dependent protein kinase activity, measured in the presence of 10 μM cAMP, increased from d18 to d20 and then decreased on d21 (Fig. 1B). Endogenous cAMP-dependent protein kinase activity, measured in the absence of cAMP, showed a similar developmental pattern (Fig. 1A). The percentage activated cAMP-dependent protein kinase

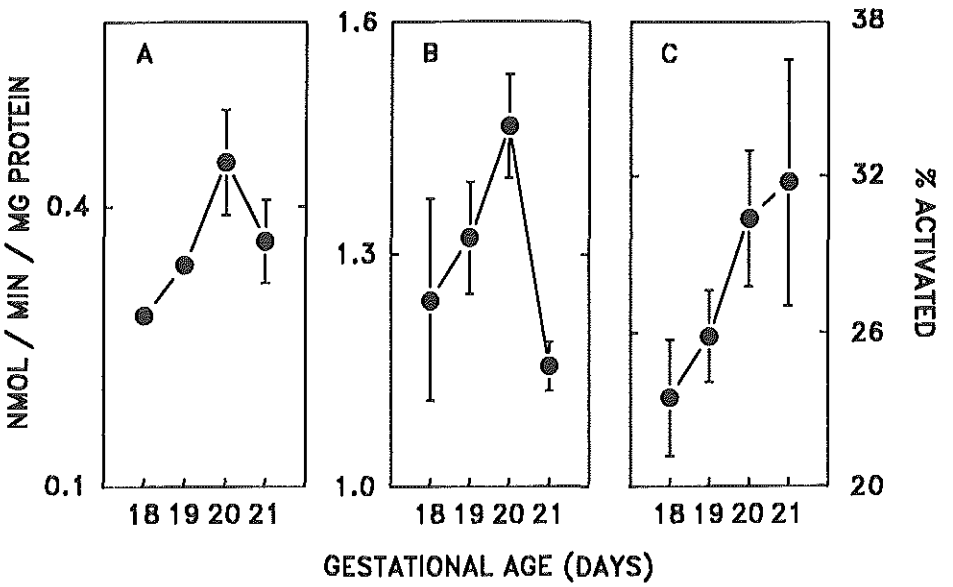

Figure 1. **Ontogeny of cAMP-dependent protein kinase activity in fetal type II cells during late gestation.** Post-mitochondrial supernatants were prepared from type II cells in primary culture, isolated from fetal rats at 18 to 21 days gestation. Total cAMP-dependent protein kinase activity was assayed in the presence of 10 μM cAMP (B) and endogenous cAMP-dependent protein kinase was assayed in the absence of cAMP (A). Activities were corrected for background activity in the presence of cAMP-dependent protein kinase inhibitor peptide. The percentage activated cAMP-dependent protein kinase was calculated as endogenous activity x 100 / total activity (C). The data represent the means $\pm$ S.E. of three independent experiments carried out in duplicate.

(endogenous x 100 / total) increased from d18 to d21 (Fig. 1C). Previously, we have reported that CT activity increases in type II cells with advancing gestation (15). This temporal relationship between cAMP-dependent protein kinase and CT supports a regulatory role for cAMP-dependent protein kinase in activating CT activity in maturing type II cells.

Effect of catalytic subunit of cAMP-dependent protein kinase on lung CT

To demonstrate that cAMP-dependent protein kinase is able to phosphorylate lung CT, CT purified from adult rat lung was first incubated with alkaline phosphatase to dephosphorylate the enzyme. Subsequently, CT was incubated in the presence of [γ-^{32}P]ATP and catalytic subunit of cAMP-dependent protein kinase and subjected to SDS-PAGE and autoradiography. Most of the radioactivity was found at the 40 kDa band where purified CT runs on the gel (not shown). Thus, lung CT seems to be a substrate for cAMP-dependent protein kinase. We further investigated the effect of cAMP-dependent protein kinase on the activity of rat lung CT. Incubation of CT (0.08 nmol/min) with 20-60 units of catalytic subunit of cAMP-dependent protein kinase in the presence of 0.1 mM ATP did not significantly alter its activity (Fig. 2A). In addition, no effect of catalytic subunit of cAMP-dependent protein kinase on CT activity (Fig. 2A) was noted when CT activity was assayed in the presence of 0.5 mM PC/OA [1:1 molar ratio] vesicles ((0.47 nmol/min), which has been shown to stimulate CT activity maximally (25). Since we did not observe any effect of cAMP-dependent protein kinase on CT activity, purified CT was first dephosphorylated with alkaline phosphatase (0.071 nmol/min vs. 0.043 nmol/min, dephosphorylated vs. control, activity measured in absence of PC/OA vesicles) and then incubated with 20 units of catalytic subunit of cAMP-dependent protein kinase in the presence of 0.1 mM ATP. However, again the activity of CT did not change (Fig. 2B). These results suggest that phosphorylation of lung CT by cAMP-dependent protein kinase does not directly affect the activity of the pulmonary enzyme.

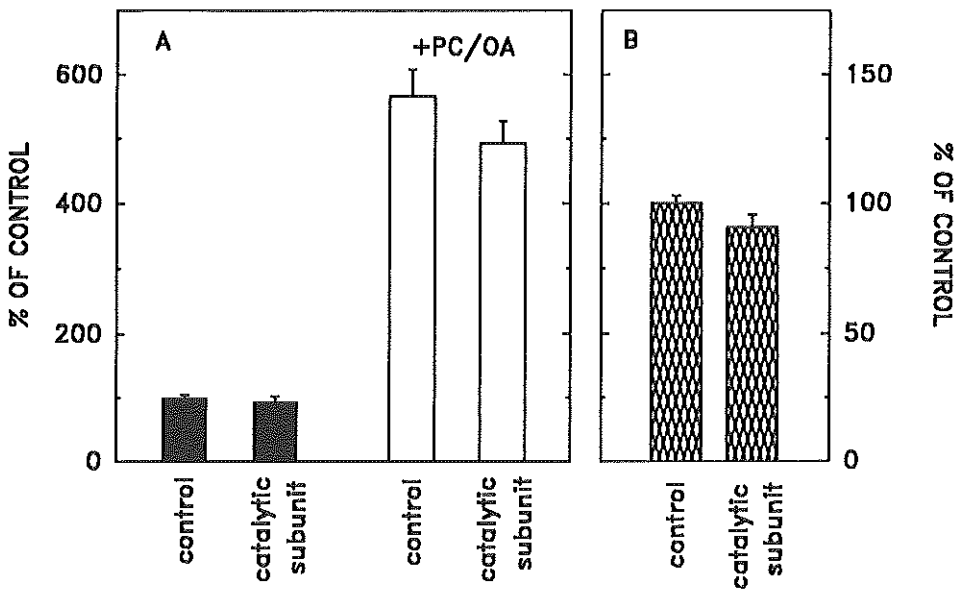

Figure 2. **Effect of catalytic subunit of cAMP-dependent protein kinase on the activity of adult rat lung phosphocholine cytidylyltransferase. A:** Purified lung cytidylyltransferase was incubated with 20 U of catalytic subunit of cAMP-dependent protein kinase (abbreviated as PKA) in the presence of 0.1 mM ATP, with (open bars) or without (filled bars) 0.5 mM phosphatidylcholine/oleic acid (1/1 molar ratio) vesicles (abbreviated as PC/OA). Subsequently cytidylyltransferase activity was measured. Controls [control (0.08 nmol/min) and PC/OA (0.47 nmol/min)] consisted of a pre-incubation with ATP but without catalytic subunit of cAMP-dependent protein kinase. Additional controls (no ATP, no catalytic subunit; or catalytic subunit but no ATP) were similar (not shown). Incubations with 40 or 60 U of catalytic subunit did also not alter the activity. **B:** Purified lung cytidylyltransferase was first dephosphorylated with alkaline phosphatase (0.071 nmol/min) and then incubated with 0.1 mM ATP, with or without 20 U of the catalytic subunit of cAMP-dependent protein kinase. Subsequently, cytidylyltransferase activity was measured. The cytidylyltransferase activities are presented as percentage of controls. Mean ± S.E. are shown of at least three separate experiments, all performed in duplicate.

Effect of cAMP on CT activity of fetal type II cells

We first investigated whether incubation of d20 fetal type II cell cytosol (0.22 nmol/min/mg) with ATP leads to inhibition of CT as has been reported for rat liver (12) and rabbit lung cytosol (22). As shown in figure 3A, cytosolic CT activity was not changed after a 30-min incubation at 37°C with 0.5 mM ATP and 8 mM Mg^{2+}. Addition of 2 mM DTT did not alter this observation. In all subsequent experiments, cytosol from d20 fetal type II cells was pre-incubated for 30 min at 37°C in the presence of 0.5 mM ATP, 8 mM $MgCl_2$ and 2 mM DTT. In order to further investigate the role of cAMP-dependent protein kinase in regulating CT activity in fetal type II cells, we added either 0.25 mM cAMP or 1 mM dibutyryl-cAMP to the pre-incubation solution. No effect on CT activity was noted (Fig. 3A). In contrast, cAMP-dependent protein kinase activity was already stimulated with 10 μM cAMP (Fig. 1B). Pre-incubation of cytosol with different amounts of catalytic subunit of cAMP-dependent protein kinase (0-50 units) also did not change the CT activity (Fig. 3A). In addition, neither cAMP-dependent protein kinase inhibitor peptide (10 μM) or H8 (50 μM), a cyclic nucleotide-dependent protein kinase inhibitor, did affect CT activity (Fig. 3B).

In line with these cytosolic findings, incubations of intact d20 fetal type II cells with increasing concentrations of cAMP (0-2 mM) did not significantly alter the CT activity (0.58 nmol/min/mg) in the post-mitochondrial supernatant. Similar results were obtained when cells were exposed to either 1 mM dibutyryl-cAMP or 1 mM 8-[4-chlorophenylthio]-cAMP (Fig. 4A). Neither the cytosolic (0.35 nmol/min/mg) nor the microsomal (1.34 nmol/min/mg) CT activity was changed (Fig. 4B). In addition, the distribution of CT activity between cytosol and microsomes was

not altered (not shown). Similar results were obtained with d19 fetal type II cells.

To exclude the possibility that cAMP-dependent protein kinase was not stimulated by cAMP or its analogues, we measured cAMP-dependent protein kinase activity in cytosol from fetal type II cells which were incubated with 1 mM dibutyryl-cAMP. After 1 h of incubation, cAMP-dependent protein kinase activity increased by 47.6±11.6% (mean ± SE) compared to control (1.4 nmol/min/mg). The activity returned to control values after 3 h of incubation (Fig. 5A). Total cAMP-dependent protein kinase activity, assayed in the presence of 10 μM cAMP, did not change significantly.

To test if this activated cytosolic cAMP-dependent protein kinase was able to influence CT activity, cytosol from dibutyryl-cAMP (1 h of incubation) stimulated cells was incubated at 37°C with purified rat lung CT (0.043 nmol/min measured in absence of lipid activators) in the presence of 0.1 mM ATP, 8 mM $MgCl_2$ and 2 mM DTT. After 30 min of incubation, CT activity was measured. No change in CT activity was found despite the increase in cAMP-dependent protein kinase activity (Fig. 5B). Taken together, these results suggest that cAMP-dependent protein kinase does not regulate CT activity in fetal type II cells.

6.5 Discussion

Although the developmental profiles of cAMP-dependent protein kinase and CT activities in fetal type II cells are compatible with a role for cAMP-dependent protein kinase in regulating CT activity, our findings with purified rat lung CT and fetal type II cells do not support such a regulatory

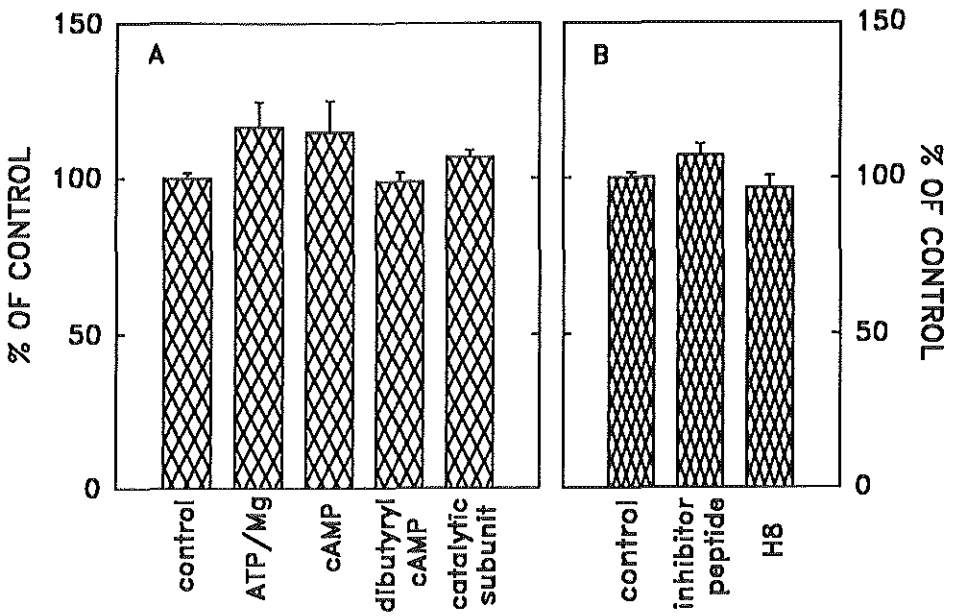

Figure 3. **Effect of activators or inhibitors of cAMP-dependent protein kinase on cytidylyltransferase activity of fetal type II cell cytosol.** Cytosol from day 20 fetal type II cells was incubated for 30 min at 37°C (controls) in the presence of 0.5 mM ATP, 8 mM $MgCl_2$ and 2 mM DTT (ATP/Mg). Further addition of one of the following was tested: 0.25 mM cAMP, 1mM dibutyryl-cAMP, 20 U of the catalytic subunit of cAMP-dependent protein kinase, 10 μM protein kinase inhibitor peptide or 50 μM H8, a cyclic nucleotide-dependent protein kinase inhibitor. Subsequently cytidylyltransferase activity was measured [presented as % of control (0.22 nmol/min)]. Data represent means $\pm$ S.E. of at least four separate experiments carried out in duplicate.

role for cAMP-dependent protein kinase. Firstly, purified adult rat lung CT is phosphorylated by the catalytic subunit of cAMP-dependent protein kinase but the activity of rat lung CT is not affected. In contrast, phosphorylation of rat liver CT by the catalytic subunit of cAMP-dependent protein kinase has been shown to be accompanied by a decrease in CT activity (17). Secondly, experiments with rat liver cytosol have shown a time-dependent

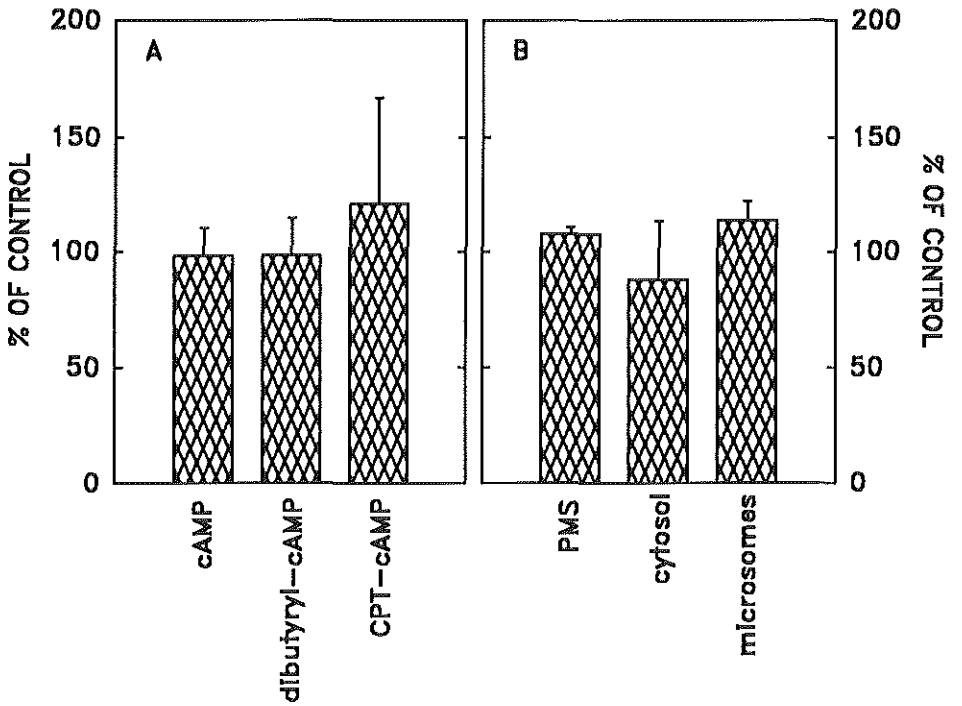

Figure 4. **Effect of cAMP on cytidylyltransferase activity of fetal type II cells in primary culture.** **A:** Day 20 fetal type II cells were incubated with MEM alone (controls) or MEM with either 0.25 mM cAMP, 1 mM dibutyryl-cAMP or 1 mM 8-[4-chlorophenylthio]-cAMP (abbreviated as CPT-cAMP). After 3 hours of incubation, cells were homogenized and cytidylyltransferase activity was assayed in post-mitochondrial supernatant as described under Materials and Methods. **B:** Day 20 fetal type II cells were incubated with MEM alone (controls) or MEM with 1 mM dibutyryl-cAMP. After 1 hour of incubation, cells were homogenized and cytidylyltransferase activity was assayed in post-mitochondrial supernatant (PMS), cytosol and microsomes as described under Materials and Methods. The cytidylyltransferase activities are presented as percentage of controls [PMS:0.58 nmol/min; cytosol:0.35 nmol/min; microsomes: 1.34 nmol/min). Means ± S.E. are shown for at least 4 separate experiments carried out in duplicate.

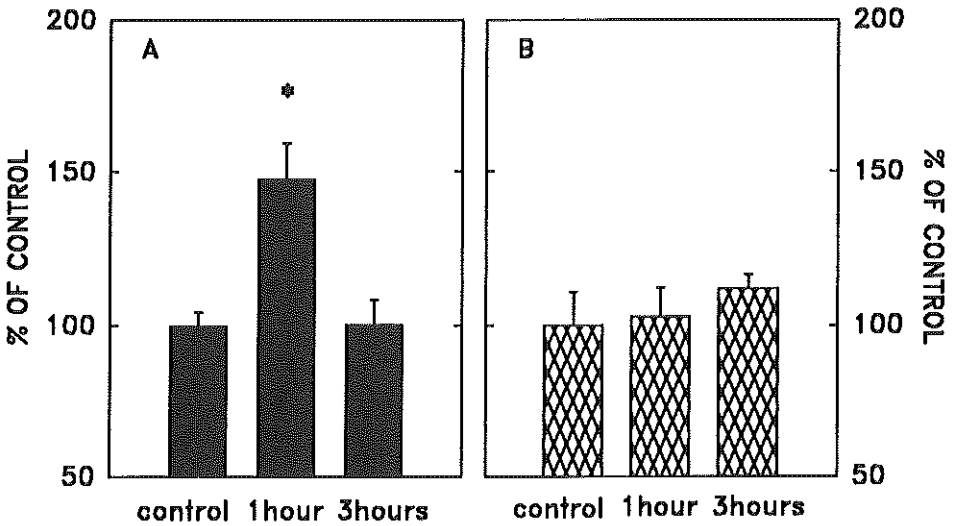

Figure 5. **The effect of dibutyryl-cAMP on the activity of cAMP-dependent protein kinase in fetal type II cells in primary culture and its subsequent effect on cytidylyltransferase activity.** **A:** Day 20 fetal type II cells were incubated with MEM alone (control) or MEM with 1 mM dibutyryl-cAMP. After 1 or 3 hours, cells were homogenized and cAMP-dependent protein kinase activity was assayed in cytosol. Endogenous cAMP-dependent protein kinase activity is shown as percentage of control (1.4 nmol/min/mg). **B:** Cells were incubated and cytosol prepared as in A. This cytosol was incubated with purified rat lung cytidylyltransferase in the presence of 0.1 mM ATP, 8 mM $MgCl_2$ and 2 mM DTT. After 30 min of incubation cytidylyltransferase activity was assayed (shown as percentage of control, 0.043 nmol/min measured in the absence of lipid activators). Data are presented as mean ± S.E. of at least 4 independent experiments performed in duplicate.

activation of CT by incubation at 37°C which was inhibited by ATP/Mg (12). The ATP/Mg inhibition was abolished by cAMP-dependent protein kinase inhibitors. This time-dependent activation of CT is not found with cytosol of fetal type II cells. This may explain the lack of inhibition of cytosolic CT of fetal type II cells by ATP/Mg. Furthermore, cAMP and cAMP-dependent protein kinase blockers do not affect cytosolic CT activity

of fetal rat type II cells. In contrast, Radika and Possmayer (22) reported that ATP/Mg inhibited CT activity in fetal rabbit lung cytosol. They showed also that the inhibition was abolished by cAMP-dependent protein kinase blockers. In line with our findings, however, they were unable to demonstrate a cAMP effect. Thirdly, our findings with intact fetal type II cells that cAMP and its analogues stimulate cAMP-dependent kinase activity without affecting CT activity and subcellular distribution, argue against a regulatory role for cAMP-dependent kinase. This is in agreement with recent studies with intact hepatocytes which showed that increased cAMP levels or increased cAMP-dependent protein kinase activity did not affect CT activity (29,30). In addition, it was shown that the phosphorylation state of CT was not altered following stimulation of cAMP-dependent protein kinase (30). In the present study, we measured the short-term effect of cAMP on fetal type II cell CT activity. The short-term inhibition of PC synthesis of hepatocytes has recently been shown to be due to a decrease in cellular diacylglycerol levels rather than by a decreased activity of CT (29). Long-term treatment (> 15 h) with cAMP of hepatocytes (31), fetal lung explants (32) and a human alveolar carcinoma cell line (A549 cells) (33), has been reported to increase PC synthesis. The effect of this long-term treatment on fetal type II cell CT remains to be elucidated. We observed that cAMP-dependent protein kinase was activated in fetal type II cells following 1-h exposure to dibutyryl-cAMP but not following a 3-h exposure. This suggests that cAMP-dependent protein kinase cannot solely be responsible for the long-term effect of cAMP on PC synthesis. It is worthwhile mentioning that our finding of an increase in cAMP-dependent protein kinase in fetal type II cells with advancing gestation is contrary to previous studies with whole fetal rat lung (21), where a decrease was noted. As cAMP-dependent protein kinase

of fetal type II cells was measured 24 h following cell isolation, it is plausible that this discrepancy in the developmental profiles of cAMP-dependent protein kinase merely reflects the cellular complexity of the lung or the *in vitro* conditions used.

6.6 References

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CHAPTER 7

THE EFFECT OF PROTEIN PHOSPHATASES AND PROTEIN KINASE C ON CTP:PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE ACTIVITY IN FETAL RAT TYPE II PNEUMOCYTES

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submitted

7.1 Abstract

CTP:phosphocholine cytidylyltransferase (CT) (EC 2.7.7.15) catalyses a rate regulatory step in the *de novo* synthesis of surfactant phosphatidylcholine (PC) in alveolar type II cells. To investigate whether CT is regulated by protein kinase C and protein phosphatases, we first studied the ontogeny of protein kinase C and protein phosphatase activities in type II cells of fetal rat lung. Protein kinase C activity increased from fetal day 18 to 20 and then decreased on day 21. Protein phosphatase activity increased from day 18 to 19, remained constant on day 20 and decreased markedly on day 21. The ontogenic pattern of either enzymatic activity is not similar to that of CT activity which continues to rise after 20 days of gestation. Secondly, we investigated whether protein phosphatases affect CT activity of fetal type II cells. Alkaline phosphatase treatment increased CT activity purified from adult rat lung in a concentration-dependent manner. Pre-incubation of fetal type II cell cytosol with alkaline phosphatase resulted also in an increase of CT activity. Under phosphorylating conditions with NaF, cytosolic CT activity was decreased. However, no decrease in activity was found when cytosol was incubated with okadaic acid, a selective phosphatase inhibitor of protein phosphatases type 1 and 2A. Thirdly, we investigated the effect of protein kinase C on CT activity in fetal type II cells. Pre-incubation of cytosol of fetal type II cells in the presence of ATP and Mg++ with phorbol ester (TPA), a protein kinase C activator, did not affect CT activity. Neither protein kinase C inhibitor peptide, nor H7 and H8, protein kinase C inhibitors, did change CT activity. Protein kinase C activity in cytosol increased or decreased after treatment with TPA and protein kinase

inhibitors, respectively. Furthermore, treatment of intact fetal type II cells with either TPA, H7 or H8 for 1 to 3 h did not alter CT activity assayed in post-mitochondrial supernatant, cytosol and microsomes. Exposure of the cells to TPA did activate protein kinase C. We conclude that CT activity in fetal type II cells may be modulated by protein phosphatase but not protein kinase C activity.

7.2 Introduction

Phosphatidylcholine (PC) is a major component of pulmonary surfactant, which is produced by alveolar epithelial type II cells. The increased production of pulmonary surfactant during the latter part of gestation is of paramount importance for the ability of the newborn to establish regular air-breathing. Surfactant deficiency due to lung immaturity is the main factor responsible for the respiratory distress syndrome in premature neonates. The increased synthesis of PC in alveolar type II cells at the end of gestation has been shown to be associated with an increased activity of the enzyme CTP:phosphocholine cytidylyltransferase (CT) (1), which is known to catalyse a rate-limiting step in the *de novo* PC synthesis (2). Several mechanisms have been proposed for the regulation of CT in the lung and isolated type II cells.

It has been demonstrated that CT is present in both cytosolic and membrane-associated cell fractions and that the membrane-bound form is the most active (3-5). Fatty acids (3-8) and phospholipids (9,10) may play an important physiologic role in the activation of CT in fetal lung. Recent studies have identified the lipid-binding domain of CT as an α-helical domain

of the protein (11,12). Although activation of CT by membrane lipids may play an important role during normal type II cell development (1,6,13), we have recently demonstrated that the increased PC formation in maturing type II cells is, in part, due to an increased gene and protein expression of CT (14). Our studies demonstrated that CT protein content and activity increased in the membrane fraction with advancing gestation with no developmental change in the cytosolic fraction (1,14). The mechanism for this increased association with the membranes during late gestation is unknown.

Studies with hepatocytes, HeLa and Chinese hamster ovary (CHO) cells have suggested a regulation of CT activity by a phosphorylation / dephosphorylation mechanism (15-20). Dephosphorylation of CT in CHO (20) and HeLa (16) cells has been shown to coincide with a redistribution of enzyme from cytosolic to membrane compartment. In contrast, a recent study with hepatocytes suggests that dephosphorylation is not a prerequisite for its translocation but occurs after insertion into the membrane (21). Collectively, these studies indicate that reversible phosphorylation plays an important role in the regulation of CT. Purified rat liver (22) and lung (23) CT can be phosphorylated *in vitro* by cAMP-dependent protein kinase. Recent studies in hepatocytes have shown that the inhibition of PC synthesis by cAMP is not due to the phosphorylation state of CT (24,25). Similarly, we have shown that CT is not regulated by cAMP-dependent protein kinase in fetal type II cells (23). Aside from cAMP-dependent protein kinase, protein phosphatases (26) and protein kinase C (27) are likely involved in protein phosphorylation and dephosphorylation. No data regarding both enzyme activities are currently available for isolated fetal type II cells. Herein, we investigated the ontogeny of protein phosphatase and protein kinase C activities and the

possible regulation of CT activity by phosphatases and protein kinase C in fetal rat type II cells.

7.3 Materials and methods

Materials

Female (200-250g) and male (250-300g) Wistar rats were purchased from Charles River (St. Constant, Quebec) and bred in our animal facilities. Cell culture media, antibiotics, trypsin and protein kinase C enzyme assay system were obtained from Gibco Canada (Burlington, Ontario). Fetal calf serum (FCS) was from Flow Laboratories (McLean, VA), collagenase and DNAse from Worthington Biochemical (Freehold, NJ). Cell culture flasks were purchased from Falcon (Becton Dickinson, Lincoln Park, NJ). [*Methyl*-^{14}C]phosphocholine was from New England Nuclear Research (Dupont Canada, Mississauga, Ontario). [*Methyl*-^{14}C]CDPcholine and adenosine 5'-[γ-^{32}P]ATP were obtained from Amersham Canada (Oakville, Canada). *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H8) and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) were from Seikagaku America (St. Petersburg, Florida). Protein kinase C substrate (MBP 4-14) , protein kinase C inhibitor peptide (19-31) and okadaic acid were from Upstate Biotechnology Inc. (Lake Placid, NY). All remaining unlabelled biochemicals were obtained from Sigma (St. Louis, MO).

Cell cultures

Timed pregnant rats were sacrificed by diethylether excess on days 18

to 21 of gestation (term = day 22). The fetuses were aseptically removed from the mothers and the fetal lungs dissected out. The epithelial cells were isolated as described in detail elsewhere (1,28). All experiments were performed 24 h after isolation. Although the term "type II cells" is used in this paper, the cuboidal epithelium which lines the acinar tubules during the late pseudoglandular and early canalicular stages of lung development does not contain lamellar bodies, the phenotypic marker for type II cells. In previous studies, we have shown that these cells do express other phenotypic features of type II cells and possess antigenic determinants of mature type II cells (28,29).

Cell fractionation

After overnight incubation in tissue culture flasks, attached epithelial cells were collected by scraping in homogenization buffer, the composition of which varied with each enzymatic assay. After homogenization with 50 strokes of a Dounce homogenizer, the homogenate was centrifuged at 300x*g* for 10 min. The resulting supernatant was centrifuged at 13,000x*g* for 10 min to obtain a postmitochondrial supernatant. Microsomal and cytosolic fractions were obtained by centrifugation at 313,000x*g* for 15 min. Microsomes were resuspended in the same homogenization buffer as initially used to scrape the cells. All steps were carried out at 4°C. Cell homogenates and cell fractions were stored at -70°C until enzyme activities were measured.

Enzyme assays

CT activity was assayed in the forward direction by measuring the rate of incorporation of [*methyl*-^{14}C]phosphocholine into CDPcholine. The incubation medium (0.2 ml) contained 20 mM Tris-succinate (pH 7.8), 6

mM $MgCl_2$, 4 mM CTP, 1.6 mM [*methyl*-^{14}C]phosphocholine (specific activity: 0.625 μCi/μmol) and up to 150 μg protein. After 30 minutes (20 min for the purified CT) of incubation at 37°C, the reaction was stopped by addition of 0.1 ml of 25% (w/v) trichloroacetic acid and 0.5 ml of charcoal suspension (6% charcoal in 50 mM phosphocholine). The samples were placed on ice and [*methyl*-^{14}C]CDPcholine was isolated as described by Weinhold et al. (30). The recovery (70-74%) of CDPcholine was determined in each set of assays by adding a known amount of [*methyl*-^{14}C]CDPcholine to a complete assay mixture. All assays were corrected for background and recovery.

For protein kinase C measurements, cells were scraped and homogenized in 0.6 ml of 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM ß-mercaptoethanol, 25 μg/ml aprotinin and 25 μg/ml leupeptin. Cellular debris was removed by a 2 min centrifugation in a microcentrifuge. When a partial purification step of protein kinase C was performed, 0.5% Triton X-100 was included in the homogenization buffer. Protein kinase C in the homogenate was partially purified on a small DEAE cellulose (Whatman DE52) column (DEAE cellulose was suspended in wash buffer (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA and 10 mM ß-mercaptoethanol)). After washing with 5 ml wash buffer, the protein kinase C was eluted with 5 ml elution buffer (wash buffer plus 0.2 M NaCl). When the partial purification step was omitted, cell homogenate was diluted 8 x with elution buffer. A 20-25 μl aliquot of diluted homogenate or partially purified protein kinase C was incubated at 30°C in 20 mM Tris, pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 20 μM [γ-^{32}P]ATP (specific activity 225 μCi/μmol), 50 μM protein kinase C substrate [MBP 4-14] (31). After 5 min of incubation, samples were spotted on phosphocellulose filters. The filters

were washed with 1% (v/v) phosphoric acid and water, transferred to scintillation vials and radioactivity was measured. All samples were assayed in the presence of lipid activators (10 μM phorbol 12-myristate 13-acetate (TPA), 0.28 mg/ml phosphatidylserine and 0.3% (v/v) Triton X-100 mixed micelles (32)) and corrected for the remaining activity measured in the presence of 20 μM protein kinase C inhibitor peptide (31). Protein kinase C activities in diluted homogenates were also assayed in the absence of lipid activators.

Protein phosphatase activity was assayed by measuring the rate of dephosphorylation of [γ-^{32}P]-labelled phosphorylase a (33). This substrate was prepared by incubating unphosphorylated 5 mg/ml of phosphorylase b at 25°C in 50 mM Tris, pH 8.2, 50 mM glycerol-2-phosphate, 1 mM [γ-^{32}P]ATP (specific activity 22.5 μCi/μmol), 10 mM Mg-acetate and 1 unit/ml phosphorylase kinase. After 60 min of incubation, the reaction was terminated by addition of an equal volume of 90% saturated $(NH_4)_2SO_4$, pH 7. The precipitate was collected by centrifugation, washed with buffer A (50 mM Tris/HCl (pH 7.0), 1 mM EDTA, 50 mM mercaptoethanol) containing 45% saturated $(NH_4)_2SO_4$ and redissolved in 1 ml of buffer A. The substrate was dialysed against 500 ml of buffer A for 16 h at 4°C (with one change of dialysis buffer), collected by centrifugation at 10,000x*g* for 10 min and redissolved in 1 ml buffer A containing 0.25 M NaCl at 25°C. Protein phosphatase activity was assayed in 40 μl post-mitochondrial supernatant of type II cells, homogenized in 50 mM Tris/HCl, pH 7, 1 mM EGTA, 50 mM mercaptoethanol and 1 mg/ml bovine serum albumin. The reaction was initiated by the addition of 20 μl of [γ-^{32}P]-labelled phosphorylase a solution (specific activity at the time of the assays was 6.7 μCi/μmol). After 20 min incubation at 30°C the reactions were terminated by the addition of 100 μl of

ice-cold trichloroacetic acid and 100 μl of 6 mg bovine serum albumin/ml. The solution was vortexed, kept on ice for 10 min and then centrifuged at 13,000x*g* for 4 min. A portion of 200 μl of the supernatant was counted for radioactivity. Assays were corrected for radioactivity measured with 40 μl homogenization buffer instead of post-mitochondrial supernatants.

In preliminary experiments we established that, under the conditions used, product formation in all assays was directly proportional to incubation time and amount of protein.

Purification of CT

Rats were sacrificed by diethylether excess and immediately afterwards a tracheostomy was performed and lungs were inflated with a pressure of 15 cm H_2O. The thorax was opened and the lungs were thoroughly perfused with normal saline via injection in the right ventricle and opening the left atrium. Lungs were immediately frozen in liquid nitrogen and kept at -70°C till the purification was started. CT was purified from 100 g lung as described by Weinhold *et al.* for liver (30). A 2278-fold purification was obtained and a yield of 9.34% of total cytosolic activity (23).

Incubation of purified CT with alkaline phosphatase

A 25 μl aliquot of purified CT (0.47 nmol/min measured in the presence of 0.5 mM (1:1 molar ratio) PC/oleic acid [OA] vesicles) was incubated at 37°C in a total volume of 155 μl of buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM DTT, 1 mM EDTA and 0.025% sodium azide, pH 7.4) with 0-10 U alkaline phosphatase and 4 mM $MgCl_2$ in the absence or presence of 0.5 mM (1:1 molar ratio, 0.25 mM each) PC/OA vesicles. After 30 min of incubation, CT activity was assayed.

Incubation of fetal type II cells in culture with TPA, H7 or H8

Day 20 type II cells were grown to confluency (about 24 hours) in Eagle's minimal essential medium (MEM) with 2% fetal calf serum and 5 μg/ml transferrin. The cells in the culture flasks were washed three times with fresh serum-free MEM prior to incubation with serum-free MEM containing either TPA (dissolved in 10% DMSO in ethanol), H7 or H8 (dissolved in water) or the corresponding amount of solvent as control. After 1 or 3 hours of incubation, cells were washed twice with phosphate-buffered saline (PBS), and scraped in Tris/saline buffer (145 mM NaCl, 50 mM NaF, 2.5 mM EDTA, 50 mM Tris-HCl, pH 7.4). Post-mitochondrial supernatant, cytosol and microsomes were prepared as described above and CT activity was assayed. When protein kinase C activity was measured, cells were homogenized in the appropriate buffers.

Protein measurements

Protein concentrations were determined by the method of Bradford (34), using bovine serum albumin as standard.

Statistical analysis

Data are presented as mean ± S.E. Statistical differences between various groups were analysed by unpaired t-test (for two groups) or by analysis of variance with the Neumann-Keuls test (for more than two groups). Statistical significance was accepted at the $p<0.05$ level (two-tailed).

7.4 Results

Ontogeny of protein kinase C and protein phosphatase activities in maturing type II cells during late gestation

Previously, we have demonstrated that CT activity increases in maturing type II cells during late gestation (1). To investigate whether protein kinase C and protein phosphatases can regulate CT activity in fetal type II cells during late gestation, we first studied the developmental profiles of the enzyme activities in homogenates of type II cells isolated from fetal rats of 18 to 21 days of gestation. The protein kinase C activity, measured in the presence (Fig. 1A) or absence (Fig. 1B) of lipid activators, increased from day 18 to day 20 of fetal gestation and then decreased on day 21. This decrease of protein kinase C activity on day 21 was more evident when assays were performed in the absence of lipid activators (Fig. 1B). Similar results were obtained when protein kinase C was first partially purified on DEAE cellulose columns and assayed in the presence of lipid activators (not shown). The average recovery of protein kinase C activity after the purification step was 64%. Protein phosphatase activity increased in type II cells from day 18 to day 19, remained constant on day 20, but then decreased on day 21 (Fig. 1C). These data demonstrate that protein kinase C and protein phosphatase activities are developmentally regulated in maturing type II cells. However, the decreased activities on day 21 do not parallel the further increase in CT activity on day 21 (1).

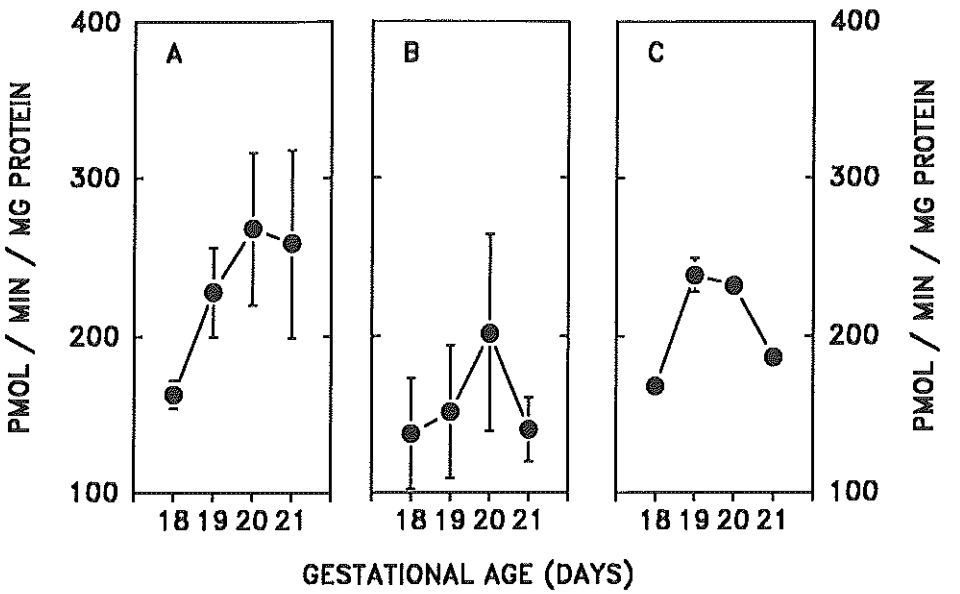

Figure 1. **Ontogeny of protein kinase C and protein phosphatase activities in fetal type II cells during late gestation.** Protein kinase C activity was assayed in the presence (A) or absence (B) of lipid activators (10 μM TPA, 0.28 mg/ml phosphatidylserine and 0.3% [v/v] Triton X-100 mixed micelles) in homogenates prepared from type II cells in primary culture, isolated from fetal rats at 18 to 21 days gestation. Activities were corrected for background activity measured in the presence of protein kinase C inhibitor peptide. Protein phosphatase activity was assayed by measuring the rate of dephosphorylation of [γ-^{32}P]-labelled phosphorylase a in postmitochondrial supernatants of day 18 to 21 fetal type II cells (C). The data represent mean $\pm$ S.E. of three independent experiments carried out in duplicate. Where error bars are not evident, they are within the plot point.

Regulation of CT in fetal type II cells by protein phosphatases

Recently, we (23) and others (22) have shown that CT can be phosphorylated *in vitro* by cAMP-dependent protein kinase. However, it appears that CT activity in fetal type II cells is not regulated by cAMP-dependent protein kinase (23). It has been shown that the liver enzyme can be dephos-

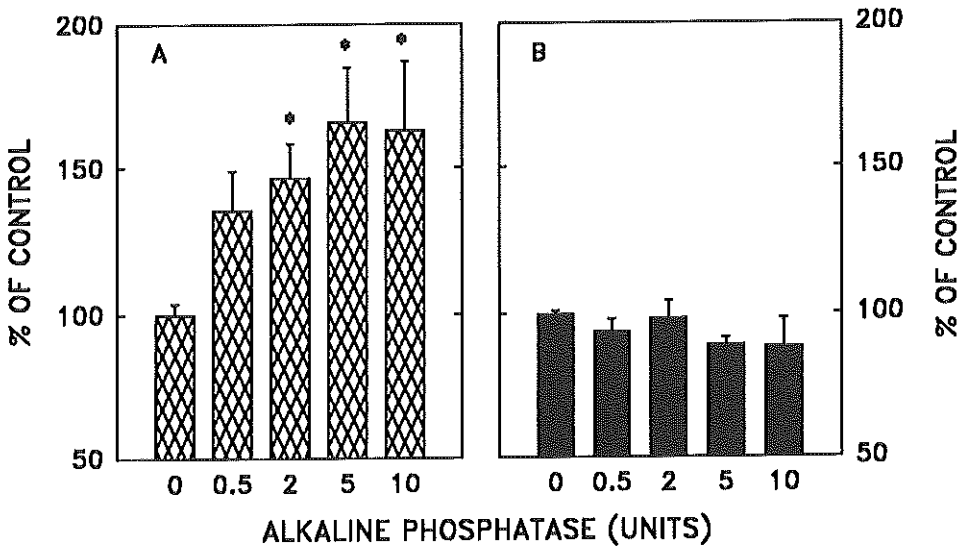

Figure 2. **Effect of alkaline phosphatase on the activity of purified rat lung cytidylyltransferase.** Purified lung cytidylyltransferase was incubated at 37°C with 0 to 10 units of alkaline phosphatase and 4 mM $MgCl_2$ in the absence (A) or presence (B) of 0.5 mM (1:1 molar ratio, 0.25 mM each) phosphatidylcholine/oleic acid vesicles. After 30 min of incubation, cytidylyltransferase activity was assayed. Control activities were 0.08 nmol/min in the absence (A) and 0.47 nmol/min in the presence (B) of phosphatidylcholine/oleic acid vesicles. Mean ± S.E. are shown of three separate experiments carried out in duplicate.

phorylated *in vitro* by alkaline phosphatase (22). Here we found that dephosphorylation of purified lung CT with alkaline phosphatase increased CT activity in a concentration-dependent manner (Fig. 2A). However, when purified lung CT was incubated with 0.5 mM PC/OA vesicles (1/1 molar ratio, 0.25 mM each) prior to alkaline phosphatase treatment, no further activation of the enzyme was observed (Fig. 2B). The most likely explanation for the latter finding is that CT is maximally stimulated by the PC/OA vesicles (13,35). We then incubated cytosol of day 20 fetal type II cells with

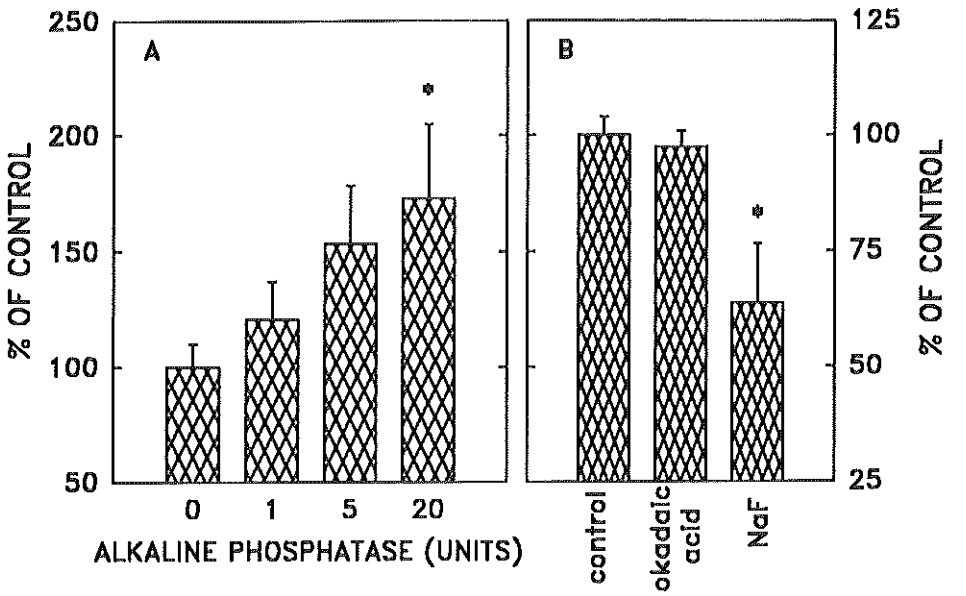

Figure 3. **Effect of alkaline phosphatase and phosphatase inhibitors on cytidylyltransferase activity of fetal type II cell cytosol.** (A) Cytosol (50-100 μg protein) from day 20 fetal type II cells (homogenized in 145 mM NaCl, 50 mM Tris-HCl, pH 7.4) was pre-incubated at 37°C with 0 to 20 units of alkaline phosphatase in the presence of 8 mM $MgCl_2$ and 2 mM DDT. (B) Cytosol (50-100 μg) was pre-incubated at 37°C in the presence of 0.5 mM ATP, 8 mM $MgCl_2$ and 2 mM DDT (control) with the addition of 5 μM okadaic acid or 50 mM NaF. The okadaic acid control, 25 μl of 20% DMSO in ethanol, was not different from regular buffer control. After 30 min of incubation, cytidylyltransferase activity was measured. The activity is presented as % of control (0.25 nmol/min/mg protein). Data represent mean ± S.E. of at least 3 separate experiments carried out in duplicate.

increasing concentrations of alkaline phosphatase. Again, we found a concentration-dependent increase in cytosolic CT activity (Fig. 3A). Incubation of cytosol of day 20 fetal type II cells with 50 mM NaF, a potent protein phosphatase inhibitor, decreased the CT activity (Fig. 3B). We did not find a significant inhibition of cytosolic CT activity after incubation with 5 μM okadaic acid, a potent inhibitor of two of the most common protein

phosphatases, type 1 and 2A. Taken together, these results are compatible with CT activity being regulated by protein phosphatase activity in fetal type II cells.

Effect of protein kinase C on CT activity of fetal type II cells

Amino acid sequence analysis of CT has revealed several potential phosphorylation sites for protein kinase C (14,36). To determine whether protein kinase C can influence CT activity in fetal type II cells, we first incubated day 20 fetal type II cell cytosol with activators or inhibitors of protein kinase C. In these experiments, cytosol was incubated for 30 min at 37°C in the presence of 0.5 mM ATP, 8 mM $MgCl_2$ and 2 mM DDT and the substance to be tested. No effect on CT activity was noted when different concentrations of TPA (10^{-8} to 10^{-4} M) were added to the incubation medium (Fig. 4A). However, cytosolic protein kinase C activity was increased by $\approx$70 % after 10^{-5}M TPA treatment. When cytosolic protein kinase C was first partially purified on a DEAE cellulose column, the protein kinase C activity was increased by 170% after incubation with 10^{-5}M TPA (not shown). Neither 20 μM protein kinase C inhibitor peptide nor 50 μM protein kinase inhibitors H7 and H8 did affect cytosolic CT activity (Fig. 4B). Although H8 has a higher affinity for cyclic nucleotide-dependent kinases (including protein kinase A) than for protein kinase C, it will inhibit protein kinase C activity at a concentration of 50 μM (37). H7 is a potent protein kinase C inhibitor (37,38). Pre-incubation of cytosol with all 3 inhibitors, indeed, decreased protein kinase C activity (not shown). Also, there was no effect on cytosolic CT activity when ATP was omitted from the incubation medium (not shown). In line with these cytosolic findings,

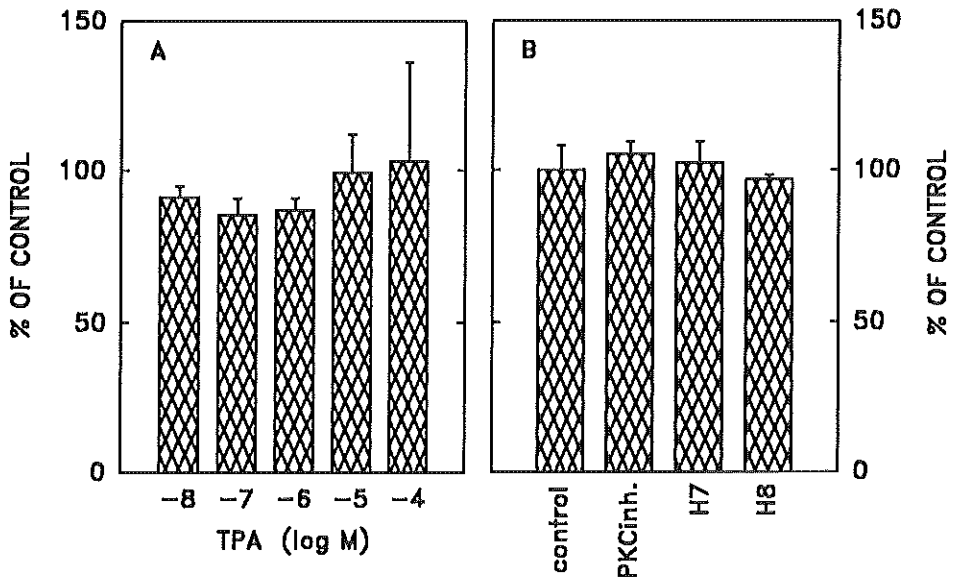

Figure 4. **Effect of phorbol ester (TPA) and protein kinase C inhibitors on cytidylyltransferase activity of fetal type II cell cytosol.** Cytosol (50-100 μg protein) from day 20 fetal type II cells (homogenized in 145 mM NaCl, 50 mM Tris-HCl, pH 7.4) was incubated for 30 min at 37°C in the presence of 0.5 mM ATP, 8 mM $MgCl_2$ and 2 mM DDT (control) with the addition of either 10^{-8} to 10^{-4} M TPA, 20 μM protein kinase C inhibitor peptide, 50 μM H7 or 50 μM H8. Subsequently cytidylyltransferase activity was measured (presented as % of control (0.25 nmol/min/mg protein)). Data represent mean ± S.E. of at least 3 separate experiments carried out in duplicate.

incubations of intact day 20 fetal type II cells with 10^{-5}M TPA for 1 hour did not alter CT activity in either post-mitochondrial supernatant, cytosol or microsomes (Fig. 5A). In addition, the distribution of CT activity between cytosol and microsomes was not altered by TPA treatment (not shown). Similar results were obtained when cells were exposed for 3 hours to different concentrations (10^{-8}M to 10^{-4}M) of TPA. It is well known that protein kinase C activation is mediated through its translocation from the

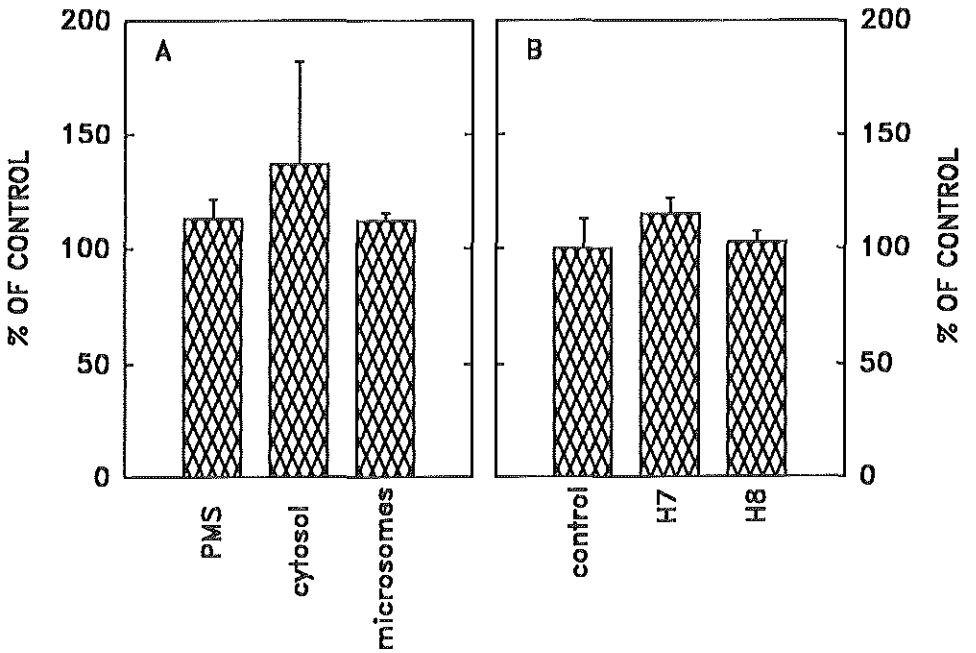

Figure 5. **Effect of phorbol ester (TPA) and protein kinase inhibitors on cytidylyltransferase activity of fetal type II cells in primary culture.** (A) Day 20 fetal type II cells were incubated with MEM plus 10 μM TPA or MEM containing an equal mount of 10% DMSO in ethanol as used for TPA (control). After 1 hour of incubation, cells were homogenized and cytidylyltransferase activity was assayed in post-mitochondrial supernatant (PMS), cytosol and microsomes. (B) Day 20 fetal type II cells were incubated with MEM alone (control) or MEM with either 50 μM H7 or H8. After 3 hours of incubation, cells were homogenized and cytidylyltransferase activity was assayed in post-mitochondrial supernatant (PMS). The cytidylyltransferase activities are presented as percentage of control (PMS: 0.9 nmol/min/mg protein; cytosol: 0.25 nmol/min/mg protein; microsomes: 1.9 nmol/min/mg protein). Data represent mean $\pm$ S.E. of 3 separate experiments carried out in duplicate.

cytosolic to the membrane fraction. As shown in Table 1, TPA induced a shift in the subcellular distribution of protein kinase C activity from cytosol to membranes in fetal type II cells after 1 h exposure. The specific activity

Table 1: **Effect of phorbol ester (TPA) on protein kinase C of fetal type II cells in primary culture.**

Treatment	Protein kinase C activity	
	% in membrane fraction	Pvalue
Control	57.3 ± 2.3	-
TPA, 1 μM	73.8 ± 5.3	<0.05
TPA, 10 μM	86.5 ± 2.7	<0.05

Day 20 fetal type II cells were incubated with MEM plus either 1 or 10 μM TPA or MEM containing an equal mount of 10% DMSO in ethanol as used for TPA (control). After 1 hour of incubation, cells were homogenized and protein kinase C activity was assayed in cytosol and microsomes. Percentage of total activity present in membrane fraction is calculated as total activity in membrane fraction (specific activity x protein) x 100, over sum of total activities in cytosol and membranes. Data represent mean ± S.E. of 3 separate experiments.

in the membranes increased 2-fold. Exposure of cells for 3 hours to 50 μM H7 or H8 also did not change the CT activity in the post-mitochondrial supernatant (Fig. 5B). Similar results were observed after 1 hour exposure to H7 or H8 (not shown). Previously, we have shown that these inhibitors decrease the basal protein kinase C activity in fetal lung cells (39). Taken together, these results suggest that protein kinase C does not regulate CT activity in fetal type II cells.

7.5 Discussion

Reversible phosphorylation is a common mechanism for the regulation of many intracellular events in eukaryotic cells (26,27, 40). Phosphorylation and dephosphorylation of proteins has been demonstrated for adult type II cells (41). In these cells, several proteins were shown to be phosphorylated by protein kinase C and dephosphorylated by protein phosphatases, suggesting key roles for these enzymes in reversible protein phosphorylation in adult type II cells. The present study shows that the activities of protein kinase C and protein phosphatase are developmentally regulated in fetal type II cells during a period which is critical for the preparation of the lung to the onset of air-breathing. The developmental profile of CT activity (1) is similar to those of protein kinase C and protein phosphatase activities till day 20 of gestation. Both protein kinase C and protein phosphatase activities decrease on day 21 while that of CT increases (1). Thus, a role for these enzymes in regulating CT activity in fetal type II cells during development is unclear.

The developmental profiles of protein kinase C and protein phosphatase activities correlate with those of growth related genes, c-myc and histone 3 (42). This is not surprising, as it is well known that protein kinases and protein phosphatases are involved in the intracellular transduction pathways of growth factors (43). Protein kinase C is involved in the cascade of intracellular events following activation of several growth factor receptors. Thus, the developmental profiles of protein kinase C and protein phosphatase activities likely reflect the balance between growth and maturation of type II cells during late fetal gestation. Studies with whole rat lung have shown that protein phosphatase type 1 and 2A activities increase

from fetal day 18 to term (45). The different ontogenic profiles of protein phosphatases in whole fetal lung and isolated fetal type II cells are most easily explained by the cellular complexity of the lung. To our knowledge, no developmental profile of protein kinase C activity in lung has been reported. Interestingly, the ontogenic pattern for protein kinase C is remarkably similar to that of cAMP-dependent protein kinase in fetal type II cells (23). This supports the idea that the activity of both protein kinases in fetal type II cells reflects a stage of cellular development.

Although no clear temporal relationship between protein phosphatase and CT activities was noted, several lines of evidence suggest that CT activity is regulated by protein phosphatase activity in fetal type II cells. First, we found that dephosphorylation of purified lung CT as well as of cytosolic CT of fetal type II cells with alkaline phosphatase stimulated its activity. Secondly, incubation of cytosol under phosphorylating conditions with NaF decreased CT activity. More or less similar findings have been reported by Radika and Possmayer with fetal rabbit lung (45). Surprisingly, okadaic acid did not inhibit cytosolic CT activity. Recent studies with hepatocytes (19), have suggested that okadaic acid-induced inhibition of PC synthesis is due to an increase in CT phosphorylation. Also, pre-incubation with Mg^{++} and ATP had no effect on CT activity in cytosol of isolated type II cells (23). Studies with purified CT (22), rat liver (17), hepatocytes (19) and CHO cells (20) have suggested that dephosphorylation of CT promotes translocation of CT from the cytosolic to membrane fraction, thereby activating the enzyme. Recent studies with HeLa cells (16) and hepatocytes (21) suggest, however, that a change in lipid composition of membranes is probably the initial event which mediates translocation of CT and that dephosphorylation of the enzyme is not required for translocation. Whether

such translocation mechanism without dephosphorylation occurs in fetal type II cells remains to be investigated.

Our findings with intact fetal type II cells and with cytosol prepared from such cells suggest that protein kinase C does not regulate CT activity in fetal type II cells. Neither protein kinase C activators nor inhibitors had any effect on the enzyme activity. Recently, Scott (46) reported that disaturated phosphatidylcholine (DSPC) synthesis increases in fetal rabbit type II cells in response to TPA. Another known activator of protein kinase C, *sn*-1-oleoyl-2-acetylglycerol (OAG), decreased, however, DSPC synthesis in these cells (46). Moreover, the activity of the enzyme CT was not studied. In HeLa cells, TPA stimulates PC synthesis (47-49). It is unclear whether the increased PC synthesis in HeLa cells is caused by a translocation of CT from cytosol to membrane (47,48,50,51). Studies with other cells are also controversial regarding TPA induction of CT activity by translocation (52-56). However, it appears that direct phosphorylation of the enzyme by protein kinase C does not account for the increase in CT activity in HeLa cells (47-49). As CT contains several potential sites for phosphorylation by protein kinase C (14,36,57), further studies are required to reveal their function in the complex regulation of CT activity in fetal type II cells.

We conclude that protein kinase C and protein phosphatase activities are developmentally regulated in fetal type II cells during late gestation. Although dephosphorylation of CT *in vitro* activates the enzyme, the importance of dephosphorylation in the overall regulation of CT activity in fetal type II cells remains to be elucidated. Moreover, it is unclear which protein kinase regulates CT activity by phosphorylation in fetal type II cells. Our present and previous (23) studies suggest that neither protein kinase C nor cAMP-dependent protein kinase are directly involved in the regulation of CT

activity in fetal type II cells.

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CHAPTER 8

MECHANISMS OF DECREASED SURFACTANT PHOSPHATIDYLCHOLINE SYNTHESIS IN RATS WITH CONGENITAL DIAPHRAGMATIC HERNIA

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submitted

8.1 Abstract

We studied the underlying mechanisms of surfactant deficiency in a rat model for congenital diaphragmatic hernia (CDH), induced by Nitrofen. Fetal type II cells and lung fibroblasts were isolated on day 21 of gestation. Phosphatidylcholine (PC) and disaturated phosphatidylcholine (DSPC) synthesis were lower in CDH than in control (no Nitrofen) type II cells. The activity of CTP:phosphocholine cytidylyltransferase (CT), a rate-regulatory step in de the novo PC synthesis, was lower in type II cells from severe CDH than in controls or than in a milder form of CDH. No difference was found in lysoPC acyltransferase activity or protein/DNA ratio. Conditioned medium from fetal lung fibroblasts stimulated with cortisol (FCM) is known to increase DSPC synthesis in fetal type II cells. FCM from control fibroblasts stimulated PC and DSPC synthesis in both control and CDH fetal type II cells, but FCM from CDH fibroblasts did not.
These data suggest that decreased PC and DSPC synthesis in CDH can be explained by a lower CT activity in fetal type II cells due to a decreased production of fibroblast pneumocyte factor by lung fibroblasts.

8.2 Introduction

Despite significant advances in the management of newborn infants with congenital diaphragmatic hernia (CDH), mortality rate remains as high as 40-60% (1). This high mortality is mainly attributed to pulmonary hypoplasia and persistent pulmonary hypertension (2). It has become clear

however, that the lungs of newborn infants with CDH are not only hypoplastic, but also show a variable morphological and biochemical immaturity (3-6). This immaturity has also been found in animal models for CDH. Besides structural immaturity (7), surfactant deficiency has been demonstrated in a fetal lamb model (8) and a fetal rat model (9) of CDH. The induction of CDH in fetal rats by the maternal administration of Nitrofen (2,4 dichlorophenyl-p-nitrophenylether) has been proven to be a reproducible and reliable model for CDH with many characteristics which are also present in children with CDH (2,10,11). By varying the timing of the Nitrofen gift during pregnancy the location of the diaphragmatic defect and the severity of pulmonary hypoplasia can be manipulated (10). In this model Suen et al. showed a decreased amount of pulmonary disaturated phosphatidylcholine (DSPC) when compared to control fetuses (9). DSPC is a major constituent of pulmonary surfactant, which is produced in alveolar type II pneumocytes. The CDPcholine pathway is the primary pathway for the de novo phosphatidylcholine (PC) synthesis in the developing lung (reviewed in 12). Pool size studies have demonstrated that the reaction catalyzed by CTP:phosphocholine cytidylyltransferase (CT) is the rate-limiting step in the CDPcholine pathway in fetal type II cells (13). Previously, we showed that the activity of CT increases in fetal type II cells during late gestation (14). There is good evidence that a considerable part of DSPC is formed by remodelling of de novo synthesized unsaturated PC (reviewed in 12). The most important mechanism for this remodelling is a deacylation of PC at the 2-position followed by a reacylation of the resulting palmitoyllysophosphatidylcholine by the enzyme lysophosphatidylcholine acyltransferase, which has a high selectivity to utilize palmitoyl-CoA as a substrate (12,15) and shows a developmental increase in fetal type II cells (16). The synthesis of DSPC in

fetal type II cells is under hormonal control. The maternal administration of glucocorticoids leads to an increased PC synthesis in type II cells isolated from rat fetuses (17). This glucocorticoid action however, is not directly on the fetal type II cells but involves the production of a fibroblast-pneumocyte factor (FPF) by the fetal lung fibroblasts (18). This FPF then, stimulates PC synthesis in fetal type II cells by increasing CT activity (18).

To gain a better insight into the mechanisms by which the amount of surfactant DSPC is reduced in CDH, we studied PC and DSPC synthesis, CT and lysophosphatidylcholine acyltransferase activities in fetal type II cells isolated from rat fetuses with Nitrofen-induced CDH. As type II cell-fibroblast interactions are important for the control of DSPC synthesis, also the capacity of lung fibroblasts isolated from rat fetuses with Nitrofen-induced CDH to stimulate PC synthesis in fetal type II cells was studied.

8.3 Materials and Methods

Materials

Female and male Sprague Dawley rats were purchased from Harlan Olac (Zeist, the Netherlands) and bred in our animal facilities. 2,4 dichlorophenyl-p-nitrophenylether (Nitrofen) was obtained from Rohm Haas Company (Philadelphia). Cell culture media, trypsin and fetal and newborn bovine serum were obtained from Gibco (Gaithersburg,MD), collagenase and DNase from Worthington Biochemical (Freehold, NJ). Cell culture flasks and multiwell plates were purchased from Costar (Cambridge,USA). [*Methyl*-^{14}C]phosphocholine, [*Methyl*-^{14}C]CDPcholine, [1-^{14}C]palmitoyl-CoA and [*Methyl*-^{3}H]choline were from Amersham (UK). Remaining

unlabelled biochemicals were obtained from Merck (Darmstadt, Germany).

Induction of CDH

Rats were mated during 1 hour. This was considered day 0 of gestation. On day 10 of gestation 100 mg Nitrofen, dissolved in 1 ml olive oil, was administered by gastric tube, resulting in a left-sided or bilateral CDH with severe pulmonary hypoplasia in the majority of fetuses (d10-CDH) (19). Controls received 1 ml of olive oil. In some experiments 60 mg Nitrofen was given on day 12 of gestation, resulting in a small right-sided CDH with only mild pulmonary hypoplasia in the majority of fetuses (d12-CDH). Food and water were supplied ad libitum during the whole period of pregnancy.

Cell cultures

The pregnant rats were sacrificed by diethylether excess on day 21 of gestation (term = day 22.5) and the fetuses were aseptically removed from the dams and sacrificed by decapitation. Median sternotomy with extension into both chest cavities by bilateral supracostal incisions was performed and the diaphragm and lungs inspected for the presence or absence of CDH before removal of the lungs. For d10-CDH only (both) lungs from fetuses with the typical large left-sided (missing more than half of the diaphragmatic crus) or bilateral CDH were pooled. For d12-CDH only (both) lungs from fetuses with the typical small right-sided CDH (missing up to 30% of the diaphragmatic crus) were pooled. Controls did not show CDH. For each culture, lungs from at least 20 (up to 50) fetuses were pooled. The epithelial cells and fibroblasts were isolated from the fetal lungs as described in detail elsewhere (20,21). Although the term 'type II cells' is used in this paper, the cuboidal epithelium which lines the acinar tubules during the late pseudo-

glandular and early canalicular stages of lung development does not contain lamellar bodies, the phenotypic marker for type II cells. In previous studies, it was shown that these cells do express other phenotypic features of type II cells and possess antigenic determinants of mature type II cells (20,22).

Fibroblast-conditioned cortisol-containing media

Fibroblast-conditioned media were prepared as described previously (23,24). In short, fibroblasts (from controls or d10-CDH) that had attached to the tissue culture flasks during the first 1-h attachment period (20) in minimal essential medium containing 10% fetal bovine serum were rinsed with serum-free MEM and grown to confluence in MEM containing 10% charcoal-stripped newborn calf serum. At confluence, fibroblasts were rinsed with serum-free MEM and subsequently incubated for 24 h in serum-free MEM containing 10^{-7} M cortisol. After exposure, the medium was collected, centrifuged to remove detached cells and incubated for 2 h at 58°C (23,24) and stored at -40°C. Portions of MEM containing 10^{-7} M cortisol but not exposed to fibroblasts, were treated in the same manner.

Measurement of PC and DSPC synthesis in fetal type II cells

At the end of the cell isolation (20) type II cells were plated in equal numbers (in high density) in multiwell plates. After overnight incubation of fetal type II cells in MEM containing 10% fetal bovine serum, cells were rinsed with serum-free MEM and subsequently incubated with the media described above pre-mixed 1:1 with serum-free MEM. To these media 0.5 μCi/ml [*Methyl*-^{3}H]choline was added. After 24 hours of incubation, the medium was removed and cells were rinsed repeatedly with phosphate buffered saline. Cells were scraped and homogenized. After a portion of the

homogenate had been kept for protein determination, an aliquot (0.2 μmol phospholipid phosphorus) of a natural surfactant (Alvofact^{R}, Boehringer Ingelheim, Germany) was added as a carrier and lipids were extracted by the method of Bligh and Dyer (25). An aliquot of the extracted lipids was evaporated to dryness in a scintillation vial. After the addition of scintillation liquid (Instagel, Packard-Becker, Groningen, Netherlands) radioactivity was counted. Although the PC-fraction was not isolated, Rooney and Motayama (26) demonstrated that almost 95% of the radioactivity incorporated from [*Methyl*-^{3}H]choline into lipid was found in PC. Therefore, this measurement will be considered a measure of PC synthesis. DSPC was isolated from the lipid extract by the method of Mason (27) and after drying, radioactivity was counted.

Enzyme assays

Enzymes were assayed in post-mitochondrial supernatants obtained from fetal type II cells as follows. After overnight incubation in tissue culture flasks and rinsing with a Tris buffer (145 mM NaCl, 50 mM Tris-HCl, pH 7.4), attached cells were collected by scraping in homogenization buffer consisting of 145 mM NaCl, 50 mM Tris-HCl (pH 7.4), 50 mM NaF and 2.5 mM EDTA. The cells were homogenized with 50 strokes of a Dounce homogenizer and the homogenate was centrifuged at 300 g for 10 min at 4°C. The resulting supernatant was centrifuged at 13000 g for 10 min at 4°C to obtain a postmitochondrial supernatant. This was stored at -70°C (14).

CT activity was assayed in the forward direction by measuring the rate of incorporation of [*methyl*-^{14}C]phosphocholine into CDPcholine.

The incubation medium (0.1 ml) contained 50 mM Tris-succinate (pH 6.5),

10 mM MgAcetate, 5 mM CTP, 1 mM [*Methyl*-^{14}C]phosphocholine (specific activity 0.45 μCi/μmol) and up to 100 μg protein. Assays were performed in the absence and presence of (egg) PC / oleic acid (OA) (0.25 mM each) vesicles, which are known to activate the enzyme (28,29). After 20 min of incubation the reaction was terminated by boiling for 2 min, and the protein was removed by centrifugation. A 50 μl aliquot was taken from the supernatant, mixed with 2 μmol of unlabelled CDPcholine and 8 μmol of unlabelled phosphocholine as carriers, and applied to silica H-plates which were developed in 0.5% NaCl in H_2O/methanol/concentrated NH_3 (50:50:5 v/v) for isolation of the reaction product, CDPcholine (30). Radioactivity was measured in a scintillation counter.

Lysophosphatidylcholine acyltransferase activity was assayed by measuring the conversion of 1-palmitoyl-*sn*-glycero-3-phosphocholine into PC using [1-^{14}C]palmitoyl-CoA as acyl donor (16).

In preliminary experiments, we established that, under the conditions used, product formation in all assays was directly proportional to incubation time and amount of protein.

Protein measurements

Protein concentrations were determined by the method of Bradford (31), using bovine serum albumin as standard.

DNA measurements

DNA concentrations were determined in cell sonicates by the method described by Labarca and Paigen (32) which is based on the enhancement of fluorescence seen when bisbenzimidazole (Hoechst 33258) binds to DNA. Herring sperm DNA was used as standard.

Statistical analysis

Statistical differences between various groups were analyzed by unpaired t-test (for two groups) or by analysis of variance with the Neumann-Keuls test (for more than two groups). Statistical significance was accepted at the $p<0.05$ level (two-tailed).

8.4 Results

Comparison between fetal type II cells from CDH and controls

When 100 mg Nitrofen was given on day 10 of gestation, large left-sided CDH with severe pulmonary hypoplasia were found in 70-80% of the fetuses (d10-CDH). When 60 mg Nitrofen was given on day 12 of gestation small right-sided CDH with only mild pulmonary hypoplasia were found in 70-80% of the fetuses (d12-CDH). Fetuses from the occasional dam which contained <60% of fetuses with the typical diaphragmatic defect were not collected. As described under 'Methods', for tissue cultures, only lungs from fetuses with the typical defect (or from control rats) were collected. No immunohistochemical identification of cells in primary culture was performed, but on phase-contrast microscopy, CDH type II cell and fibroblast cultures had the typical aspect of those cells (20).

The protein/DNA ratio was not different between d10-CDH and control fetal type II cells (Table 1). PC and DSPC synthesis were measured in confluent type II cells in primary culture incubated with previously prepared cortisol-containing MEM (heated at 58°C for 2 hours) diluted 1:1 with fresh serum-free MEM. PC and DSPC synthesis were significantly lower in d10-CDH compared to control fetal type II cells (Table 1).

Table 1. **Comparison between day-21 fetal type II cells isolated from control fetuses and fetuses with severe CDH (d10-CDH).**

	control	d10-CDH
protein/DNA (μg/μg)	6.65 ± 1.44	6.99 ± 1.72
PC synthesis (dpm/well)	469057 ± 128298#	331771 ± 83568#
DSPC synth. (dpm/well)	127233 ± 31584*	69203 ± 16722*

Results are expressed as mean ± S.D. For protein/DNA n=5 performed in duplicate. PC and DSPC synthesis were measured in confluent type II cells in primary culture incubated with previously prepared cortisol-containing MEM (heated at 58°C for 2 hours) diluted 1:1 with fresh serum-free MEM. For PC and DSPC synthesis n=10 (with a similar protein content of 142±78 μg/well in controls and d10-CDH); * significantly different with $p<0.001$; # $p=0.011$.

CT and lysophosphatidylcholine acyltransferase activity in fetal type II cells from controls and from fetuses with mild or severe CDH

In order to better understand why the amount of DSPC is decreased in CDH, we elected to measure the activity of CT and lysophosphatidylcholine acyltransferase because they catalyse important steps in the synthesis of DSPC and are known to be developmentally regulated in normal fetal type II cells (14,16). CT, assayed both in the absence or presence of PC/OA vesicles, was decreased in fetal type II cells isolated from d10-CDH but not in type II cells from fetuses with d12-CDH (Fig. 1), which have a much milder degree of pulmonary hypoplasia. The stimulation of type II cell CT activity by PC/OA vesicles is low (around 20%) and not significantly different between control and CDH groups (Fig. 2).

Lysophosphatidylcholine acyltransferase activity in fetal type II cells, although lowest in d10-CDH, was not significantly different between controls

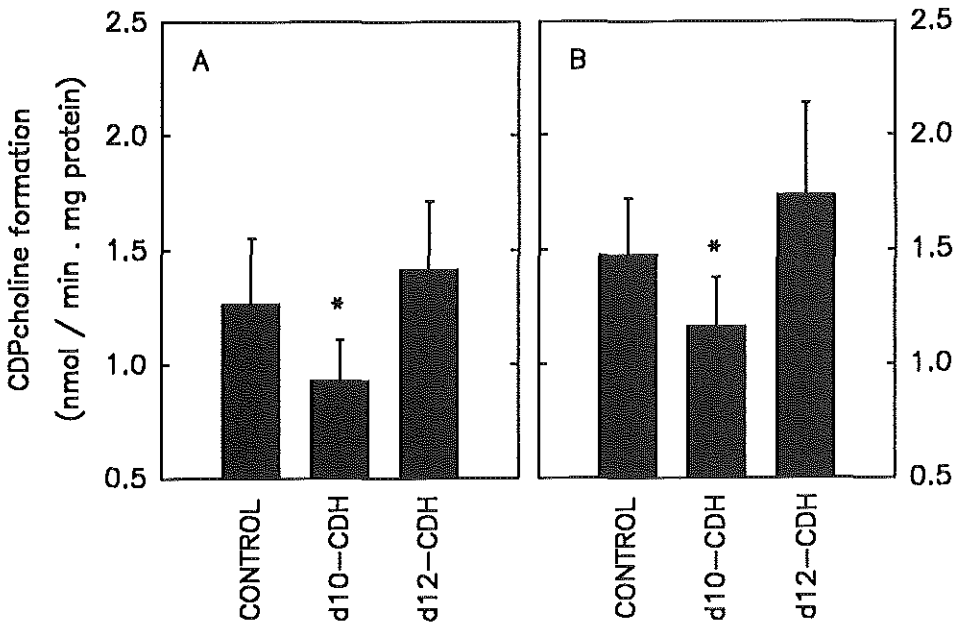

Figure 1. **CTP:phosphocholine cytidylyltransferase activity in fetal type II cells from CDH compared to controls.** Cytidylyltransferase activity was assayed in the absence (A) or presence (B) of phosphatidylcholine/oleic acid (0.25 mM each) vesicles in post-mitochondrial supernatant of day-21 fetal type II cells isolated from lungs of controls, severe CDH (d10-CDH) and small CDH (d12-CDH). Data represent means±S.D. of 8 independent experiments for controls and d10-CDH and of 4 independent experiments for d12-CDH. Each measurement was performed in quadruplicate. * significantly different from control and d12-CDH by ANOVA ($p<0.01$) followed by Neumann-Keuls test.

and both CDH groups (Fig. 3).

PC and DSPC synthesis in fetal type II cells incubated with fibroblast-conditioned cortisol-containing medium (FCM) from lung fibroblasts of controls and of fetuses with severe CDH

CT activity and thus PC and DSPC synthesis in fetal type II cells are regulated by a fibroblast-pneumocyte factor (FPF) produced by fetal lung

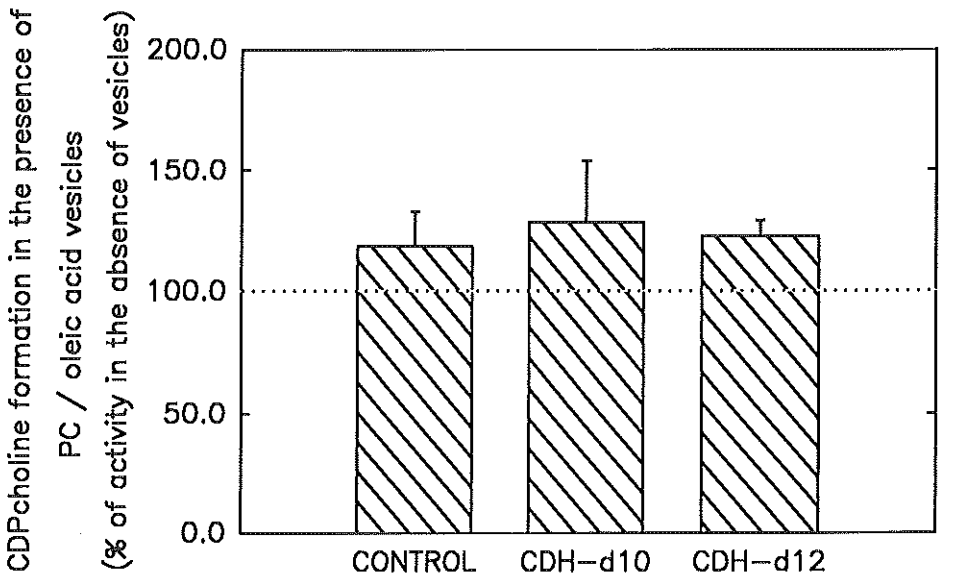

Figure 2. **Stimulation of CTP:phosphocholine cytidylyltransferase by activating lipids in fetal type II cells from CDH compared to controls.** Data from figure 1 were used to calculate the activity in the presence of phosphatidylcholine/oleic acid vesicles as a percentage of the activity in the absence of the lipid vesicles. Phosphatidylcholine/oleic acid vesicles in the concentration used, are known to maximally activate cytidylyltransferase. Data represent means±S.D. of 8 independent experiments for controls and d10-CDH and of 4 independent experiments for d12-CDH. Each measurement was performed in quadruplicate.

fibroblasts (18). Therefore, to better understand the regulation of CT activity and PC and DSPC synthesis in CDH, we studied type II cell-fibroblast interactions in the Nitrofen model. FCM from fibroblasts of controls stimulated PC and DSPC synthesis significantly in both control and d10-CDH fetal type II cells (Table 2). FCM from fibroblasts of d10-CDH did not stimulate PC and DSPC synthesis significantly in either control or d10-CDH fetal type II cells (Table 2). We conclude that fetal type II cells from d10-

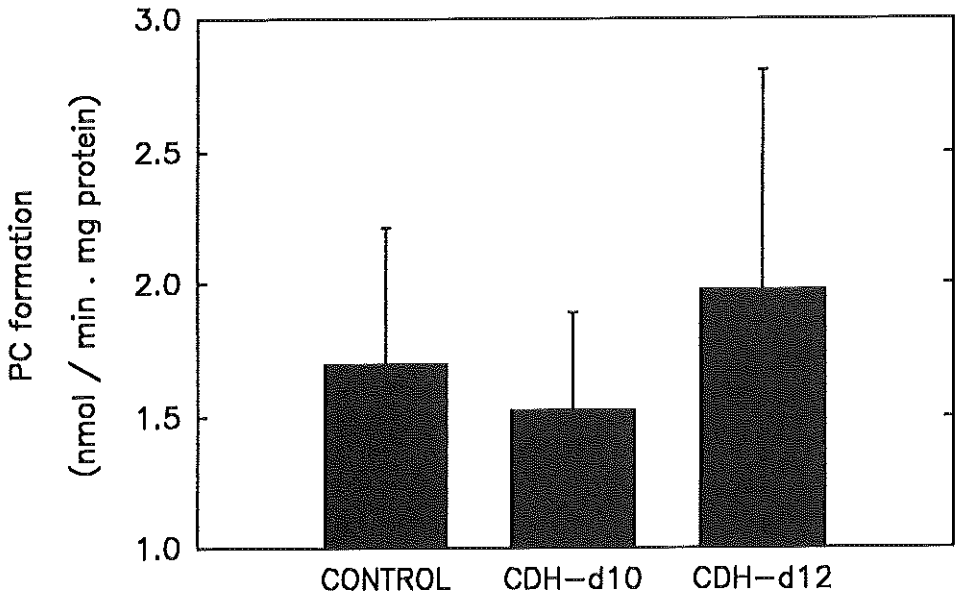

Figure 3. **Lysophosphatidylcholine acyltransferase activity in fetal type II cells from CDH compared to controls.** Acyltransferase activity was assayed in post-mitochondrial supernatant of day-21 fetal type II cells isolated from lungs of controls, severe CDH (d10-CDH) and small CDH (d12-CDH). Data represent means±S.D. of 6 independent experiments for controls and d10-CDH and of 4 independent experiments for d12-CDH. Each measurement was performed in quadruplicate.

CDH are able to respond well to stimulation by FCM, but fibroblasts from d10-CDH do not produce adequate amounts of FPF-like activity in response to cortisol.

8.5 Discussion

The present study is the first to describe the underlying mechanisms of the decreased surfactant concentration in the lungs of fetuses with CDH.
Firstly, we studied protein/DNA ratio in type II cells from control and CDH fetuses because two studies using the Nitrofen-induced CDH rat model did find a significantly lower protein/DNA ratio in CDH lungs compared to control lungs, although the difference was small (9,33). We did not find a difference in protein/DNA ratio between CDH and control fetal type II cells in primary culture (Table 1). This is in agreement with the study of Sluiter et al. (34) who did not find a difference in protein/DNA ratio between lungs from control fetuses and lungs from fetuses with CDH, using the same Nitrofen-induced CDH rat model. Hosoda et al. (35) also found no difference in protein/DNA ratio between control lungs and hypoplastic lungs in two different models for pulmonary hypoplasia, a rabbit chronic amniotic leak model and a CDH fetal lamb model. These data suggested that cell size in the hypoplastic lungs was unchanged and that the hypoplasia was due to a decreased cell population (hypoplasia but not atrophy) (35). We demonstrated previously that also during normal fetal rat development, protein/DNA ratio in whole lung (34) or isolated type II cells (14) did not change with advancing gestation.
Secondly, we investigated PC and DSPC synthesis in fetal type II cells from control and CDH fetuses because previous studies had demonstrated that phospholipid and especially DSPC was decreased in lungs of fetuses with Nitrofen-induced CDH compared to controls (9,33). We found a decreased PC and DSPC synthesis in type II cells from CDH fetuses compared to

controls (Table 1). Type II cells isolated at fetal day-21 from rats with severe CDH were responsive to conditioned medium from cortisol-treated control lung fibroblasts: PC and DSPC synthesis increased in the type II cells in response to FCM. The magnitude of this response was at least equal to that in control fetal type II cells (Table 2). Caniggia et al. (20) observed an at least equally good response to FCM with regard to increasing DSPC synthesis in type II cells from day-20 rat fetuses as in those from day-21 fetuses, while type II cells from day-19 fetuses showed less response to FCM. According to their counting our experiments were performed on day 20.5. Thus the observation that type II cells from CDH fetuses respond to FCM (Table 2) suggests that in lungs of CDH fetuses the immaturity of type II cells is not very pronounced. The ability of FCM obtained from fibroblasts isolated from normal fetal rat lungs at day 18 of gestation to increase DSPC synthesis in day-20 fetal type II cells was shown to be less than that of FCM from lung fibroblasts from fetuses at day 19 to day 21 of gestation (20). Similar developmental changes in fibroblasts, with regard to production of FCM stimulatory to type II cells, were shown in studies on mouse lung (36). Thus, the absence of a significant stimulatory effect of conditioned medium from cortisol-stimulated fetal lung fibroblasts isolated from rats with severe CDH on PC and DSPC synthesis by type II cells (Table 2), suggests a more pronounced immaturity of the fibroblasts than of the type II cells in the lungs of fetuses with CDH.

Thirdly, we found a decreased CT activity in fetal type II cells isolated from d10-CDH, which, at least partially, explains the decreased PC synthesis. This is consistent with a more immature state of these fetal type II cells, as CT activity has been shown to increase in fetal type II cells with advancing gestation (14). The relatively small difference between the CT activity in the

Table 2. **Phosphatidylcholine (PC) and disaturated phosphatidylcholine (DSPC) synthesis in day-21 fetal type II cells incubated with fibroblast-conditioned cortisol-containing medium (FCM) from fibroblasts of controls and of fetuses with severe CDH (d10-CDH).**

lipid synthesized	Rate of synthesis (% of synthesis in MEM+cortisol)		
	MEM +cortisol	FCM control	FCM d10-CDH
control type II cells			
PC	100 ± 16.5*	120.6 ± 25.7*	109.9 ± 26.3
DSPC	100 ± 18.4*	116.6 ± 20.5*	105.9 ± 17.4
d10-CDH type II cells			
PC	100 ± 19.7*	129.7 ± 36.1*	110.8 ± 40.8
DSPC	100 ± 23.2*	129.3 ± 47.9*	104.2 ± 33.3

[*Methyl*-^{3}H]choline incorporation into PC and DSPC was measured in control and d10-CDH fetal type II cells incubated with MEM+cortisol or fibroblast-conditioned cortisol-containing medium from control or d10-CDH fibroblasts (all diluted 1:1 with fresh serum-free MEM). PC and DSPC synthesis per μg cell protein (mean ± S.D.) were expressed as % of the average value after exposure to MEM+cortisol. In each type II cell culture the 3 conditions MEM+cortisol, FCM control and FCM d10-CDH were tested in parallel. Each value represents 18 FCM's (or MEM+cortisol) tested on 4 separate type II cell cultures. Results were similar when synthesis rate per culture well instead of per μg protein was used to calculate the percentages.
* The conditions MEM+cortisol and FCM control were significantly ($p<0.05$) different from each other by one way analysis of variance (comparing the 3 conditions) followed by Neumann-Keuls test.

fetal type II cells from fetuses with CDH and controls, again suggests that type II cell immaturity in lungs from fetuses with CDH is not very pronounced. CT activity in type II cells from day-21 control fetuses was not stimulated very much by PC/OA vesicles, which is in agreement with our previous findings of a more activated state of the enzyme at this stage of

development (29). However, the same low degree of stimulation by lipid vesicles was found in CDH fetal type II cells, also suggesting a highly activated state of the enzyme. These data suggest that the decrease in CT activity in type II cells from CDH fetuses is most likely explained by a decreased amount of the enzyme (37). FCM has been demonstrated to increase mRNA for CT in fetal type II cells (23). Our data can be explained by an immaturity of lung fibroblasts in CDH with a lower FPF production and as a result a lower amount of CT in the fetal type cells, resulting in decreased PC and DSPC synthesis. In a study on isolated fetal rat type II cells (16) it was found that the activity of lysophosphatidylcholine acyltransferase was lower when the cells were less mature. However, in the present study, we found no clear evidence for a lowered lysophosphatidylcholine acyltransferase activity in type II cells from CDH fetuses (Fig. 3).

Our observations can be summarized as follows. We found a decreased activity of CT in fetal type II cells isolated from rats with severe CDH. This decreased activity of CT in fetal type II cells from fetuses with CDH may, at least partially, be due to a decreased capacity of lung fibroblasts to produce fibroblast-pneumocyte factor. Indeed, conditioned medium from cortisol-stimulated fetal lung fibroblasts isolated from rats with severe CDH did not increase PC and DSPC synthesis significantly in fetal type II cells. On the other hand, the fetal type II cells isolated from rats with severe CDH were responsive to conditioned medium from cortisol-treated control lung fibroblasts. This suggests that in lungs of CDH fetuses the primary cause of decreased PC synthesis in the type II cells is immaturity of the fibroblasts.

This conclusion has implications for future research and potential treatment options in CDH. Research involving CDH should not only focus on epithelial maturation, but also on the maturation of the lung mesenchyme and

epithelial-mesenchymal interactions. Treatment of CDH, preferably started antenatally, would ideally advance both type II cell and fibroblast maturation. Epidermal growth factor has been shown to advance the production of FPF by lung fibroblasts in response to corticosteroids by one day in fetal mice (36). Thus, epidermal growth factor may be a possible candidate for antenatal treatment of CDH in combination with the recently studied antenatal administration of corticosteroids (38). The combined administration of antenatal corticosteroids and thyrotropin releasing hormone (TRH) was shown to improve pulmonary biochemical immaturity in the Nitrofen-induced CDH model (39). However, in the light of the recent finding of an increased perinatal and maternal morbidity in a large multicenter clinical trial with maternal administration of corticosteroids and TRH to prevent respiratory distress syndrome in premature infants (40), more experimental evidence is needed before the combination of corticosteroids and TRH for antenatal treatment of CDH can be used in clinical practice.

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CHAPTER 9

SUMMARY AND PERSPECTIVES

9.1 Summary

Chapter 1 (Introduction). Neonatal respiratory distress syndrome (RDS) is caused by lung immaturity associated with surfactant deficiency. Despite the success of the treatment of RDS with exogenous surfactant, many problems remain. Surfactant treatment is clearly not a substitute for attempts to increase fetal lung maturation. Therefore, it is necessary to have a profound understanding of the regulation of surfactant synthesis during fetal lung maturation. Lung development is usually described in five phases. During the last stages of fetal lung development cell proliferation slows down and cell differentiation becomes more important. The maturation of the type II cell during this stage of development is of special importance, because it is the producer of surfactant. Surfactant is essential for air breathing after birth. It helps to open alveoli during inspiration and prevent alveolar collapse during expiration. This function of surfactant is due to its capacity to reduce surface tension at the air-liquid interface of the alveoli and to vary surface tension with varying alveolar inflation. Surfactant consists of about 5-10% protein and >90% lipids of which phosphatidylcholine (PC) is by far the most abundant. The dipalmitoyl-form of PC has been shown to be the main surface tension lowering component. Together with the increased surfactant PC production towards the end of gestation, the activity of the enzyme CTP:phosphocholine cytidylyltransferase (CT) increases. This enzyme has been shown to be a rate-limiting step in the de novo PC synthesis and to be regulated by hormones. Therefore, to understand the regulation of surfactant production during late gestation, it is important to understand the mechanisms of regulation of CT activity in type II cells during fetal development.

However, the restrictions of studying the regulation of one enzyme in one cell type should be kept in mind.

Chapter 2 (Review of the literature). The primary pathway for the de novo PC synthesis is the CDPcholine pathway which has an increased activity towards the end of gestation. In this pathway, choline is phosphorylated by choline kinase. Phosphocholine is then converted to CDPcholine by CT. Finally, the phosphocholine moiety is transferred to diacylglycerol by the CDPcholine:1,2-diacylglycerol phosphocholinetransferase to form PC. Under most circumstances, the activity of CT has been shown to be rate limiting in fetal type II cells. This enzyme has been purified and the cDNA cloned from rat liver and lung. The central region is thought to be the catalytic region. The carboxy-terminal part can be phosphorylated and dephosphorylated on serine residues and contains an amphipathic α-helical domain, which has been shown to be the lipid-binding domain. Indeed, the interaction of CT with lipids is essential for CT activity. The active, lipid-bound forms of the enzyme are either CT bound to membranes, especially membranes of the endoplasmic reticulum, or large CT aggregates in the presence of phospholipids (so-called H-forms) in the cytosol. CT catalyses a reversible reaction but it is thought that in intact cells the reverse reaction is insignificant. The largest part of the review (chapter 2) describes the mechanisms of regulation of CT with emphasis on fetal type II cells in the developing lung.

Chapter 3. In fetal type II cells during late gestation, we demonstrated an increased PC synthesis from several precursors. CT was found to be the only enzyme in the CDPcholine pathway with a developmental increase similar to that of PC synthesis. When CT activity was assayed in different subcellular

fractions, the greatest increase, as a function of development, was found in microsomes. This developmental increase was accompanied by a shift in subcellular distribution of CT activity from cytosol to microsomes in fetal type II cells during late gestation. This can be explained by a subcellular translocation of CT from cytosol to microsomes or by an increased CT gene expression.

Chapter 4. We cloned the CT cDNA from fetal rat type II cells and showed 99% sequence homology with rat liver CT cDNA. CT mRNA increased threefold in fetal type II cells with advancing gestation, whereas CT mRNA levels in lung fibroblasts remained constant. Western blotting revealed that CT protein content increased threefold in the microsomal fraction, but not in cytosol of type II cells with advancing gestation. It is concluded that the developmental increase in CT activity in fetal type II cells is, at least in part, due to increased CT gene expression resulting in a higher amount of CT protein.

Chapter 5. The degree of activation of cytosolic CT by lipid vesicles (PC/oleic acid [OA] vesicles) was found to be gestation dependent in fetal rat type II cells. In contrast, microsomal CT activation by lipid vesicles remained constant with advancing gestation. Cytosolic lipids extracted from day 21 (term = 22 days) fetal type II cells were able to activate CT more than cytosolic lipids extracted from day 18 fetal type II cells. This developmental difference in the ability to activate CT was not found with lipids extracted from microsomes. We conclude that cytosolic but not microsomal lipids are involved in the developmental activation of cytosolic CT in fetal type II cells at late gestation.

Chapter 6. We found that cAMP-dependent protein kinase activity in fetal type II cells had a developmental profile similar to that of CT activity. Purified lung CT was found to be phosphorylated in vitro by cAMP-dependent protein kinase. These findings suggested a potential role of cAMP-dependent protein kinase in the regulation of CT activity in fetal rat type II cells during development. However, despite phosphorylation by cAMP-dependent protein kinase the activity of the purified CT did not change. Incubating fetal type II cell cytosol in vitro with cAMP-dependent protein kinase or stimulators or inhibitors of this kinase also did not alter CT activity. Treatment of intact fetal type II cells in culture with cAMP analogues increased cAMP-dependent protein kinase but did not alter CT activity. We conclude that the increase in CT activity in fetal type II cells at late gestation is not regulated by cAMP-dependent protein kinase.

Chapter 7. Protein phosphatase and protein kinase C activities were found to be regulated during fetal rat type II cell development at late gestation. Both activities first increased but then decreased on day 21 of gestation. In vitro, pre-incubation of purified CT or fetal type II cell cytosol with alkaline phosphatase increased CT activity in a concentration-dependent manner. Under phosphorylation conditions with sodium fluoride, a phosphatase inhibitor, cytosolic CT was decreased. Pre-incubation of fetal type II cell cytosol with a protein kinase C activator (the phorbol ester TPA) or protein kinase inhibitors altered protein kinase C activity but not CT activity. Furthermore, treatment of intact fetal type II cells in culture with either TPA, which did activate protein kinase C, or protein kinase C inhibitors did not alter CT activity. We conclude that CT activity in fetal type II cells may be modulated by protein phosphatases but not by protein kinase C.

Chapter 8. Surfactant deficiency was recently demonstrated in a rat model for congenital diaphragmatic hernia (CDH), induced by Nitrofen. We found that PC and disaturated phosphatidylcholine (DSPC) synthesis were lower in fetal type II cells from fetuses with CDH than from controls and this was associated with a lower CT activity. Conditioned medium from fetal lung fibroblasts stimulated with cortisol (FCM) is known to increase DSPC synthesis (through a fibroblast-pneumocyte factor) in fetal type II cells. In our experiments, FCM from control fibroblasts stimulated PC and DSPC synthesis in both control and CDH fetal type II cells, but FCM from CDH fibroblasts did not. Our data suggest that reduced PC synthesis in CDH is, at least in part, due to a lower CT activity in fetal type II cells which is caused by a decreased production of fibroblast-pneumocyte factor by lung fibroblasts.

9.2 General conclusions and perspectives

The synthesis of PC, the most abundant component of pulmonary surfactant, increases in maturing fetal type II cells at late gestation. This increased PC production is associated with an increased activity of CT, the rate-limiting step in the de novo PC synthesis. The increased activity of CT in developing type II cells can be partially explained by an increase in CT gene expression with a resulting higher amount of CT protein. This is in accordance with studies investigating spontaneous maturation of fetal rat lung explants (1) or fetal type II cells (2) in culture. A spontaneous increase in CT mRNA was found. The effect of corticosteroids on CT gene expression is controversial at the moment, but a study with fetal type II cells showed a

small but significant increase in CT mRNA in response to cortisol-containing fibroblast-conditioned medium (3). After stimulation with corticosteroids, an activation of CT is probably more important than an increased enzyme mass (4,5).

The increased CT protein in fetal type II cells during normal development was especially found in the microsomal fraction. This correlated with CT activities which were also found to increase more in the microsomal fraction. The developmental increase in activity of microsomal CT was also found in another study with fetal type II cells (6) and in several other studies with whole lung (7-10). From our data it is difficult to judge whether an activation of CT by a translocation from cytosol to microsomes also plays a role. It is possible that the newly synthesized CT remains bound to the microsomal membranes. One study with whole lung found good evidence for an activation of CT by subcellular translocation from cytosol to microsomes following birth (10).

From our studies it is clear that besides an increase in CT protein, also the activation of CT is important during fetal type II cell development. Cytosolic lipids play a very important role in this developmental activation of CT. These data are in agreement with findings in whole lung which demonstrate the importance of phospholipids in the developmental activation of cytosolic CT (11). Cytosolic CT has been shown to exist in two forms, a low molecular weight L-form, which is inactive in the absence of added phospholipids, and a high molecular weight H-form, which is active in the absence of added phospholipids (12,13). The H-form is a lipoprotein complex, consisting of CT and phospholipids, and is the predominant form in adult lung. Maternal administration of corticosteroids has been shown to induce the conversion of inactive cytosolic L-form to active H-form in fetal

lung and this effect is mediated by intracellular fatty acids (14). It is likely that a similar mechanism of L-form/H-form conversion plays an important role during fetal type II cell development but definite experimental evidence is lacking. However, in whole lung an increase in H-form following birth has been demonstrated (9).

Our data support the regulation of CT activity by phosphorylation and dephosphorylation of CT in fetal type II cells. CT is dephosphorylated by protein phosphatases and thereby activated. It is not clear which protein kinases phosphorylate CT in fetal type II cells. Protein kinase C and cAMP-dependent protein kinase are very unlikely candidates for CT phosphorylation, as was demonstrated by our studies. These findings with fetal type II cells are similar to recent findings with other cell types (15-22). Further studies are necessary to identify the protein kinases involved in CT phosphorylation.

As discussed in chapter 2, several intriguing studies with different cell types, especially with hepatocytes, suggest that the different mechanisms of CT regulation are interdependent. For example, fatty acids have been shown to increase cytosolic CT activity by a conversion of L-form to H-form (14,23,24), to decrease the phosphorylation state of CT (25,26) and to promote the activation of CT by subcellular translocation from cytosol to microsomes (10,27-31). In addition, the cytosolic H-form and the microsomal form of CT have been shown to be very similar forms of the enzyme (32). A possible mechanism for activation is the dephosphorylation of CT prior to a translocation (25,33,34) of L-form to microsomal membranes (32). Inactivation would involve a reversed translocation of CT from membranes to cytosol, where the dephosphorylated form exists (25,34) as H-form, followed by a further deactivation by phosphorylation (25) and

dissociation of the H-form into inactive L-form (32)(Fig. 3, chapter 2). These interactions and their role in the regulation of CT during type II cell development need further study.

Other possible mechanisms of CT regulation, as shown in other cell types, such as an activation by diacylglycerols and a feedback regulation by the amount of PC in membranes (see chapter 2) need further study in fetal type II cells.

The relative contribution of the activation of CT versus the increase in CT enzyme mass during developmental and hormonal regulation of CT activity in fetal type II cells remains to be established. It is expected that the gene sequence for CT will be known soon, which will make it much easier to study the regulation of gene expression. Structure-function relationships will be further investigated with the help of site-directed mutagenesis and transgenic animals. New laboratory techniques will help to resolve the question of the recently suggested and intriguing possibility of the channelling of intermediates of the CDPcholine pathway from one enzyme to the next. The role of the cytoskeleton in this channelling will be further examined.

Apart from the molecular mechanisms of regulation of CT in fetal type II cells, it is important to understand how hormonal factors, growth factors, extracellular matrix and other extracellular influences affect CT activity through these intracellular pathways of CT regulation. Fetal type II cell-fibroblast interactions have been shown to be very important in modulating the effect of hormones and growth factors on CT activity and thus PC synthesis. There is good evidence for the following sequence of exogenous corticosteroid stimulation in fetal lung (Fig. 4, chapter 2): corticosteroids induce the production of fibroblast-pneumocyte factor (FPF) in lung fibroblasts adjacent to the alveolar epithelial cells (35) at a pre-translational

level (review 36); this FPF induces fatty acid synthase and other enzymes involved in fatty acid synthesis in fetal type II cells at a pre-translational level (3); this leads to an increase in fatty acid biosynthesis, and fatty acids, their metabolites, or lipids into which they become incorporated ultimately activate CT (37) by increasing cytosolic H-form and possibly translocation of CT from cytosol to microsomes. Our study using a rat model for CDH, demonstrates that this sequence can be disturbed by an abnormal lung development and reinforces the idea that it is at least as important to study mesenchyme and epithelial-mesenchymal interactions as it is to study epithelial cell functions. Several other hormones such as thyroid hormones, insulin and sex hormones, and also several growth factors such as epidermal growth factor (EGF) and transforming growth factor beta (TGF-ß) have been shown to influence this sequence of events either by influencing the production of FPF in lung fibroblasts or by modulating the effect of FPF on fetal type II cells (for review see 38). That such regulatory mechanisms are not only important in cell culture or organ culture systems but also in clinical practice is illustrated by the success of the prenatal use of corticosteroids to prevent respiratory distress syndrome in premature infants (editorials 39,40). The antenatal administration of thyrotropin-releasing hormone (TRH) in combination with corticosteroids to accelerate lung maturity (41-43) originates from the insight gained from culture work and subsequent animal studies (review 38). The recent suggestion to use antenatal TRH and corticosteroids for lung maturation in CDH (44,45) also originates from laboratory data showing lung immaturity in different models of CDH (Chapter 8)(46-48). The same is true for the possible use of EGF (review 38) as is currently being tested in baboons. The increased incidence of RDS in infants born to women with diabetes (49) can also be explained, at least in

part, by the insulin inhibition of the glucocorticoid-induced production of FPF by fetal lung fibroblasts (50).

Our studies can help in the understanding of the mechanisms involved in these modulations of lung maturity. From such basic studies as the regulation of one enzyme in one cell type it is difficult to expect a direct clinical implication. The implications should be seen in the more general perspective of understanding underlying mechanisms that eventually lead to clinical applications. Some direct ideas can however be generated from our studies and similar studies by others. The importance of choline as a substrate for PC, and the influence of choline depletion on CT activity, make it worth to evaluate the importance of choline intake, and its precursors, by premature infants with RDS. The importance of lipids and fatty acids in the regulation of CT activity pose questions about the in vivo administration of lipid emulsions and their composition. Some studies demonstrate that certain fatty acids may stimulate CT activity better than others (14,23,24) and in vivo studies have demonstrated that the intravenous administration of lipids (Intralipid) at least influences the fatty acid composition of surfactant lipids (51). Phosphorylation and dephosphorylation mechanisms are frequently involved in the intracellular transduction of hormonal and growth factor signals. Thus, the influence of new (experimental) therapies with growth hormone, insuline-like growth factors, EGF etc.. on surfactant PC should be studied. The purification and potential clinical use of FPF would of course be very interesting. Our findings in the CDH model suggest that the role of the mesenchyme should be further explored and that new strategies to enhance lung maturity in CDH should take into account the possible ineffective FPF production by fibroblasts. It may be worth to study the effect of EGF in CDH because EGF has been shown to advance immature fibroblasts

to the production of FPF (52). Finally, the cloning of CT cDNA from rat lung (chapter 4) and the recent cloning of a human CT cDNA (53) suggest that we are not so far away from finding genetic variations in the CT gene that may explain some of the variation in the susceptibility to RDS in premature infants. This is currently being investigated for the surfactant protein A gene (54).

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Samenvatting en Conclusies

Samenvatting

Hoofdstuk 1 (Inleiding): Hyaliene membraanziekte (HMZ), de belangrijkste reden van longproblemen bij preterme pasgeborenen, wordt veroorzaakt door onrijpheid van de longen die gepaard gaat met een tekort aan surfactant. Ondanks het succes van de behandeling van HMZ met exogeen toegediend surfactant, blijven vele problemen onopgelost. Het is duidelijk dat de behandeling met surfactant alléén onvoldoende is en de pogingen om de foetale longrijpheid te bevorderen niet kan vervangen. Daarom is het nodig dat we beter begrijpen hoe de regulatie van de surfactantsynthese verloopt gedurende de foetale longontwikkeling. De longontwikkeling wordt gewoonlijk in vijf fasen beschreven. Tijdens de laatste fasen van de ontwikkeling vertraagt de celgroei en komt de differentiatie van de cellen op de voorgrond. Vooral de rijping van de type II cel is gedurende deze ontwikkelingsfase bijzonder belangrijk,m omdat deze cel het surfactant aanmaakt. Surfactant is essentieel voor de ademhaling na de geboorte. Het helpt om de alveolen te ontplooien bij inspiratie en voorkomt het samenvallen van de alveolen bij expiratie. Dit is mogelijk dankzij de oppervlaktespanningsverlagende werking van surfactant ter hoogte van het lucht-water oppervlak in de alveolen en dankzij de mogelijkheid van surfactant deze oppervlaktespanning aan te passen aan de inflatietoestand van de alveolus. Surfactant bestaat voor meer dan 90% uit vetten, waarvan het fosfatidylcholine veruit het belangrijkste is, en voor 5-10% uit eiwitten. Het is aangetoond dat de dipalmitoylvorm van fosfatidylcholine de voornaamste oppervlaktespanningsverlagende component is. Tegelijkertijd met de toename van de produktie van het

surfactantfosfatidylcholine aan het einde van de zwangerschap neemt de activiteit van het enzym CTP:fosfocholine cytidylyltransferase (CT) toe. Dit enzym katalyseert een snelheidsbepalende stap tijdens de *de novo* fosfatidylcholinesynthese. De regulatie van de enzymactiviteit van CT staat onder hormonale invloed. Om de regulatie van de surfactantproduktie aan het einde van de zwangerschap goed te verstaan, moeten we dus de mechanismen begrijpen die de activiteit regelen van CT in type II cellen gedurende de foetale ontwikkeling. Het doel van dit proefschrift is de studie van de regulatie van de activiteit van CT in foetale type II cellen aan het einde van de zwangerschap. We moeten wel steeds voor ogen houden dat de studie van één enzym in één celtype uiteraard zijn beperkingen heeft, en dat de resultaten niet zonder meer te extrapoleren zijn naar de klinische situatie.

Hoofstuk 2 (Overzicht van de literatuur): De voornaamste syntheseweg voor de *de novo* fosfatidylcholineproduktie is de CDPcholine-syntheseweg die een verhoogde activiteit vertoont aan het einde van de zwangerschap. In deze syntheseweg wordt choline gefosforyleerd door cholinekinase. Fosfocholine wordt dan omgezet tot CDPcholine door CT. Tenslotte wordt de fosfocholinegroep getransfereerd naar diacylglycerol door het CDPcholine:1,2-diacylglycerol fosfocholinetransferase zodat fosfatidylcholine gevormd wordt. Onder de meeste omstandigheden is de activiteit van CT snelheidsbepalend voor deze syntheseweg in type II cellen. Vrij recent is CT volledig gezuiverd en het cDNA ervan gekloneerd uit de lever en longen van ratten. Het centrale deel van het CT-molecule wordt aanzien als het katalytische deel. Het C-terminale deel kan gefosforyleerd en gedefosforyleerd worden op serine residuen en bevat ook een amfipathische α-helix, waarvan aangetoond werd dat deze het vet-bindende deel van het enzym is. Inderdaad is de

interactie van CT met vetten essentieel voor de enzymactiviteit van CT. De actieve, vet-gebonden vormen van CT zijn: CT gebonden aan membranen, vooral membranen van het endoplasmatisch reticulum, en de grote CT aggregaten met fosfolipiden (zogenaamde H-vormen) in het cytosol. In principe katalyseert CT een reversibele reactie maar men denkt dat de reactie in omgekeerde richting in intacte cellen onbelangrijk is. Hoofdstuk 2 beschrijft verder de mechanismen van de regulatie van CT met de nadruk op de regulatie in foetale type II cellen van de zich ontwikkelende long.

Hoofstuk 3: We toonden aan dat de fosfatidylcholinesynthese vanuit meerdere precursoren toeneemt in foetale type II cellen bij een vorderende zwangerschapsduur. CT was het enige enzym van de CDPcholine-syntheseweg met een toename in activiteit gedurende de longontwikkeling die gelijklopend was met de toename van de fosfatidylcholineproduktie. Wanneer de activiteit van CT gemeten werd in verschillende celfracties werd de grootste toename, als functie van de ontwikkeling, gevonden in de microsomen. Tegelijkertijd met deze toename van CT activiteit in foetale type II cellen aan het einde van de zwangerschap, verschoof de subcellulaire verdeling van CT activiteit van het cytosol naar de microsomen. Dit kan verklaard worden door een subcellulaire translocatie (verplaatsing) van CT van het cytosol naar de microsomen ofwel door een verhoogde expressie van het CT gen met een verhoogde aanmaak van het enzym.

Hoofstuk 4: We kloneerden het CT cDNA van foetale type II cellen van de rat en vonden een overeenkomst van 99% met de sequentie van het CT cDNA van de lever van de rat. Het CT mRNA in de foetale type II cellen nam drievoudig toe met een vorderende zwangerschapsduur, terwijl

het CT mRNA in longfibroblasten constant bleef. Met behulp van Western blotting vonden we ook een drievoudige toename van het CT eiwit in de microsomen van type II cellen met vorderende zwangerschapsduur, maar niet in het cytosol van deze cellen. We concluderen dat de toename van de CT activiteit in foetale type II cellen gedurende de ontwikkeling tenminste voor een deel veroorzaakt wordt door een verhoogde expressie van het CT gen met een grotere hoeveelheid CT eiwit als gevolg.

Hoofstuk 5: We vonden dat de mate van activatie van cytosolisch CT door vetten (fosfatidylcholine / oleïnezuur vesikeltjes) in foetale type II cellen afhankelijk was van de zwangerschapsduur. De activatie van microsomaal CT door vetten, daarentegen, bleef constant bij een vorderende zwangerschapsduur van de type II cellen. Vetten in het cytosol die onttrokken werden aan foetale type II cellen van dag 21 in de zwangerschap (à terme = dag 22) activeerden CT meer dan vetten in het cytosol onttrokken aan foetale type II cellen van dag 18. Een dergelijke ontwikkelingsafhankelijke activatie van CT werd niet gevonden met microsomale vetten. We concluderen dat de vetten in het cytosol, maar niet de vetten van de microsomen, betrokken zijn bij de ontwikkelingsafhankelijke activatie van cytosolisch CT in foetale type II cellen.

Hoofstuk 6: We vonden dat het activiteitspatroon van het cAMP-afhankelijke proteinekinase in foetale type II cellen gedurende de ontwikkeling gelijkaardig verliep aan dit van CT. CT gezuiverd uit longen kon in vitro gefosforyleerd worden door het cAMP-afhankelijke proteinekinase. Deze bevindingen suggereren een rol voor het cAMP-afhankelijke proteinekinase in de regulatie van de CT activiteit in foetale type II cellen tijdens de

longontwikkeling. Ondanks de fosforylering door het cAMP-afhankelijke proteinekinase vonden we echter geen verandering in de activiteit van het gezuiverde CT. Tevens vonden we geen verandering in de activiteit van het cytosolisch CT bij incubatie in vitro van cytosol van foetale type II cellen met het cAMP-afhankelijke proteinekinase of met stimulatoren of inhibitoren van dit kinase. Bij incubatie van intacte type II cellen in kweek met cAMP-analogen verhoogde wel de activiteit van het cAMP-afhankelijke proteinekinase maar niet deze van CT. We concluderen dat de verhoogde activiteit van CT in foetale type II cellen aan het einde van de zwangerschap niet bepaald wordt door het cAMP-afhankelijke proteinekinase.

Hoofdstuk 7: We vonden dat er een regulatie bestaat van de activiteiten van de proteinefosfatases en van het proteinekinase C in foetale type II cellen aan het einde van de zwangerschap. De beide enzymactiviteiten namen eerst toe bij een vorderende zwangerschapsleeftijd maar namen af op dag 21 van de zwangerschap. In vitro incubatie van gezuiverd CT of van cytosol van foetale type II cellen met alkalische fosfatase verhoogde de CT activiteit op een concentratie-afhankelijke manier. Onder fosforylerende omstandigheden met natriumfluoride, een inhibitor van fosfatases, vonden we een verlaagde activiteit van het cytosolische CT. Pre-incubatie van het cytosol van foetale type II cellen met een activator van het proteinekinase C (forbolester TPA) of met inhibitoren van het proteinekinase C veranderden wel de activiteit van het proteinekinase C maar niet die van CT. Wanneer we intacte type II cellen in kweek incubeerden met TPA (hetgeen de proteinekinase C activiteit deed toenemen) ofwel met inhibitoren van proteinekinase C, zagen we ook geen verandering in de activiteit van CT in de cellen. We concluderen dat de activiteit van CT in foetale type II cellen mogelijk gemoduleerd wordt door

proteinefosfatases maar niet door het proteinekinase C.

Hoofdstuk 8: Recent werd een tekort aan surfactant aangetoond in een rattenmodel voor congenitale hernia diafragmatica (CDH) geïnduceerd door Nitrofen. We vonden een lagere aanmaak van fosfatidylcholine en van tweevoudig verzadigd fosfatidylcholine in type II cellen van foetale ratten met CDH dan van controle foetussen. Dit ging gepaard met een lagere activiteit van CT in type II cellen van foetale ratten met CDH. Het is bekend dat FCM (kweekmedium dat geconditioneerd werd door foetale longfibroblasten die gestimuleerd werden door cortisol) de aanmaak verhoogt van tweevoudig verzadigd fosfatidylcholine in foetale type II cellen. In onze proeven vonden we dat FCM van controlefibroblasten de fosfatidylcholine en de tweevoudig verzadigd fosfatidylcholine produktie verhoogde zowel in controle als in CDH foetale type II cellen, maar dat FCM van CDH fibroblasten dit niet deed. Deze bevindingen suggereren dat de verlaagde aanmaak van fosfatidylcholine in CDH tenminste ten dele kan verklaard worden door een lagere activiteit van CT in de foetale type II cellen en dat dit het gevolg is van een verminderde produktie van de zogenaamde "fibroblast-pneumocyte factor" door de longfibroblasten.

Conclusies

Surfactant is nodig voor het open houden van de longblaasjes en wordt aangemaakt in de zogenaamde type II cellen van de long. De synthese van fosfatidylcholine, de belangrijkste component van surfactant, in foetale type II cellen neemt toe naar het einde van de zwangerschap. Dit gaat gepaard aan een toename van de activiteit van het enzym CTP:fosfocholine cytidylyltransferase (CT), dat een snelheidsbepalende stap katalyseerd in de aanmaak van fosfatidylcholine. Deze toegenomen activiteit van CT in foetale type II cellen wordt vooral gevonden in het endoplasmatisch reticulum. Dit kan tenminste ten dele verklaard worden door een toegenomen CT genexpressie met een verhoogde aanmaak van het CT eiwit tot gevolg. Niet alleen wordt het enzym meer aangemaakt, het blijkt ook meer geactiveerd te worden bij een toenemende zwangerschapsduur. Vooral de vetten in het cytosol spelen een belangrijke rol in de activatie van CT in de foetale type II cellen gedurende de longontwikkeling. Mogelijk speelt ook een activering door verplaatsing van het enzym van cytosol naar endoplasmatisch reticulum een rol. CT in foetale type II cellen kan geactiveerd worden door defosforylatie door proteinefosfatases en geinactiveerd door fosforylatie door proteinekinases. Welke proteinekinases hierin een rol spelen blijft echter onduidelijk. Onze studies sluiten een belangrijke rol voor proteinekinase C en cAMP-afhankelijk proteinekinase vrijwel uit.

De laatste jaren is het duidelijk geworden dat de verschillende mechanismen voor de regulatie van de activiteit van CT niet onafhankelijk van elkaar functioneren maar elkaar beïnvloeden. Zo stimuleren vetten in het cytosol niet alleen rechtstreeks de activiteit van CT maar ze bevorderen ook de

defosforylatie van CT en de translocatie van CT van het cytosol naar het endoplasmatisch reticulum. Deze complexe regulatie van de activiteit van CT in foetale type II cellen staat onder controle van hormonen en groeifactoren. In deze hormonale controle spelen fibroblast-type II cel interacties een zeer belangrijke rol. Een goed voorbeeld is de regulatie van CT activiteit door corticosteroiden. Deze induceren de productie van een "fibroblast-pneumocyte factor" (FPF) in fibroblasten die dicht tegen de type II cellen aanliggen. Dit FPF induceert vervolgens in de type II cellen een hogere aanmaak van het enzyme vetzuursynthase wat aanleiding geeft tot verhoogde productie van vetzuren. Deze vetzuren, of de vetten waarin ze ingebouwd worden, stimuleren de activiteit van CT in de type II cellen wat leidt tot een hogere aanmaak van surfactantfosfatidylcholine. Vele andere hormonen en groeifactoren hebben hun invloed via beinvloeding van deze sequentie. Dat dit niet alleen belangrijk is in celkweken maar ook voor de kliniek wordt geïllustreerd door het succes van het prenataal toedienen van glucocorticoiden aan prematuren ter voorkoming van HMZ. Recent wordt (experimenteel) "thyrotropin releasing hormone (TRH)" prenataal toegediend samen met glucocorticoiden om de longrijping van de prematuur te bevorderen. Het idee hiervoor komt voort uit basaal wetenschappelijk onderzoek. Hetzelfde geldt voor het dierexperimenteel gebruik van 'epidermal growth factor' (EGF) ter bevordering van de longrijpheid in apen. De verhoogde incidentie van HMZ bij kinderen van moeders met diabetes kan ook deels verklaard worden door de insuline-inhibitie van de FPF productie door de longfibroblasten. In ons onderzoek toonden we aan dat de verminderde aanmaak van surfactantfosfatidylcholine in type II cellen geïsoleerd van longen van ratten met een hernia diafragmatica ook kan verklaard worden door een verminderde FPF produktie door de longfibroblasten.

Het belangrijkste doel van studies zoals beschreven in dit proefschrift is het verkrijgen van een beter inzicht in de mechanismen van de regulatie van de longrijping. Van dergelijke studies kan natuurlijk niet verwacht worden dat ze direct leiden tot klinische toepassingen. Het verkregen inzicht zal uiteindelijk wel leiden tot verbetering van het klinisch beleid. Toch geven onze studies, en gelijkaardige studies door anderen, aanleiding tot enkele ideeën voor de kliniek. De rol van choline (substraat voor fosfatidylcholine) en de vetsamenstelling van parenterale en orale voeding op de produktie en samenstelling van surfactant dienen nader bekeken worden. Aangezien hormonen en groeifactoren veelal hun effect uitoefenen door fosforylatie en defosforylatie van eiwitten in de cel, moet de invloed van deze factoren op de surfactantproduktie bestudeerd worden. De zuivering van FPF en het mogelijk klinisch gebruik ervan zou natuurlijk zeer interessant zijn. De stoornis in FPF produktie in hernia diafragmatica geeft aanleiding tot verder onderzoek en tot behandelingen die hiermee rekening proberen te houden. Tenslotte zal de klonering van CT cDNA leiden tot het bestuderen van genetische variaties in het CT gen die mogelijk kunnen verklaren waarom sommige pasgeborenen gevoeliger zijn voor het krijgen van HMZ. Er is nog veel werk te doen...

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