CHANGES IN CDP-DIGLYCERIDE:INOSITOL TRANSFERASE ACTIVITY DURING RABBIT LUNG DEVELOPMENT

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SUMMARY

During fetal and neonatal development the specific activity of the enzyme, CDP-diglyceride:inositol transferase in rabbit lung tissue increased 8- to 9-fold from day 17 of gestation to adulthood. This developmental increase in enzyme activity was not specific for lung tissue since a similar increase was also observed in liver tissue. The properties of CDP-diglyceride:inositol transferase in microsomes prepared from either fetal or adult rabbit lung tissue appeared to be the same. The increase in CDP-diglyceride:inositol transferase activity in rabbit lung tissue during development appeared to be reflective of synthesis of new enzyme rather than activation of pre-existing enzyme.

SPECULATION

CDP-diglyceride:inositol transferase activity in fetal rabbit lung tissue increases at a stage in development when the phosphatidylinositol content of lung surfactant is decreasing. In the presence of CMP, CDP-diglyceride:inositol transferase catalyzes the synthesis of CDP-diglyceride from phosphatidylinositol. We speculate that the increased CDP-diglyceride:inositol transferase activity, in conjunction with elevated intracellular CMP levels resulting from accelerated phosphatidylcholine biosynthesis, provides a pool of CDP-diglyceride for phosphatidylglycerol biosynthesis during the latter stages of fetal lung maturation.

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CDP-diglyceride:inositol transferase activity of rabbit lung tissue increases during development. This developmental increase in enzyme activity was also observed in liver tissue. The increased enzyme activity in lung tissue appeared to be reflective of synthesis of new enzyme rather than activation of pre-existing enzyme.

INTRODUCTION

During the latter stage of gestation, fetal lungs begin to synthesize and secrete a surface-active lipoprotein. This lung lipoprotein, surfactant, is largely responsible for reducing surface tension at the air-tissue interface of the alveoli preventing alveolar collapse during expiration (5, 19). It has been known for some time that the principal surface-active component of surfactant is dipalmitoylphosphatidylcholine (DP-PC) (14), and the metabolism and function of this lipid in lung have been studied extensively (for reviews see 6, 10, 20). Inadequate production of surfactant is the major cause of the respiratory distress syndrome of the newborn, and many investigators have sought to identify the mechanism by which augmented DP-PC biosynthesis is initiated late in gestation.

More recently the role(s) of the other lipids present in lung surfactant has been investigated. In most species studied, the second most abundant lipid in surfactant is phosphatidylglycerol (PG), and this lipid affects the surface-active properties of DP-PC in surfactant (4, 11, 16). In human and rabbit fetal lungs, elevated surfactant-PG levels appear after DP-PC synthesis has commenced (9, 11). Moreover, there is evidence that the increase in the level of PG in surfactant is accompanied by a decrease in the level of phosphatidylinositol (PI) (11). However, the relationship between PG and PI metabolism in maturing fetal lung is not understood. As part of a study of PG and PI metabolism in fetal rabbit lung tissue, we have recently characterized CDP-diglyceride:inositol transferase (EC 2.7.8.11), the enzyme catalyzing the final reaction in PI biosynthesis (3). We report here the finding of increases in the activity of CDP-diglyceride:inositol transferase in rabbit lung and liver tissue during gestation and a continued increase in the activity of this enzyme after birth.

METHODS AND MATERIALS

The animals utilized for this study were pregnant New Zealand White rabbits (4-6 Kg) which were obtained on either the 15th or 21st day of pregnancy from Pel-Freez BioAnimals, Rogers, Arkansas. The day of mating was set as day zero of pregnancy. Rabbits were caged individually and fed rabbit chow *ad libitum*. Myo-[2-³H]inositol (12.5 Ci/mole) in 90% ethanol was obtained from New England Nuclear, Boston, Mass. CDP-dioleoylglycerol was purchased from Serdary Research Laboratories, London, Ont. Canada. Bovine serum albumin (Fraction V) and myo-inositol were obtained from Sigma Chemical Company, St. Louis, Mo. All other chemicals were commercially available, reagent grade.

Enzyme Preparation. Previously we found that most of the CDP-diglyceride:inositol transferase activity of rabbit lung tissue was associated with the microsomal fraction (3). For this reason, washed rabbit lung microsomes were employed in the present study to characterize CDP-diglyceride:inositol transferase and to evaluate gestational changes in the activity of this enzyme. Pregnant rabbits were anesthetized by injection of sodium pentobarbital (Nembutal, approx. 25 mg/Kg body weight) intravenously. The lungs and livers were removed rapidly from the does and from the fetuses. When neonatal rabbits were employed, anesthesia was achieved by the intraperitoneal injection of Nembutal (approx. 100 mg/Kg body weight). Tissues were rinsed with ice-cold 0.154 M NaCl solution, blotted with filter paper and weighed. After mincing with scissors, tissues were homogenized in ice-cold 0.25 M sucrose containing EDTA (0.1 mM) and Tris-HCl (1 mM, pH 7.4), [buffered sucrose, 4 ml/gm tissue]. Homogenization was conducted employing a teflon/glass Potter-Elvehjem homogenizer (0.13-0.18 mm clearance, 1,000 rpm). Each homogenate was further diluted with 2 ml of buffered sucrose/gm tissue before filtration through two layers of cheesecloth. The residue on the cheesecloth was washed once with buffered sucrose (2 ml/gm tissue); the filtrates were combined and centrifuged at 750 x g for 10 min. After centrifugation the supernatant fraction was centrifuged at 12,000 x g for 15 min and the resulting supernatant fraction was further centrifuged at 105,000 x g for 60 min. The 105,000 x g pellet was washed once in buffered sucrose solution, recentrifuged at 105,000 x g for 60 min and the pellet was resuspended in Tris-HCl buffer (175 mM, pH 8.5) to achieve a final concentration of approximately 15 mg protein/ml. This freshly prepared subcellular fraction was used in enzyme assays.

Assay of CDP-diglyceride:inositol transferase. The assay method employed was based on that described by Wootton and Kinsella (21). Standard assays were conducted in a total volume of 0.5 ml containing Tris-HCl (175 mM, pH 8.5), CDP-dioleoylglycerol (0.3 mM), mercaptoethanol (5 mM), MnCl₂ (3.8 mM), myo-[2-³H]inositol [1.4 mM (approx. 2 μ Ci/ μ mole)] and microsomal enzyme at 2 different concentrations (approx. 300 μ g and 600 μ g protein/ml). Incubations were initiated by the addition of microsomes followed immediately by the addition of freshly prepared MnCl₂ and were conducted for 30 min at 37°. Termination of incubations and extraction of products were accomplished as described elsewhere (3). In the routine assays ³H incorporation into the total lipid

extract was measured since we had established that greater than 93% of [³H]inositol incorporated was contained in a single product that co-chromatographed with authentic phosphatidylinositol in two different chromatographic systems (3). The assay was found to be linear for at least 30 min with protein concentrations up to 1 mg/ml. In other studies (unpublished) we have observed that rabbit lung microsomes readily incorporate [³H]inositol into phosphatidylinositol by a phosphatidylinositol:inositol exchange reaction. This exchange does not constitute net synthesis of phosphatidylinositol and can be inhibited by CDP-diglyceride (20 μ M). Since in the present study the standard assay mixture contained CDP-diglyceride (300 μ M), phosphatidylinositol:inositol exchange is unlikely to interfere with the measurement of CDP-diglyceride:inositol transferase activity.

Other methods. Protein was measured by the procedure of Lowry et al. (15), using bovine serum albumin as the reference standard. Lipid phosphorus was measured according to the method of Ames and Dubin (1).

RESULTS

Increases in CDP-diglyceride:inositol transferase specific activity were observed in rabbit lung tissue from day 17 of gestation to day 18 of life. The results obtained are depicted in Figure 1. On day 17 of gestation the CDP-diglyceride:inositol transferase specific activity of fetal lung tissue was only 12% of that found in adult lung tissue. The specific activity in fetal lung tissue increased as gestation advanced but at birth it was still only 29% of the adult value of 86.4 nmoles inositol incorporated $\times$ mg⁻¹ protein $\times$ h⁻¹. It is noteworthy that this developmental change in the specific activity of CDP-diglyceride:inositol transferase was not specific for lung tissue; indeed, a similar trend was observed in the specific activity of this enzyme in rabbit liver tissue (Fig. 2). The increase in the specific activity of CDP-diglyceride:inositol transferase may reflect new enzyme synthesis, or it may be due to activation of pre-existing enzyme. Alternatively, it is possible that optimal *in vitro* conditions for assaying the enzyme in fetal lung tissue are different from those favoring the assay of the activity of the adult form of the enzyme. With respect to the possibility of activation of pre-existing enzyme we found that the composition of fetal lung microsomes, as judged from the lipid phosphorus/protein ratio, differed from that of adult lung microsomes (Table I). In other experiments (data not shown) differences in lipid composition of the two preparations of microsomes were also detected. Most notably, the fetal lung tissue microsomes contained lysophosphatidylcholine in concentrations 2- to 3-times that found in adult lung tissue microsomes. Others have found that developmental changes in CTP:phosphocholine cytidyltransferase (EC 2.7.7.15) of fetal rat lung tissue were largely attributable to lipid-activation of previously synthesized enzyme (18). The lipid-activation of CTP:phosphocholine cytidyltransferase in the cytosolic fraction of rat lung tissue, has been ascribed to the effects of PG (7). In the present study a total lipid extract was prepared from adult rabbit lung tissue microsomes using an acidified solvent system (8), and the lipids were resuspended in Tris-HCl buffer (175 mM, pH 8.5) by sonicating for 60 seconds (Bransonic 220 bath-sonicator, 125 watts; Branson, Shelton, Conn.). This lipid suspension, when added to either fetal lung microsomes or adult lung microsomes (1 μ mole lipid/mg microsomal protein), had a negligible effect on CDP-diglyceride:inositol transferase activity. Similarly, lysophosphatidylcholine at concentrations up to 8 μ g/ml did not affect CDP-diglyceride:inositol transferase activity in microsomes from adult rabbit lung tissue. These findings do not preclude activation of previously synthesized enzyme during lung development or even a role of lipids in such a process, but rather are indicative that if such an activation occurred, it differed qualitatively from the activation of rat lung tissue CTP:phosphocholine cytidyltransferase.

For optimal *in vitro* activity, the fetal lung tissue enzyme appeared to have similar requirements to those of the adult form of the enzyme (Table I). Each has the same pH optimum, the same Mn²⁺ requirement and similar apparent Km values for both CDP-diglyceride and inositol. Fetal lung CDP-diglyceride:inositol transferase activity is localized predominantly in the microsomal fraction, as it is in adult lung tissue (3). These several findings are supportive of the view that enhanced synthesis of CDP-diglyceride:inositol transferase accounts for the increased activity of the enzyme observed during lung development. The enzyme specific activities in this study were expressed as activity per mg protein, thus the increased enzyme levels actually represented synthesis of enzyme over and above the general increase in protein synthesis that occurred in lung tissue during the developmental period studied. The mean fresh tissue weight of a fetal lung is approximately 15 mg at day 17 of gestation, increasing to about 600 mg at the time of birth and to approximately 7 gm in the adult. At these three times during development, total CDP-diglyceride:inositol transferase activity per lung (nmoles inositol incorporated $\times$ h⁻¹) was found to be approximately 0.1, 20 and 2000, respectively.

DISCUSSION

From day 17 of gestation until adulthood there was a gradual increase in the activity of CDP-diglyceride:inositol transferase in rabbit lung tissue. At birth this developmental increase was only 33% complete and even by the 9th day of life, it was only 53% complete. When expressed as total units of CDP-diglyceride:inositol transferase activity per lung, an increase of approximately 2×10^4 -fold in enzyme activity was observed over the developmental period studied. In this study we sought to determine whether this increased enzyme activity was due to causes other than synthesis of new enzyme. At day 25 of gestation, fetal lung tissue microsomes contain only 44% of the lipid found in adult lung tissue microsomes per mg protein. However, the addition of lipids extracted from adult lung tissue microsomes to fresh fetal lung tissue microsomes was not followed by stimulation of CDP-diglyceride:inositol transferase activity. The optimum *in vitro* assay conditions for CDP-diglyceride:inositol transferase appeared to be the same when either fetal lung or adult lung tissue microsomes were employed. We conclude that the developmental increase in rabbit lung CDP-diglyceride:inositol transferase activity is the result of synthesis of new enzyme. An increase in phosphatidate phosphohydrolase (PAPase, EC 3.1.3.4) activity was observed during rabbit lung maturation (17). However, the pattern of increase in PAPase specific activity differed from that of CDP-diglyceride:inositol transferase activity in two ways. First, the increase in PAPase specific activity during gestation was restricted to fetal lung, whereas CDP-diglyceride:inositol transferase activity increased in both lung and liver tissues (Fig. 2). Second, the increase in lung PAPase specific activity occurred over a finite period of fetal development, whereas the increase in CDP-diglyceride:inositol transferase specific activity was more protracted in time, reaching even to adulthood.

A developmental increase in CDP-diglyceride:inositol transferase specific activity has been described in maternal rabbit mammary tissue during pregnancy and lactation (21). It was proposed that the increased enzyme activity in mammary gland occurred in response to elevated prolactin levels in the maternal blood. Other similarities between lipid metabolism in the lactating mammary gland and in lung have been pointed out elsewhere (13). However, it is not yet established whether prolactin plays a role in the biochemical maturation of the fetal lung (2, 12, 13).

In this study microsomes from whole lung tissue were employed but the type II pneumocytes which produce surfactant comprise only 5-15% of all lung cell types. Since type II pneumocytes secrete PI (in surfactant) they may contain more CDP-diglyceride:inositol transferase than do other lung cells and so part of the developmental increase in lung CDP-diglyceride:inositol transferase activity may reflect proliferation of type II pneumocytes. Nevertheless it is likely that much of the developmental increase in lung CDP-diglyceride:inositol transferase activity is associated with lung functions other than surfactant production, especially since a similar trend was detected in liver tissue. Indeed the importance of CDP-diglyceride:inositol transferase in surfactant production may relate not to the developmental increase in its activity, but

rather to the fact that this enzyme catalyzes a reversible reaction (3). In the presence of sufficiently high concentrations of CMP, CDP-diglyceride:inositol transferase catalyzes the synthesis of CDP-diglyceride from PI. It is envisioned that CMP for this reaction may arise as a by-product of DP-PC biosynthesis and that CDP-diglyceride so formed can be utilized for PG biosynthesis. Stimulation by CMP of the reverse reaction catalyzed by CDP-diglyceride:inositol transferase ensures that more of the limited pool of CDP-diglyceride is utilized for PG biosynthesis at the expense of PI biosynthesis. This mechanism would account for the observed sequence of events leading to the production of mature surfactant viz., a surge in DP-PC biosynthesis followed in time by a restriction of PI biosynthesis and a concomitant stimulation of PG biosynthesis.

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Table 1

PROPERTIES OF CDP-DIGLYCERIDE:INOSITOL TRANSFERASE IN FETAL AND ADULT RABBIT LUNG TISSUE MICROSOMES

	DAY 25 FETAL	ADULT
pH optimum	8.8-9.4	8.8-9.4
Mn ²⁺ optimum conc.	0.3 mM	0.3 mM
Km (inositol)	0.12 mM	0.10 mM
Km (CDP-diglyceride)	0.12 mM	0.18 mM
lipid composition of microsomes (nmoles lipid P/mg protein)	481	1091

For determination of the pH optimum, the following buffers were used: Tris-maleate (175 mM, pH 5.5-8.5), 2-amino-2-methyl propan-1-ol/HCl (175 mM, pH 8.0-10.0).

Fig. 1

Changes in CDP-diglyceride:inositol transferase activity during rabbit lung development. Fetal or neonatal lungs from animals of the same litter were combined and a lung microsomal fraction was prepared from the lung tissues of each litter (mean litter size = 7). Enzyme activity in the microsomal fraction was measured in duplicate at 2 different protein concentrations as described under Methods. Data points are representative of the mean values of tissues from several litters (3 to 8) $\pm$ standard error of the mean (SEM). Where no SEM is given, the data are from tissues of either 2 litters or 1 litter (day 29 only). The mean CDP-diglyceride:inositol transferase activity of adult rabbit lung tissue was 86.4 nmoles inositol incorporated $\times$ mg⁻¹ protein $\times$ h⁻¹.

Fig. 2

Changes in CDP-diglyceride:inositol transferase activity during rabbit liver development. Fetal or neonatal livers from animals of the same litter were combined and CDP-diglyceride:inositol transferase activity in the microsomal fraction was determined as described for Figure 1. Data points are representative of the mean values of liver tissue from several litters (3 to 7) $\pm$ SEM. Where no SEM is given, the data are from tissues of either 2 litters or 1 litter (day 29 of gestation and day 18 post-partum only). The mean CDP-diglyceride:inositol transferase activity of adult rabbit liver tissue was 174.0 nmoles inositol incorporated $\times$ mg⁻¹ protein $\times$ h⁻¹.