Regulation of CTP:phosphocholine cytidyltransferase activity in type II pneumonocytes

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Phosphatidylcholine synthesis by rat type II pneumonocytes was altered either by depleting the cells of choline or by exposing the cells to extracellular lung surfactant. Effects of these experimental treatments on the activity of a regulatory enzyme, CTP:phosphocholine cytidyltransferase, were investigated. Although choline depletion of type II pneumonocytes resulted in inhibition of phosphatidylcholine synthesis, cytidyltransferase activity (measured in cell homogenates in either the absence or presence of added lipids) was greatly increased. Activation of cytidyltransferase in choline-depleted cells was rapid and specific, and was quickly and completely reversed when choline-depleted cells were exposed to choline (but not ethanolamine). Choline-dependent changes in enzymic activity were apparently not a result of direct actions of choline on cytidyltransferase and they were largely unaffected by cyclic AMP analogues, oleic acid, linoleic acid or cycloheximide. The K_m value of cytidyltransferase for CTP (but not phosphocholine) was lower in choline-depleted cells than in choline-repleted cells. Subcellular redistribution of cytidyltransferase also was associated with activation of the enzyme in choline-depleted cells. When measured in the presence of added lipids, $66.5 \pm 5.0\%$ of recovered cytidyltransferase activity was particulate in choline-depleted cells but only $34.1 \pm 4.5\%$ was particulate in choline-repleted cells. An increase in particulate cytidyltransferase also occurred in type II pneumonocytes that were exposed to extracellular surfactant. This latter subcellular redistribution, however, was not accompanied by a change in cytidyltransferase activity even though incorporation of [^3H]choline into phosphatidylcholine was inhibited by approx. 50%. Subcellular redistribution of cytidyltransferase, therefore, is associated with changes in enzymic activity under some conditions, but can also occur without a resultant alteration in enzymic activity.

INTRODUCTION

Disaturated phosphatidylcholine is the most abundant component of lung surfactant which is produced by type II pneumonocytes of the alveolar epithelium (Klaus *et al.*, 1961; Pattle & Thomas, 1961). Type II pneumonocytes, because of their large capacity for disaturated phosphatidylcholine synthesis, are convenient cells for investigations of the regulation of phosphatidylcholine synthesis, and afford the added advantage that disaturated phosphatidylcholine destined for secretion can be distinguished from molecular species of phosphatidylcholine for other cell functions. Based on results from pulse–chase experiments, it was concluded that the rate-limiting reaction in the incorporation of [^3H]choline into phosphatidylcholine by isolated type II pneumonocytes was the formation of CDP–choline catalysed by CTP:phosphocholine cytidyltransferase (EC 2.7.7.15) (cytidyltransferase) (Post *et al.*, 1984a). This finding was consistent with earlier observations that cytidyltransferase activity increases at a time during lung development when there is increased synthesis of phosphatidylcholine for surfactant (Rooney *et al.*, 1976; Stern *et al.*, 1976; Farrell, 1977; Chan *et al.*, 1983), and is also compatible with evidence that this enzyme is a regulatory enzyme in the synthesis of phosphatidylcholine in other tissues (Pelech & Vance, 1984).

Weinhold and coworkers investigated the mechanisms of regulation of cytidyltransferase in rat lung and observed that the activity of this enzyme increases when a latent form of the enzyme in the cytosol undergoes either aggregation in the presence of lipids (Feldman *et al.*, 1978) or translocation to the endoplasmic reticulum (Weinhold *et al.*, 1981, 1984). Regulation of cytidyltransferase activity by subcellular translocation of this enzyme in other cells has been observed (Sleight & Kent, 1983a, b, c; Pelech & Vance, 1984). Some effectors of the subcellular translocation of cytidyltransferase have been identified, but the molecular mechanism of this process remains unknown (Pelech & Vance, 1984). Furthermore, the regulation of cytidyltransferase in isolated type II pneumonocytes has not been investigated.

In the present investigation, type II pneumonocytes were maintained under two sets of experimental conditions that were known to result in alteration of phosphatidylcholine synthesis, and changes in cytidyltransferase activity were examined. The first set of experimental conditions was based on the observation that the synthesis of phosphatidylcholine by isolated type II pneumonocytes could be rendered responsive to changes in the extracellular concentration of choline if the cells were first depleted of choline (Anceschi *et al.*, 1984). Homogenates of choline-depleted type II pneumonocytes were found to have a greater activity of cytidyltransferase

Abbreviation used: cytidyltransferase, CTP:phosphocholine cytidyltransferase (EC 2.7.7.15).

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than did homogenates of untreated cells (Anceschi *et al.*, 1984). Under the second set of experimental conditions, isolated type II pneumonocytes were exposed to purified lung surfactant. It was observed recently that extracellular surfactant was taken up by type II pneumonocytes and synthesis of phosphatidylcholine was inhibited (Tesan & Bleasdale, 1985).

The objectives of the present investigation were to characterize the mechanism of regulation of cytidylyltransferase activity employing the choline-depleted type II pneumonocyte as an experimental model and then to determine if such a regulatory mechanism was involved in the physiological response of type II pneumonocytes to extracellular lung surfactant. Some of the results of this investigation were presented in abstract form (Anceschi & Bleasdale, 1984).

MATERIALS AND METHODS

Materials

The animals used in this investigation were male Sprague-Dawley rats (200–225 g) that were free from specific pathogens and were obtained from SASCO Inc., Omaha, NE, U.S.A. Rats were housed inside a laminar-flow hood and fed *ad libitum* until killed. Choline oxidase (EC 1.1.3.17) that was purified from *Arthrobacter globiformis* (specific activity: 11.5 μmol of H_2O_2 formed $\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein, pH 8.0, 25 °C) was purchased from Toyo Jozo Co., Tokyo, Japan. Catalase (EC 1.11.1.6) that was purified from bovine liver (specific activity 40 mmol of H_2O_2 consumed $\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein, pH 7.0, 25 °C) was supplied by Worthington Biochemical Corporation, Freehold, NJ, U.S.A. 2-Hydroxy-3,5-dichlorobenzenesulphonate (sodium salt) was from Research Organics Inc., Cleveland, OH, U.S.A. Dulbecco's modified Eagle's medium, with or without choline, was prepared using chemicals from Sigma Chemical Co. This medium was supplemented with 1% (v/v) antibiotic/antimycotic solution [containing penicillin (10000 units/ml), fungizone (25 $\mu\text{g}/\text{ml}$) and streptomycin (10 mg/ml)] obtained from GIBCO, Grand Island, NY, U.S.A. Medium was sterilized by ultrafiltration before use.

Dibutyl cyclic AMP, 8-bromo cyclic AMP and cycloheximide were obtained from Sigma Chemical Co. Oleic acid and linoleic acid were supplied by Serdary Research Laboratories, London, Ontario, Canada. The sources of all other materials have been described previously (Bleasdale *et al.*, 1982, 1983).

Isolation and choline depletion of type II pneumonocytes

Type II pneumonocytes were isolated by a modification of the method of Mason *et al.* (1977a,b) as described elsewhere (Bleasdale *et al.*, 1982, 1985). Purity of the final cell suspension was assessed by light microscopy using a modified Papanicolaou stain (Kikkawa & Yoneda, 1974) and by electron microscopy (Snyder *et al.*, 1981). Approx. 5×10^7 cells, of which more than 80% were type II pneumonocytes, were isolated routinely from six rats.

Isolated cells were collected by centrifugation (250 g for 6 min) and washed twice with choline-free Dulbecco's modified Eagle's medium that was supplemented with antibiotics and antimycotics (referred to subsequently as choline-free medium). Washed cells (approx. 5×10^7) were resuspended in 10 ml of choline-free medium that contained choline oxidase (1.3 mg of protein) and

catalase (0.08 mg of protein) and the suspension was incubated at 37 °C for 3 h. Cells were then collected by centrifugation (250 g for 6 min) and washed twice in choline-free medium before being used in the experiments described below. In most experiments, choline-repleted cells (rather than untreated cells) were used as controls. Choline repletion was accomplished by the addition of choline (either 0.05 mM or 0.10 mM) to choline-depleted cells that were suspended in choline-free medium. Although choline repletion appeared to be complete within 1 h at 37 °C, the standard choline repletion procedure involved exposure to choline for 3 h at 37 °C.

Assay of CTP:phosphocholine cytidylyltransferase activity

Activity of CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) was assayed by measurement of the incorporation of [^{14}C]phosphocholine into CDP-choline by using a modification of the procedures of Pelech *et al.* (1981) and Weinhold *et al.* (1981) as described elsewhere (Anceschi *et al.*, 1984). For the experiments in which the subcellular distribution of CTP:phosphocholine cytidylyltransferase activity was investigated, the assay buffer [Tris/succinate solution (30 mM, pH 6.5)] was replaced with Tris/HCl (50 mM, pH 7.4). Assays were performed in both the absence and presence of total lipids from lung (1 μmol of lipid phosphorus/ml of assay mixture). Lipids for the assay were extracted (Bleasdale *et al.*, 1979) from rat lungs that had been perfused to remove blood.

Subcellular distribution of CTP:phosphocholine cytidylyltransferase activity

Because of the limited yield of cells for analysis, only the distribution of CTP:phosphocholine cytidylyltransferase between a total particulate fraction and a soluble fraction was investigated. Choline-depleted cells and choline-repleted cells were collected by centrifugation (250 g for 6 min), washed twice with ice-cold phosphate-buffered saline and then resuspended in ice-cold homogenization medium [NaCl (0.154 M) that contained Tris/HCl (50 mM, pH 7.4) and dithiothreitol (1 mM)]. Cell suspensions were subjected to ultrasound (three 10 s periods in a Bransonic 220 bath-type sonicator, 125 W) and then homogenized manually in a glass/glass conical homogenizer (radial clearance, 0.065 mm; 12 strokes). This procedure was found previously to result in lysis of most type II pneumonocytes without disruption of mitochondria (Bleasdale *et al.*, 1985). When the use of ultrasound was omitted from the procedure, the distribution of cytidylyltransferase among subcellular fractions was not significantly altered, but the number of cells that were not lysed increased greatly (results not shown). Cell homogenates were centrifuged at 750 g for 10 min and the supernatant fluid that was obtained was centrifuged further (105000 g for 60 min). The pellet that resulted was resuspended in homogenization medium and is referred to subsequently as the particulate fraction. The supernatant fluid is referred to subsequently as the soluble fraction. CTP:phosphocholine cytidylyltransferase activity in the particulate and soluble fractions was assayed immediately after the preparation of the fractions.

Quantification of choline

Intracellular amounts of choline were measured enzymically. Cells were disrupted with ultrasound (as

described above) and then immersed in a boiling-water bath for 10 min. The disrupted cells were centrifuged at 10000 *g* for 5 min and aliquots of the supernatant fluid were taken for the assay of choline as described by Muneshige *et al.* (1983), except that phenol was replaced by 2-hydroxy-3,5-dichlorobenzenesulphonate (1.9 mM) (McGowan *et al.*, 1983) and the concentration of 4-aminoantipyrine that was employed was 0.8 mM.

Other methods

Activities of choline kinase (EC 2.7.1.32) and choline-phosphotransferase (EC 2.7.8.2) were assayed by use of the methods recommended by Ulane (1982) and van Golde (1982) respectively.

Surfactant was purified from the material lavaged from the lungs of adult rats by use of the procedure described by Katyal *et al.* (1977), and then quantified by measurement of lipid phosphorus (Bleasdale *et al.*, 1979).

Incorporation of [³H]choline into phosphatidylcholine by type II pneumonocytes was measured as described elsewhere (Anceschi *et al.*, 1984) except that the cells were maintained in Dulbecco's modified Eagle's medium that was supplemented with fetal bovine serum (10%, v/v). Exposure to [³H]choline (approx. 10 Ci/mol) was for 2 h.

Protein was measured as described by Lowry *et al.* (1951) using bovine serum albumin for calibration.

Statistical significance was assessed by Student's *t*-test for paired values (Zar, 1974) and differences were considered significant at *P* < 0.05.

RESULTS

Treatment of type II pneumonocytes for 3 h with choline-free medium that contained choline oxidase and catalase was found previously to result in a loss of intracellular choline (Anceschi *et al.*, 1984). The activity of cytidylyltransferase in homogenates of type II pneumonocytes that were depleted of choline was much greater than that in homogenates of untreated cells

(Table 1). The increased activity of cytidylyltransferase in choline-depleted cells was apparent when enzymic activity was assayed under optimal conditions *in vitro* in either the absence or presence of added lipids (Table 1). The major effect of added lipids is apparently to allow expression of latent cytidylyltransferase activity in the cytosol (Pelech & Vance, 1984). When choline-depleted cells were returned to medium that contained choline (0.05 mM) for 3 h, cytidylyltransferase activity fell to a value similar to that found in untreated cells (Table 1). Direct addition of either choline or betaine (up to 2 mM) to assay mixtures, however, did not influence the measured value of cytidylyltransferase activity (results not shown). In subsequent experiments, cytidylyltransferase activity in choline-depleted cells was compared with that in cells that had been first depleted of choline and then exposed to medium that contained choline (i.e., choline-repleted cells) which were considered more appropriate than untreated cells as controls.

There was a close temporal relationship between the loss of intracellular choline and the increase in cytidylyltransferase activity (Fig. 1). Effects of the depletion procedure on intracellular amounts of phosphocholine and CDP-choline were not investigated. In a previous investigation, however, choline-depleted type II pneumonocytes were incubated in the presence of [³H]choline at various concentrations and the radioactivity associated with intracellular choline and its derivatives was measured after isotopic equilibrium had been attained (Anceschi *et al.*, 1984). It was found that the depletion procedure resulted in a loss of not only choline but also phosphocholine, CDP-choline and betaine, and that in choline-depleted cells the relative amounts of these choline derivatives were altered. In the present investigation, although it was possible to deplete type II pneumonocytes of more than 85% of their intracellular choline by prolonged incubation of the cells in depletion medium, the increase in cytidylyltransferase activity was essentially complete within 3 h. Experimental variability

Table 1. Effects of choline depletion and choline repletion on CTP:phosphocholine cytidylyltransferase activity in type II pneumonocytes

CTP:phosphocholine cytidylyltransferase activity was assayed in homogenates of type II pneumonocytes that were freshly isolated and either used immediately or first subjected to choline depletion/repletion. Cells were depleted of choline for 3 h as described in the Materials and methods section. Choline repletion was for 3 h and was performed immediately after the period of choline depletion. Assays were performed in either the absence or presence of total lipids extracted from rat lung (1 μ mol of lipid P/ml). Data are mean values $\pm$ S.E.M. that were derived from four experiments. Values of total enzymic activity are not corrected for loss of cells during harvesting and washing. In the series of experiments reported in Table 1, CTP:phosphocholine cytidylyltransferase activity in untreated type II pneumonocytes was not assayed in the presence of lipids. In another series of three experiments, however, specific activity of CTP:phosphocholine cytidylyltransferase (nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹ of protein) in untreated type II pneumonocytes was 0.68 ± 0.03 in the absence of lipids and 0.94 ± 0.16 in the presence of lipids (mean values $\pm$ S.E.M.). In the same cells, total activity of CTP:phosphocholine cytidylyltransferase (nmol $\cdot$ min⁻¹ $\cdot$ 10⁻⁶ cells) was 0.11 ± 0.051 in the absence of lipids and 0.15 ± 0.027 in the presence of lipids (mean values $\pm$ S.E.M.). †Different from the corresponding value for untreated cells (*P* < 0.025); different from the corresponding value for choline-depleted cells, ***P* < 0.01, **P* < 0.05.

Experimental condition	CTP:phosphocholine cytidylyltransferase activity			
	Specific (nmol $\cdot$ min ⁻¹ $\cdot$ mg ⁻¹ of protein)		Total (nmol $\cdot$ min ⁻¹ $\cdot$ 10 ⁻⁶ cells)	
	Assay without lipid	Assay with lipid	Assay without lipid	Assay with lipid
Untreated	0.76 ± 0.10	—	0.13 ± 0.015	—
Choline-depleted	$1.64 \pm 0.10^\dagger$	2.02 ± 0.24	$0.27 \pm 0.017^\dagger$	0.32 ± 0.022
Choline-repleted	$0.86 \pm 0.11^{**}$	$1.14 \pm 0.14^*$	$0.14 \pm 0.018^{**}$	$0.18 \pm 0.025^*$

Fig. 1. Temporal relationship between choline depletion and activation of CTP:phosphocholine cytidyltransferase

Freshly isolated type II pneumonocytes were washed twice with choline-free Dulbecco's modified Eagle's medium and then suspended in choline-free Dulbecco's modified Eagle's medium that contained choline oxidase and catalase (depletion medium). After various periods of incubation at 37 °C, cells were collected by centrifugation (250 *g* for 6 min), washed, resuspended in NaCl (0.154 M) that contained Tris/HCl (50 mM, pH 7.4) and then disrupted by use of ultrasound. Aliquots of cell homogenates were taken for the enzymic measurement of choline (○) and for the assay of cytidyltransferase activity (in the absence of added lipids) (●). Data are mean values $\pm$ S.D. that were derived from three experiments.

in the magnitude of the increase in cytidyltransferase activity when type II pneumonocytes were depleted of choline was found to be due largely to variations in the enzymic activity present in freshly isolated cells and may reflect detrimental effects of the isolation procedure on the cells (Finkelstein & Mavis, 1979). Activities of the other enzymes that are involved in the incorporation of choline into phosphatidylcholine (i.e. choline kinase and cholinephosphotransferase) when measured in cell homo-

Fig. 2. Time-course of choline-dependent activation and deactivation of CTP:phosphocholine cytidyltransferase in type II pneumonocytes

At zero time (first arrow), type II pneumonocytes were transferred to the medium for choline depletion and at various intervals cells were harvested, washed, and the activity of cytidyltransferase in cell homogenates was assayed in the absence of added lipids. After 3 h, cells were washed twice with choline-free Dulbecco's modified Eagle's medium (choline-free medium) and then were resuspended in choline-free medium at a density of approx. 10^6 cells/ml. At the time indicated (second arrow), medium (●), choline (0.05 mM) (○), or ethanolamine (0.05 mM) (▲), was added to some cells and after various intervals of further incubation cytidyltransferase activity in cell homogenates was assayed. Data are mean values that were derived from three experiments.

genates under optimal conditions *in vitro*, were unaffected by choline depletion (Table 2).

When cells were depleted of choline for 3 h and then transferred to choline-free medium (that lacked choline oxidase and catalase), cytidyltransferase activity remained elevated but did not increase further (Fig. 2). When choline-depleted cells were transferred to medium that contained choline (0.05 mM), however, cytidyltransferase activity returned to levels found in untreated cells within 1–2 h (Fig. 2). Intracellular amounts of choline also returned to pretreatment levels within 1–2 h of choline repletion (results not shown). Ethanolamine was unable to substitute completely for choline in

Table 2. Choline-dependent changes in specific activities of the enzymes that catalyse incorporation of choline into phosphatidylcholine

Activities of choline kinase, CTP:phosphocholine cytidyltransferase and cholinephosphotransferase in homogenates of choline-depleted cells and choline-repleted cells were assayed under optimal conditions *in vitro* as described (Materials and methods section). Assays of CTP:phosphocholine cytidyltransferase activity were conducted in the absence of added lipids. Data are mean values $\pm$ S.E.M. that were derived from the number of experiments given in parentheses. *Different from the corresponding value for choline-depleted cells, $P < 0.01$.

Enzyme	Specific activity (nmol · min ⁻¹ · mg ⁻¹ of protein)	
	Choline-depleted cells	Choline-repleted cells
Choline kinase	0.48 ± 0.04	0.39 ± 0.07 (3)
CTP:phosphocholine cytidyltransferase	1.64 ± 0.10	0.86 ± 0.11 (4)*
Cholinephosphotransferase	3.80 ± 0.35	3.50 ± 0.10 (3)

Table 3. Choline-dependent changes in Michaelis constants of CTP:phosphocholine cytidylyltransferase

Type II pneumonocytes were subjected to either choline depletion (3 h) or choline depletion (3 h) followed immediately by choline repletion (3 h). Cell homogenates were then prepared and initial rates of CTP:phosphocholine cytidylyltransferase activity in homogenates were assayed at various concentrations of one of the substrates while the other substrate was maintained at a concentration that supported a maximal reaction rate. K_m values were computed after linear transformation (Eadie-Hofstee) of the data. The data are mean values $\pm$ S.E.M. that were derived from four experiments. *Different from the corresponding value for choline-depleted cells, $P < 0.05$.

Experimental condition	K_m (mM)	
	Assay without lipid	Assay with lipid
CTP		
Choline-depleted	1.06 ± 0.13	1.32 ± 0.05
Choline-repleted	$1.88 \pm 0.30^*$	1.41 ± 0.25
Phosphocholine		
Choline-depleted	0.43 ± 0.09	0.39 ± 0.10
Choline-repleted	0.38 ± 0.06	0.35 ± 0.08

reversing the increase in cytidylyltransferase activity, but nevertheless elicited a small, reproducible decrease in cytidylyltransferase activity in choline-depleted cells (Fig. 2).

The increased activity of cytidylyltransferase activity in choline-depleted cells that was measured under optimal assay conditions (Tables 1 and 2, Figs. 1 and 2) was still evident when either phosphocholine or CTP was reduced to sub-saturating concentration. Choline depletion had no discernible effect on the affinity of cytidylyltransferase for phosphocholine (as reflected by the K_m value) when enzymic activity was assayed in either the absence or presence of added lipids (Table 3). The affinity of cytidylyltransferase for CTP, however, appeared to be increased during choline depletion when enzymic activity was assayed in the absence of lipids. When enzymic activity was assayed in the presence of lipids, the K_m value for CTP was unaltered by choline depletion (Table 3).

In previous investigations, it was found that the activity of cytidylyltransferase in some cell types was influenced by cyclic AMP and by unesterified fatty acids (Pelech & Vance, 1982; Pelech *et al.*, 1982, 1984; Feldman *et al.*, 1981). In the present investigation, however, it was found that the increase in cytidylyltransferase activity during choline depletion and its reversal when cells were repleted of choline were not greatly affected by either cyclic AMP analogues or unesterified fatty acids (Fig. 3). Under a variety of other experimental conditions (results not shown), 8-bromo cyclic AMP, dibutyryl cyclic AMP, linoleic acid, or oleic acid failed to either block or greatly alter choline-dependent changes in cytidylyltransferase activity in type II pneumonocytes. Similarly, under a variety of experimental conditions, cycloheximide failed to prevent choline-dependent changes in cytidylyltransferase activity (Fig. 3).

The effect of choline depletion on the subcellular distribution of cytidylyltransferase in type II pneumonocytes also was investigated. Because of the limited yield of type II pneumonocytes, the distribution of cytidylyltransferase activity between a particulate fraction (105000 g pellet) and a soluble fraction (105000 g supernatant fluid) was determined. When assays were performed in either the absence or presence of added lipids, the portion of total cytidylyltransferase activity that was particulate increased as a result of choline

Fig. 3. Effects of cyclic AMP analogues, unesterified fatty acids and cycloheximide on choline-dependent changes in CTP:phosphocholine cytidylyltransferase activity in type II pneumonocytes

Type II pneumonocytes were subjected to choline depletion as described (Materials and methods section) except that some cells received one of the following additions at the beginning of the depletion period: 8-bromo cyclic AMP (1 mM) plus 3-isobutyl-1-methylxanthine (0.1 mM), dibutyryl cyclic AMP (1 mM) plus 3-isobutyl-1-methylxanthine (0.1 mM), oleate (2 mM) plus bovine serum albumin (10 mg/ml), linoleate (2 mM) plus bovine serum albumin (10 mg/ml), cycloheximide (1 μ M). At the end of the depletion period (3 h) cells were harvested and cytidylyltransferase activity in cell homogenates was assayed. Other cells were first depleted of choline for 3 h (in the absence of any test compounds) and then subjected to choline repletion for 3 h in the absence or presence of one of the test compounds. Cytidylyltransferase activity in homogenates of choline-repleted cells was assayed. Enzymic activity in cells that were incubated for 6 h without either choline depletion or choline repletion was also assayed. Data are averages of duplicate measurements that were derived from two typical experiments.

depletion. This effect was most pronounced when the latent cytosolic form of the enzyme was assayed by the inclusion of lipids in the reaction mixture (Fig. 4). The

Fig. 4. Choline-dependent changes in the distribution of CTP:phosphocholine cytidyltransferase activity between a particulate fraction and a soluble fraction of type II pneumonocytes

Choline depletion and choline repletion of type II pneumonocytes were performed as described and cell homogenates were prepared (Materials and methods section). Cell homogenates were centrifuged at 750 *g* for 10 min and the resultant supernatant fluid was further centrifuged at 105000 *g* for 60 min to obtain a particulate fraction and a soluble fraction. Cytidyltransferase activity in particulate fractions and in soluble fractions was assayed in both the absence and presence of lung total lipids (1 μ mol of lipid P/ml). Data are mean values $\pm$ S.E.M. that were derived from four experiments. The increased portion of total enzymic activity that was recovered in the particulate fraction of choline-depleted cells (compared with choline-repleted cells) was statistically significant ($P < 0.05$, Student's *t* test for paired values) when the assay for enzymic activity was conducted in either the absence or presence of lung total lipids. Recovery of total enzymic activity was $68.1 \pm 2.6\%$ for choline-depleted cells and $73.0 \pm 2.2\%$ for choline-repleted cells.

composition of the 750 *g* pellet was not defined and cytidyltransferase activity in this fraction was not routinely assayed. In experiments where cytidyltransferase activity in the 750 *g* pellet was assayed, however, specific activity and choline-dependent changes in specific activity were greater in this fraction than in the cell homogenate. Although the ratio of soluble to particulate activities of cytidyltransferase could be altered by changing the conditions of homogenization, a subcellular redistribution of cytidyltransferase activity during choline depletion was still apparent. In a typical experiment in which cells were homogenized in deionized water instead of homogenization medium, soluble cytidyltransferase activity (measured in the presence of added lipids) was 14.9% of total recovered activity in choline-depleted cells and 34.2% of total recovered activity in choline-repleted cells.

Although the mechanism of the increase in particulate cytidyltransferase in choline-depleted type II pneumonocytes has not been elucidated, translocation to the particulate fraction was itself apparently insufficient to

produce a change in cytidyltransferase activity. This conclusion is based on the results of an investigation of the involvement of subcellular redistribution of cytidyltransferase activity in the inhibition of phosphatidylcholine synthesis in type II pneumonocytes that are exposed to purified surfactant. Extracellular surfactant at a concentration of 0.15 μ mol of lipid P/ml reduced the incorporation of [3 H]choline into phosphatidylcholine from 9239 ± 490 to 4683 ± 426 d.p.m. $\cdot$ h $^{-1}$ $\cdot$ 10 $^{-6}$ cells (mean values $\pm$ S.E.M. derived from four experiments). This effect of extracellular surfactant was not due to either general toxicity or to a reduction in the specific radioactivity of intracellular [3 H]choline (Tesan & Bleasdale, 1985; M. Tesan, N. E. Tyler, N. R. Thakur, L. Hamilton & J. E. Bleasdale, unpublished work). Cytidyltransferase activity was assayed in the presence of added lipids in homogenates of cells that had been exposed to surfactant and was found to be not significantly different from that in homogenates of untreated cells (Table 4). On the other hand, exposure to surfactant resulted in increased recovery of cytidyltransferase activity in the particulate fraction (Table 4).

DISCUSSION

Type II pneumonocytes synthesize large amounts of disaturated phosphatidylcholine that is secreted in the form of lung surfactant. While there is evidence that CTP:phosphocholine cytidyltransferase is a regulatory enzyme in the synthesis of phosphatidylcholine by type II pneumonocytes (reviewed by Rooney & Brehier, 1982; Pelech & Vance, 1984), the mechanisms by which activity of this enzyme are controlled are not well understood. In the present investigation, the choline-depleted type II pneumonocyte was employed as an experimental model for examining the regulation of cytidyltransferase activity. Incubation of type II pneumonocytes in medium that lacked choline but contained choline oxidase and catalase resulted in a rapid loss of intracellular choline. The mechanism of this choline depletion was not examined but it is likely that choline leaks from the type II pneumonocyte into the medium where it is oxidized to betaine and is thus unavailable for active uptake back into the cell. Betaine formed extracellularly was not taken up by type II pneumonocytes (Anceschi *et al.*, 1984). Associated with the loss of intracellular choline was a large, rapid increase in cytidyltransferase activity that was specific and could be reversed quickly by exposure of choline-depleted cells to medium that contained choline.

The mechanism of the choline-dependent changes in cytidyltransferase activity did not appear to involve protein synthesis because the changes were rapid and relatively insensitive to cycloheximide. Acute changes in cytidyltransferase activity in other cells are similarly insensitive to inhibitors of protein synthesis (Pelech & Vance, 1984). The changes in cytidyltransferase activity in type II pneumonocytes were apparently not due to direct allosteric effects of choline on the enzyme. Since choline-depleted type II pneumonocytes appear also to contain reduced amounts of phosphocholine, CDP-choline and betaine (Anceschi *et al.*, 1984), the possibility that activation of cytidyltransferase is a response to the loss of choline derivatives can not be discounted. Assuming, however, that cytidyltransferase activity responds to changes in the intracellular concentration of

Table 4. Effect of extracellular surfactant on activity and subcellular distribution of CTP:phosphocholine cytidylyltransferase in type II pneumonocytes

Type II pneumonocytes were incubated for 15 h in Dulbecco's modified Eagle's medium that was supplemented with fetal bovine serum (10%, v/v) with or without lung surfactant (approximately 0.15 μmol of lipid P/ml) that was purified as described by Katyal *et al.* (1977). The pellet of purified surfactant was resuspended in Dulbecco's modified Eagle's medium (without serum) by use of ultrasound (20 s in a Bransonic 220 bath-type sonicator, 125 W) prior to its addition to cells. After the incubation period, cells were harvested and separated from surfactant by washing three times with homogenization medium (250 g for 10 min). Washed cells were homogenized and subcellular fractions were isolated as described (Materials and methods section). Cytidylyltransferase activity was assayed [in the presence of total lipids from lung (1 μmol of lipid P/ml)] in cell homogenates and in a 105000 g pellet (particulate fraction) and a 105000 g supernatant fluid (soluble fraction) that were derived from the 750 g supernatant fraction (Materials and methods section). Surfactant, at a concentration of 0.15 μmol of lipid P/ml, when added directly to the assay mixture did not affect cytidylyltransferase activity. Data are expressed as the specific and total activities of cytidylyltransferase in cell homogenates and as the percentages of recovered activity that are found in the particulate fraction and the soluble fraction. Data are mean values $\pm$ S.E.M. that were derived from the number of experiments given in parentheses. Recovery of cytidylyltransferase activity from the 750 g supernatant fraction was $69.6 \pm 6.1\%$ for control cells and $71.6 \pm 3.4\%$ for surfactant-treated cells. *Significantly different from corresponding value in control cells, $P < 0.05$.

Incubation condition...	Activity of CTP:phosphocholine cytidylyltransferase	
	Without surfactant	With surfactant
Cell homogenate		
Specific activity (nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ of protein)	0.95 $\pm$ 0.06 (6)	0.92 $\pm$ 0.09 (6)
Total activity (nmol $\cdot$ min $^{-1}$ $\cdot$ 10 $^{-6}$ cells)	0.15 $\pm$ 0.012 (6)	0.16 $\pm$ 0.021 (6)
Subcellular distribution (%)		
Soluble	75.4 $\pm$ 4.0 (4)	58.0 $\pm$ 2.9 (4)*
Particulate	24.6 $\pm$ 4.0 (4)	42.0 $\pm$ 2.9 (4)*

choline and that this response is mediated by another enzyme or protein that is choline-sensitive, it is interesting to note that after only 1 h of depletion treatment cytidylyltransferase activity had already increased, even though the intracellular concentration of choline was still approx. 0.36 mM [assuming a mean cell volume of 0.55 $\mu\text{l}/10^6$ cells (Fisher *et al.*, 1980) and an even distribution of intracellular choline]. Such a concentration of choline is probably sufficient to support maximal activity of choline kinase, since the K_m of this enzyme for choline is in the range 0.01–0.03 mM (Weinhold *et al.*, 1981; Ulane, 1982). In contrast, the other enzymes that metabolize choline in mammalian cells, i.e. choline dehydrogenase (EC 1.1.99.1) and base-exchange enzymes, have lower affinities for choline (Hatefi & Stiggall, 1976; Saito *et al.*, 1975) and their activities would be expected to be affected when intracellular choline is decreased to 0.36 mM. Type II pneumonocytes from lungs of adult rats contain activities of both choline dehydrogenase (Anceschi *et al.*, 1984) and base-exchange enzyme (M. M. Anceschi & J. E. Bleasdale, unpublished work). Although the involvement of either choline dehydrogenase or base-exchange enzyme in the mechanism of choline-dependent changes in cytidylyltransferase activity remains to be investigated, we speculate that acute changes in base-exchange activity may mediate the effect of choline depletion on cytidylyltransferase activity. Localized changes in lipid composition that result from altered base-exchange activity may signal binding of cytidylyltransferase to the endoplasmic reticulum, and possibly other events, that culminate in increased cytidylyltransferase activity.

Activation of cytidylyltransferase in choline depleted cells resulted in an increased affinity of the enzyme for

CTP but not phosphocholine. Since the concentration of CTP in type II pneumonocytes from adult rat lungs is approx. 2 mM (Post *et al.*, 1984b), a decrease in the K_m for CTP from 1.88 mM to 1.06 mM (Table 3) would be expected to result in increased activity of cytidylyltransferase. It has been observed previously that activation of cytidylyltransferase (i.e. increased V_{max}) is accompanied by increased affinity of the enzyme for CTP in some tissues and cell types but not in others (Pelech & Vance, 1984; Hill *et al.*, 1984). There is evidence that during activation of cytidylyltransferase the mechanism of these changes in kinetic parameters of the enzyme involves a subcellular translocation of latent cytosolic enzyme to the endoplasmic reticulum where it is more active. Latent activity of cytidylyltransferase in cytosolic fractions is expressed when assays are performed in the presence of added lipids (Feldman *et al.*, 1978). It has been proposed that lipids in the assay mixture activate cytosolic cytidylyltransferase by acting as a binding matrix that *in vivo* is provided by the endoplasmic reticulum (Pelech & Vance, 1984; Feldman *et al.*, 1985). Pelech & Vance (1984) suggested advantages of subcellular translocation to the endoplasmic reticulum as a regulatory mechanism.

Although subcellular translocation has only been demonstrated in cells that were broken (either mechanically or by use of detergents) and the molecular mechanism of translocation is unknown, some effectors of the translocation process have been identified. In hepatocytes, the acute responses to cyclic AMP analogues include a reduction in phosphatidylcholine synthesis and an inhibition of cytidylyltransferase activity that is associated with an increased recovery of the enzyme in the cytosol (Pelech *et al.*, 1981, 1983). In contrast, unesterified fatty

acids activate cytidylyltransferase in a variety of cell types apparently by increasing the amount of cytidylyltransferase bound to the endoplasmic reticulum (Pelech & Vance, 1984; Weinhold *et al.*, 1984). Weinhold and co-workers observed that latent cytidylyltransferase in a cytosolic fraction of whole lung can be activated either by aggregation of the enzyme or by translocation of the enzyme to a microsomal fraction (Feldman *et al.*, 1978; Weinhold *et al.*, 1981). The translocation *in vitro* of cytidylyltransferase to microsomes resulted in increased enzymic activity and was found to be promoted by unesterified fatty acids (Weinhold *et al.*, 1984). In the present investigation, choline-dependent subcellular redistribution of cytidylyltransferase was observed. Subcellular translocation of cytidylyltransferase in type II pneumonocytes was still apparent when the conditions of either homogenization or assay were altered. Neither cyclic AMP analogues nor unesterified fatty acids affected greatly the choline-dependent changes in cytidylyltransferase activity. The failure of either oleate or linoleate to greatly influence cytidylyltransferase activity in type II pneumonocytes is unlikely to be due to an inability of the cells to take up these fatty acids. Type II pneumonocytes isolated from the lungs of adult rats were found previously to take up and to metabolize both oleate and linoleate (Stantcheva *et al.*, 1982).

The choline-depleted type II pneumonocyte was employed only as an experimental model for investigating the regulation of cytidylyltransferase activity and it is unlikely that changes in choline availability are involved in the physiological regulation of phosphatidylcholine synthesis (Pelech & Vance, 1984). Pathological changes in phosphatidylcholine synthesis, however, have been observed in the livers of rats that were fed a choline deficient diet (Schneider & Vance, 1978) and in Chinese hamster ovary cells that were maintained in medium in which choline analogues replaced choline (Sleight & Kent, 1983c). The livers of rats that were deprived of dietary choline became depleted of choline and there was a loss of cytidylyltransferase activity from the cytosolic fraction (although without any apparent change in the amount of cytidylyltransferase measured by immunoassay) (Choy *et al.*, 1978). The influence of choline-deprivation on the amount of cytidylyltransferase activity that was particulate was not investigated. In Chinese hamster ovary cells, substitution of choline analogues for choline resulted in an increase in particulate cytidylyltransferase activity and a concomitant decrease in soluble cytidylyltransferase (Sleight & Kent, 1983c).

In previous studies, and in the present investigation, the mechanism of subcellular translocation of cytidylyltransferase was not defined. Evidence from the present investigation, however, is suggestive that increased recovery of cytidylyltransferase in a particulate fraction does not always result in increased activity of cytidylyltransferase. Extracellular surfactant is taken up by isolated type II pneumonocytes and synthesis *de novo* of phosphatidylcholine by these cells is inhibited (Tesan & Bleasdale, 1985). This inhibition was apparently not a consequence of decreased activity of cytidylyltransferase. Although total cytidylyltransferase activity was unaffected, in type II pneumonocytes that were exposed to surfactant there was an increase in particulate cytidylyltransferase activity. The proposition that extracellular surfactant influences the subcellular distribution of

cytidylyltransferase is also supported by the observations of Feldman *et al.* (1980) who found that the recovery of cytidylyltransferase activity in a cytosolic fraction prepared from rat lung is decreased if surfactant is removed from the alveoli prior to processing the lungs. The mechanism by which choline depletion of type II pneumonocytes leads to increased recovery of cytidylyltransferase activity in a particulate fraction and how this differs from movement of cytidylyltransferase to the particulate fraction induced by extracellular surfactant requires further investigation.

The procedure that was devised for depleting type II pneumonocytes of choline is applicable to 3T3-L1 adipocytes (M. Tesan & J. E. Bleasdale, unpublished work) and possibly other cells. The large, rapid and reversible increase in cytidylyltransferase activity that occurs in choline-depleted type II pneumonocytes provides a convenient model for further investigations of the regulation of cytidylyltransferase activity.

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