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ACTIVATION OF CTP : PHOSPHOCHOLINE CYTIDYLYLTRANSFERASE IN RAT LUNG BY FATTY ACIDS

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CTP : phosphocholine cytidylyltransferase activity exists in both the microsome and cytosol fractions of adult lung, 36 and 59%, respectively. Although these enzyme activities are stimulated in vitro by added lipid activators (i.e. phosphatidylglycerol), there are significant levels of activity in the absence of added lipid. We have removed endogenous lipid material from microsome and cytosol preparations of rat lung by rapid extraction with isopropyl ether. The extraction procedure did not cause any loss of cytidylyltransferase activity in the cytosol. After the extraction the enzyme was almost completely dependent upon added lipid activator. Isopropyl ether extraction of microsome preparations produced a loss of 40% of the cytidylyltransferase activity, when measured in the presence of added phosphatidylglycerol. Lipid material extracted into isopropyl ether restored the cytidylyltransferase activity in cytosol. The predominant species of enzyme activator in the isopropyl ether extracts was fatty acid. A variety of naturally occurring unsaturated fatty acids stimulated the cytidylyltransferase to the same extent as phosphatidylglycerol. Saturated fatty acids were inactive.

Introduction

The enzyme CTP : phosphocholine cytidylyltransferase catalyzes the formation of CDPcholine in the pathway for the synthesis of phosphatidylcholine. During the course of our investigation on the activity and properties of cytidylyltransferase during lung development, we have found rather striking differences between the properties of the enzyme in cytosol preparations from fetal and adult lung [1–3]. Cytidylyltransferase enzyme in the cytosol from fetal lung exists predominantly as a species having a molecular weight of 190 000 (L-form). Measurement of this enzyme activity in vitro requires the addition of specific phospholipids, including phosphatidylglycerol and lysophosphatidylethanolamine, for maximal activity. Cytidylyltransferase in the cytosol from adult lung exists predominantly in a wide range of high molecular weight species ($(5–50) \cdot 10^6$, H-form). The activity of preparations of the H-form of cytidylyltransferase is relatively independent of added phospholipid.

We have previously suggested [1–3] that some process, as yet unknown, occurs during the last stage of lung development which leads to changes in the physical and catalytic properties of cytidylyltransferase. Presumably, these developmental processes involve the interaction of the L-form of cytidylyltransferase with lipids, lipoproteins or intracellular membranes. One approach to obtain a further understanding of these processes is to examine the nature of the stimulatory lipids present in the cytosol and microsomes of adult lung and to compare the properties of the enzyme before and after removal of stimulatory lipids. We have developed methods for the extraction of lipid activators without affecting the total cytidylyltransferase activity in the cytosol. These experiments have led to the finding that cyti-

dylyltransferase activity in adult lung is stimulated by endogenous free fatty acids.

Materials and Methods

Materials

Female rats (160–180 g) were obtained from Holtzman Co. Phosphorylcholine, CTP (Type V), phosphatidylglycerol (egg yolk) and all fatty acids were purchased from Sigma Chemical Co. Phospho-[*methyl*-^{14}C]choline and [1-^{14}C]oleic acid were from England Nuclear. Pre-coated TLC plates of silica gel 60 (0.25 mm) were from E. Merck. Neutral lipid standards for thin-layer chromatography were purchased from Sigma Chemical Co.

Preparation of subcellular fractions

Lung was minced thoroughly with scissors and homogenized in 5 vol. (w/v) of 50 mM Tris-HCl/0.15 M NaCl, pH 7.4. The homogenate was centrifuged at 1 000 × *g* for 5 min to remove cellular debris and pieces of connective tissue. A mitochondria-fraction was pelleted by centrifugation at 20 000 × *g* for 30 min. The 20 000 × *g* supernatant was centrifuged at 250 000 × *g* for 60 min to obtain the pelleted microsomes and 250 000 × *g* supernatant (cytosol). The microsome fraction was suspended in a volume of Tris/NaCl buffer equal to the original volume of the 20 000 × *g* supernatant. In some experiments a 30 000 × *g* supernatant was used. This was prepared by centrifuging the 1 000 × *g* supernatant at 30 000 × *g* for 30 min. This 30 000 × *g* supernatant contained 85–90% of the total cytidylyltransferase activity.

Extraction with isopropyl ether

Tissue preparations were mixed with isopropyl ether at a ratio of 1 : 2, (v/v) by constant rotation at a speed of 18 rev./min for 5 min at room temperature [4]. The upper isopropyl ether layer was removed and residual ether in the enzyme preparation was evaporated with a stream of nitrogen. The isopropyl ether extracts were dried with a stream of nitrogen, redissolved in chloroform, and stored at –40°C.

Preparation of lipid suspensions

Aliquots of isopropyl ether extract from subcellular fractions were dried with nitrogen. The lipid was resuspended in 50 mM Tris-HCl/0.15 M NaCl, pH 7.4, by sonication for 10 min at 4°C. A turbid, milky white suspension was obtained. The amount of lipid extracted from 50–80 mg of subcellular fraction protein was suspended in 1.0 ml of Tris/NaCl buffer. Phosphatidylglycerol and fatty acids were suspended in Tris/NaCl buffer at a final concentration of 1.0 mM and were sonicated for 10 min. The suspensions of unsaturated fatty acids were generally translucent. Homogeneous solutions of saturated fatty acids were more difficult to prepare. Brief heating at 80°C facilitated suspension in the aqueous buffer. All lipid solutions were used within 1 h.

Enzyme assay

Cytidylyltransferase activity was measured in the 'forward' direction (CDPcholine formation) as described previously [3]. Phosphatidylglycerol was added at a final concentration of 0.26 mM unless otherwise indicated. The assay volume was 100 µl.

Isolation of lipids for stimulation of cytidylyltransferase

Lipid material that was extracted by isopropyl ether was dissolved in chloroform and measured aliquots were applied to silica gel TLC plates under N_2. The plates were developed in the solvent system benzene/chloroform/methanol (85 : 15 : 5, v/v). Individual classes of lipids were identified by comparison to the migration of lipid standards. Lipid material was recovered from the silica gel by three extractions with 2 ml of chloroform/methanol (2 : 1, v/v). The extraction solvents were evaporated under N_2. The extracted lipid materials were resuspended in Tris/NaCl buffer by sonication for 10 min at 4°C, within 1 h before use in the enzyme assay.

Analytical method

Protein was determined using the method of Lowry et al. [5] with bovine serum albumin as standard. Free fatty acids were measured by the colorimetric method described by Nixon and Chan [6].

Results

Subcellular distribution of cytidylyltransferase activity

Although most of the studies on cytidylyltransferase in lung have involved the enzyme recovered in

TABLE I

CYTIDYLYLTRANSFERASE SUBCELLULAR DISTRIBUTION IN ADULT LUNG

Lung was homogenized and separated into subcellular fractions as described in Materials and Methods. The homogenate fraction was the supernatant after a 5 min centrifugation at 1000 × *g*. The mitochondrial and microsomal fractions were pelleted at 20 000 × *g* × 30 min and 250 000 × *g* × 60 min, respectively. The cytosol fraction is the 250 000 × *g* supernatant. Activity was determined in the presence of 0.26 mM phosphatidylglycerol. The values are averages ± S.D. for three separate experiments

Fraction	nmol/min per g lung	%	nmol/min per mg protein
Homogenate	86 ± 5	100	1.1 ± 0.02
Mitochondria	6 ± 2	7	0.4 ± 0.11
Microsome	41 ± 5	48	3.3 ± 0.15
Cytosol	67 ± 9	77	1.1 ± 0.11

the 100 000 × *g* supernatant fraction. Significant activity has been reported in both adult and fetal lung microsomes [1,7]. Since we wished to examine endogenous lipid activators in both cytosol and microsome preparations, we investigated the subcellular distribution of cytidylyltransferase in order to establish fractionation conditions to obtain optimal activities in microsome and cytosol fractions. A fractionation method was developed which uses a 250 000 × *g* × 60 min centrifugation to sediment the maximal amount of membrane material from a 20 000 × *g* supernatant. Under these conditions, 36% of the recovered enzyme activity was in the microsomes and 59% was in the cytosol (Table I). The specific activity, (nMol/min per mg protein) was highest in the microsome fraction. Approximately 30% more activity was recovered in the combined subfractions than was present in the 1 000 × *g* supernatant. Thus, an inhibitor was removed during the fractionation or the activity in the 1 000 × *g* supernatant was measured under somewhat suboptimal conditions. The activity in the microsomal and supernatant fractions was linear with the amount of preparation added to the assay, up to 40 μl. However, the activity in the 1 000 × *g* supernatant was linear only up to 10 μl. Thus, the measurement of the amount of enzyme activity in the 1 000 × *g* supernatant was less reliable and may be an underestimate of the total activity.

TABLE II

EFFECTS OF ISOPROPYL ETHER EXTRACTION ON CYTIDYLYLTRANSFERASE ACTIVITY

The enzyme activity in subcellular fractions of adult lung was determined in untreated samples and in samples that were extracted with isopropyl ether. The activity was measured in the absence (−) and presence (+) of 0.25 mM phosphatidylglycerol. Enzyme activity is calculated as the average nmol/min per g lung ± S.E. with the number of separate experiments in parenthesis. Statistical significance of difference between groups was determined with the paired *t*-test. n.s., not significant

Fraction		Untreated	Extracted	*P*
30 000 × *g*	(−)	37 ± 5 (8)	11 ± 1 (8)	<0.001
supernatant	(+)	72 ± 9 (8)	56 ± 8 (8)	n.s.
250 000 × *g*	(−)	26 ± 2 (4)	9.5 ± 3 (4)	<0.01
particulate	(+)	42 ± 2 (4)	26 ± 4 (3)	<0.01
250 000 × *g*	(−)	18 ± 3 (4)	3 ± 2 (4)	<0.01
supernatant	(+)	68 ± 4 (4)	65 ± 6 (4)	n.s.

Effects of isopropyl ether extraction

The method developed by Cham and Knowles [4] for the selective extraction of lipids from plasma, without protein denaturation, was applied to subcellular fractions from lung. Extraction of a 30 000 × *g* × 30 min supernatant for 5 min with isopropyl ether produced a 70% loss in enzyme activity measured without the addition of phosphatidylglycerol to the assay (Table II). However, only 20% of the enzyme activity was lost when measured in the presence of phosphatidylglycerol. Extraction of the microsome fraction with isopropyl ether produced a 60% reduction in activity when measured without the addition of phosphatidylglycerol and a 40% decrease in activity when measured with phosphatidylglycerol. The activity in the 250 000 × *g* supernatant decreased by 90% after isopropyl ether extraction but the activity was completely recovered when measured in the presence of phosphatidylglycerol. Apparently, lipid activator can be removed from the soluble enzyme, without destruction of the enzyme. However, in both the 30 000 × *g* supernatant and the microsome fractions approximately 16 units of activity per g lung were not recovered after isopropyl ether extraction. Thus, a portion of the activity associated with membrane components (approximately 40%) apparently is

irreversibly destroyed by the isopropyl ether extraction.

Effects of extracted activator material on cytidylyltransferase

The lipid material that was extracted into isopropyl ether from a preparation of 250 000 × *g* × 60 min supernatant was tested as a source of activator for the cytidylyltransferase activity that remained after the extraction. The lipid in isopropyl ether extract was suspended in the Tris/NaCl buffer by sonication and varying amounts were added to enzyme assays. The amount of lipid material added to each assay was designated as the amount extracted from a measured amount of cytosol protein. Thus, the amount of lipid extracted from 1.0 mg of cytosol protein restored the enzyme activity in 0.2 mg cytosol protein (Fig. 1). Increasing amounts of lipid produced increased activity. Maximal stimulation was achieved with lipid from 2.5 mg of cytosol protein.

Identification of activators in the isopropyl ether extract

Thin-layer chromatographic analysis of a portion of a isopropyl ether extract revealed that the extract contained triacylglycerol, diacylglycerol, monoacylglycerol and free fatty acids. Visual inspection of the chromatogram after exposure to iodine vapor indicated that triacylglycerol was the major lipid component, with small amounts of diacylglycerol, monoacylglycerol and free fatty acids. An estimation of the ability of each lipid fraction to activate cytidylyltransferase showed that 85% of the recovered activity migrated with free fatty acids (Table III).

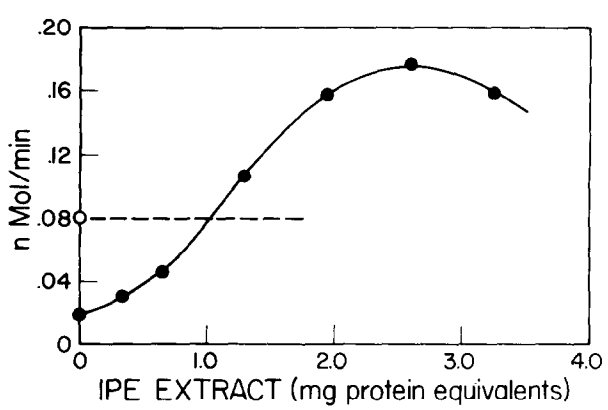

Fig. 1. Activation of cytidylyltransferase by isopropyl ether extracts of 250 000 × *g* supernatant. Aliquots (0.2 mg protein) of the extracted (•) and untreated (○) 250 000 × *g* supernatant were used as the source of enzyme. The amount of isopropyl ether (IPE) extract added to the enzyme assay is represented as the corresponding amount of cytosol protein from which it was extracted. The horizontal dotted line represents the activity of the untreated supernatant measured without the addition of lipid activator.

TABLE III

ACTIVATION OF CYTIDYLYLTRANSFERASE BY LIPID COMPONENTS ISOLATED FROM THE ISOPROPYL ETHER EXTRACTS

1 unit of activation is defined as the amount of increase in enzyme activity equivalent to that obtained with 40 μM phosphatidylylglycerol. The lipid bands were extracted with chloroform/methanol (2 : 1, v/v) (see Materials and Methods) and the amount of activation units in each determined

Lipid fractions	Activation units	Percentage
Isopropyl ether extract	82	100
Triacylglycerol	2	2
Diacylglycerol	2	2
Fatty acids	44	53
Monoacylglycerol	1.8	1
Origin	3	4
Total recovery	52	62

An amount of isopropyl ether extract that exhibited 82 units of enzyme activation contained approximately 0.4 μmol of lipid phosphorous. If this phospholipid was primarily phosphatidylglycerol and/or other acidic phospholipids, it could contribute significantly to the activation produced by the isopropyl ether extract. However, only a relatively small amount of activation was recovered from the origin of the thin-layer chromatogram (Table III), where phospholipid would be located. Furthermore, examination of the isopropyl ether extract by thin-layer chromatography with a solvent system designed to separate phospholipids (chloroform/methanol/acetic acid/H_2O, 100 : 60 : 16 : 8) indicated that the major detectable phospholipid was phosphatidylcholine. Phosphatidylcholine does not stimulate cytidylyltransferase activity [1,8]. Since we recovered only about 60% of the original stimulation activity after thin-layer chromatography, the presence of phospholipid and/or other types of activator material in the isopropyl ether extracts remains a possibility.

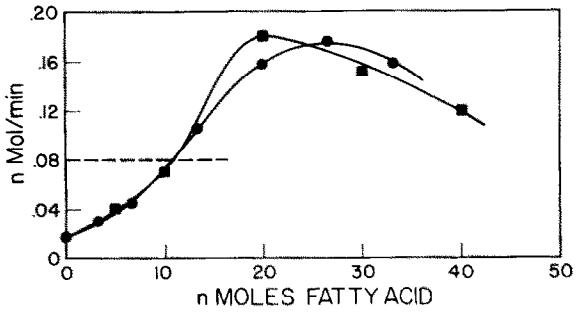

Fig. 2. Activation of cytidylyltransferase by isopropyl ether extract of 250 000 × *g* supernatant and by linoleic acid. Isopropyl ether-extracted supernatant (0.2 mg protein) was the source of enzyme. The amount of fatty acids in the isopropyl ether extract was determined by the method of Nixon and Chan [6]. The dotted line represents the enzyme activity in untreated supernatant. ▪, Linoleic acid; •, isopropyl ether extract represented in terms of its fatty acid content.

The concentration of free fatty acids in a isopropyl ether extract of cytosol was measured and the activation by this extract was compared with that obtained with linoleic acid (Fig. 2). The two activation curves are nearly identical. These results strongly suggest that free fatty acids are the only activator material in the isopropyl ether extract. According to the data in Fig. 2, the enzyme activity in extracted cytosol is restored to the activity in nonextracted cytosol by the addition of the fatty acids obtained from 1.0 mg of cytosol protein. In these experiments, 0.2 mg cytosol protein was used in the enzyme assays. Apparently a 4–5-fold excess of fatty acid is required for reactivation of the extracted enzyme, according to our current assay conditions. The need for the addition of high amounts of fatty acid for reactivation of enzyme and the apparent lag in the onset of activation produced by increasing concentrations of fatty acid may be due to nonspecific binding of fatty acids by proteins in the cytosol.

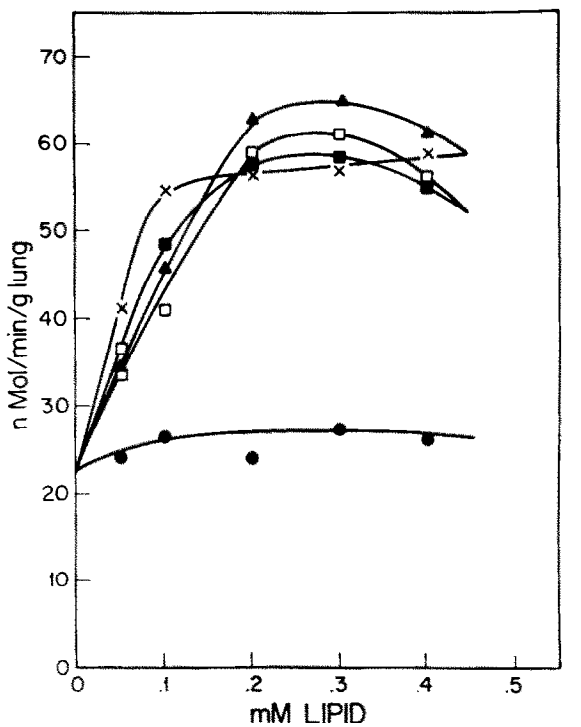

Fig. 3. Activation of cytidylyltransferase in lung cytosol by fatty acids. Commercially prepared fatty acids and phosphatidylglycerol were suspended in Tris/NaCl buffer as described in Materials and Methods. Cytidylyltransferase was prepared on the day of assay by centrifugation of a 20% homogenate of rat lung at 250 000 × *g* × 60 min. ×, Phosphatidylglycerol; •, 16 : 0; ▪, 18 : 1; □, 18 : 2; ▲, 18 : 3.

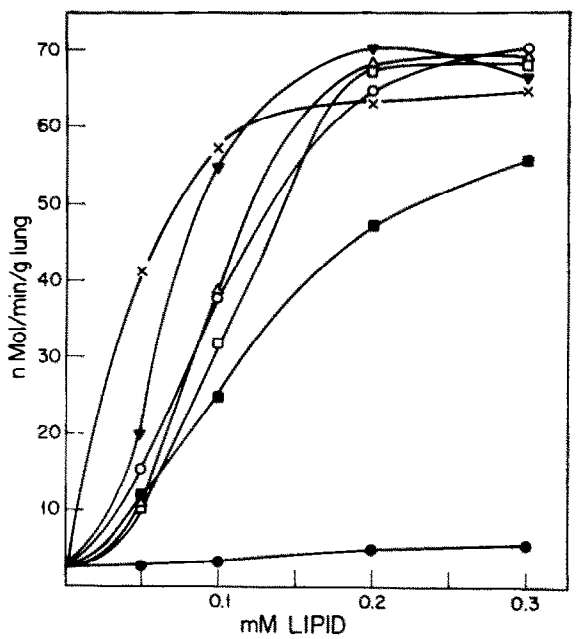

Fig. 4. Activation of cytidylyltransferase in isopropyl ether-extracted cytosol by fatty acids. Cytidylyltransferase was prepared as described in the legend to Fig. 3. The cytosol fraction was extracted with 2 vol. of isopropyl ether before utilizing in enzyme assays. ×, phosphatidylglycerol; •, 16 : 0, 18 : 0 and 20 : 0; ○, 16 : 1; ▪, 18 : 1; □, 18 : 2; △, 18 : 3, ▼, 20 : 4.

Fatty acid specificity of enzyme activation

Commercially prepared fatty acids were suspended and sonicated in Tris/NaCl buffer at a concentration of 1.0 mM immediately before addition to the enzyme assays. All unsaturated fatty acids can act as potent stimulators of the cytosol form of cytidylyltransferase (Figs. 3 and 4). Fatty acids are able to stimulate cytidylyltransferase activity from freshly prepared cytosol and cytosol extracted with isopropyl ether to the same extent as phosphatidylglycerol. The decreased stimulation by saturated fatty acids may be partially due to the difficulty of obtaining homogeneous aqueous suspensions of these species. Individual species of unsaturated fatty acids do not differ significantly in their stimulatory activity. However, highly unsaturated fatty acids, such as 18 : 3 or 20 : 4, appear to be slightly more potent.

Discussion

Previous studies of cytidylyltransferase have demonstrated that the enzyme is stimulated in vitro by a variety of phospholipids, including phosphatidylglycerol [2], phosphatidylserine, phosphatidylinositol [1,2], phosphatidic acid [3] and lysophosphatidylethanolamine [8]. The present results show that cytidylyltransferase activity in cytosol preparations of adult lung is also stimulated by unsaturated fatty acids. Furthermore, the use of a nondestructive extraction method leads to a decrease in endogenous enzyme activity which appears to be primarily due to the removal of fatty acids from the preparation. Thus, not only will fatty acids stimulate the enzyme activity, but fatty acids present in cytosol preparations are directly related to the amount of endogenous activity in the preparation. The effect of isopropyl ether extraction of microsomal preparations is less clear. Extraction of microsomes produced a reduction in endogenous activity which could be partially recovered by the addition of phosphatidylglycerol. Some enzyme activity was irreversibly lost due to a disruption of membrane structure or a direct inactivation of the enzyme. An overall assessment of the significance of the apparent requirement of fatty acids for cytidylyltransferase activity and the relationship between fatty acid requirements and stimulation by acidic phospholipids requires additional exploration.

There is precedent for a regulatory role of fatty acid in intermediary metabolism and glycerolipid synthesis. Several enzyme activities have been found to be inhibited in vitro by free fatty acids. These include glycerol-3-phosphate dehydrogenase in rat liver and muscle [9], glycerophosphate acyltransferase and 1-acylglycerophosphate acyltransferase in yeast [10], phosphatidate phosphatase in rat adipose tissue cytosol [11] and adenylate cyclase and hormone sensitive-triacylglycerol lipase in rat adipose tissue [12]. Unsaturated fatty acids have been found to stimulate diacylglycerol cholinephosphotransferase in chicken liver [13], rat brain and liver [14], phosphatidylinositol phosphodiesterase in rat brain [15], fructose-1,6-diphosphate in rabbit liver [16], and pyruvate oxidase in *Escherichia coli* [17]. A formulation of these diverse results into a rational pattern of regulatory control by fatty acids is difficult. However, a coordinated regulation of enzyme activities that produce and/or utilize diacylglycerol with a key regulatory enzyme responsible for the synthesis of CDP-choline (cytidylyltransferase) would provide a mechanism for a positive feedback control of the de novo synthesis of phosphatidylcholine.

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