Lipoprotein deprivation stimulates transcription of the CTP:phosphocholine cytidylyltransferase gene

Alan J. Ryan, Diann M. McCoy, Satya N. Mathur, F. Jeffrey Field, and Rama K. Mallampalli[1]

Department of Internal Medicine and Veterans Affairs Medical Center, University of Iowa College of Medicine, Iowa City, IA 52242

Abstract We examined the effect of lipoprotein deprivation on the expression of the rate-regulatory enzyme involved in phosphatidylcholine (PtdCho) synthesis, phosphocholine cytidylyltransferase (CCT), within an alveolar type II epithelial cell line (MLE-12). Compared with cells exposed to 10% fetal bovine serum (FBS, control), cells cultured with lipoprotein-deficient serum (LPDS) for 72 h had a 150% increase in CCT activity. Stimulation of CCT activity after LPDS exposure was associated with a 2-fold increase in immunoreactive CCT content and a corresponding increase in [^{35}S]methionine incorporation into newly synthesized CCT. LPDS induction of CCT protein was reversible, as it was suppressed to baseline levels by the addition of low density lipoproteins to the culture medium. Northern blotting revealed that LPDS increased CCT mRNA levels 2-fold compared with control. The induction of CCT mRNA by LPDS was not associated with an increase in mRNA half-life. Nuclear run-on assays revealed that LPDS-induced expression of CCT was due, at least in part, to an increase in gene transcription. These studies reveal that lipoprotein deprivation upregulates the activity of a key enzyme involved in the PtdCho biosynthetic pathway. LPDS induction of CCT protein might serve as a novel compensatory mechanism in response to lipid deprivation by increasing cellular transcription of the CCT gene.—Ryan, A. J., D. M. McCoy, S. N. Mathur, F. J. Field, and R. K. Mallampalli. **Lipoprotein deprivation stimulates transcription of the CTP:phosphocholine cytidyltransferase gene. *J. Lipid Res.* 2000.** 41: **1268–1277.**

Supplementary key words phosphatidylcholine • lipoprotein • cytidylyltransferase • choline kinase • cholinephosphotransferase • MLE-12

Phosphatidylcholine (PtdCho) is the major phospholipid in eukaryotic cellular membranes. It also serves as an important component of serum lipoproteins and secretory products such as pulmonary surfactant (1). The biosynthesis of PtdCho is regulated closely within cells by the cytidine diphosphocholine (CDP-choline) pathway. The four steps involved in this pathway involve cellular uptake of choline, choline phosphorylation by choline kinase (CK; EC 2.7.1.32), conversion of cholinephosphate to CDP-choline by cholinephosphate cytidylyltransferase (CCT; EC 2.7.7.15), and, finally, generation of PtdCho by cholinephosphotransferase (CPT; EC 2.7.8.2) (1). Much of the previous work investigating PtdCho synthesis has focused on the regulation and properties of CCT, in part because maneuvers that alter CCT activity might also alter the pool size of PtdCho, thereby impacting a specific cellular function. Thus, changes in membrane phospholipid composition may affect processes such as cellular growth, and membrane protein transport (2, 3); in the lung, modification of total cellular PtdCho content impacts surfactant balance (4).

To date, CCTα protein has been purified to homogeniety, and the cDNAs from several species have been identified and cloned (5–7). The primary structure of CCTα in mammals consists of four functional domains, including an amino-terminal sequence that targets the enzyme for nuclear membrane localization, a catalytic core conferring enzyme activity, a hydrophobic domain that interacts with lipids, and a carboxy-terminal phosphorylation domain (7). Within mammalian cells, CCTα has been localized to the cytosol, the nucleus, and to the endoplasmic reticulum, Golgi, and transport vesicles (8, 9). Two additional isoforms of CCT, CCTβ1, and CCTβ2, have been identified (10, 11). Characterization of the physiologic role of these additional isoforms requires further investigation.

The primary mechanisms by which CCT activity is controlled in cells include lipid regulation, reversible phosphorylation, and regulation at the level of mRNA (8). Of these mechanisms, studies investigating regulation of CCT by lipid factors have been most extensive (12–14). Initial studies demonstrated that enzyme activity can be markedly stimulated by unsaturated fatty acids, diacylglycerol, and anionic phospholipids (12, 15, 16). These stimulatory lipids increase CCT activity, in part, by promoting the

Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; CDP-choline, cytidine diphosphocholine; CK, choline kinase; CPT, cholinephosphotransferase; DSPtdCho, disaturated phosphatidylcholine; FBS, fetal bovine serum; LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; LysoPtdCho, lysophosphatidylcholine; PtdCho, phosphatidylcholine; SREBP, sterol regulatory element-binding proteins; VLDL, very low density lipoproteins.

[1] To whom correspondence should be addressed.

Downloaded from www.jlr.org by guest, on June 14, 2012

ASBMB JOURNAL OF LIPID RESEARCH

translocation of enzyme from the cytosol to the membrane fractions (17). Lipids delivered to cells in the form of low density lipoproteins (LDL) or very low density lipoproteins (VLDL) can serve as potent stimulators of CCT activity (18, 19). Lipids released from these lipoproteins (cholesterol or fatty acids released from triglycerides) may increase CCT activity in vitro. Not all lipids upregulate CCT activity, however, as negative regulatory lipids such as sphingosine and lysophosphatidylcholine (LysoPtdCho) have been identified (20, 21). Further, the degree to which these lipids alter CCT activity and the ability of the enzyme to associate with the membrane environment appear to be regulated by enzyme phosphorylation (22). Independent of lipid activation and phosphorylation, an additional regulatory mechanism for CCT involves changes at the level of enzyme protein and mRNA (23–25). Studies showing changes in CCT mRNA are attributed to changes in mRNA stability, whereas transcriptional regulation of CCT remains largely unknown (23, 24, 26, 27).

Although CCT activity and PtdCho synthesis can be modulated by exogenous lipid factors, overall cellular PtdCho content is generally maintained within a physiologic range presumably because of a balance between the PtdCho biosynthetic and hydrolytic pathways (28). Other membrane lipids, such as cholesterol, are also closely regulated in cells by intricate transcriptional feedback mechanisms (29). For example, cholesterol depletion triggers transcription of HMG-CoA reductase and HMG-CoA synthase genes via activation of specific transcription factors, the endoplasmic reticulum-associated sterol regulatory element-binding proteins (SREBPs) (29). In addition, the cholesterol synthesis inhibitors compactin and 25-hydroxycholesterol not only decrease cholesterol production, but also suppress PtdCho synthesis in cells cultured in lipid-depleted medium (30). Cells defective in the transcriptional activation of genes required for cholesterol synthesis also exhibit reduced PtdCho synthesis (31). Thus, the availability of cholesterol not only regulates de novo cholesterol synthesis, but cholesterol and PtdCho synthesis also appears to be coordinately regulated (32). Collectively, these studies provide some evidence of the existence of a feedback control mechanism that acts to maintain PtdCho homeostasis in response to lipid deprivation.

In the present study, we investigated feedback control mechanisms for PtdCho synthesis in lung alveolar epithelial cells after long-term lipid deprivation. The lung is highly dependent on the availability of lipid substrates that are utilized for the biosynthesis of PtdCho (33). As mentioned above, substrates such as LDL-cholesterol or fatty acids released from VLDL may serve either as activators of CCT or as components that are incorporated into PtdCho directly (33). PtdCho generated within alveolar epithelial cells can be utilized for cell membrane formation, a vital process that leads to an enormous alveolar surface area allowing for effective gas exchange within the lung. To maintain this surface area, alveolar type II epithelial cells must also have the capacity to adequately synthesize and secrete surfactant PtdCho in response to rapid changes in lipid substrate availability. In the process of studying PtdCho synthesis, we observed that CCT activity is markedly upregulated in response to long-term lipoprotein deprivation. Our studies reveal that this increase in CCT activity was due to an increase in transcription of the CCT gene, perhaps serving as a novel compensatory mechanism in the setting of lipid deprivation.

MATERIALS AND METHODS

Materials

Intermediate-size VLDL with S_f 60–100 (from the d < 1.006 g/mL fraction of plasma), LDL, actinomycin D, and Sepharose CL-B4 were from Sigma (St. Louis, MO). Lipoprotein-deficient serum (LPDS, d > 1.21 g/mL) was isolated by ultracentrifugation (34). Carbon-stripped fetal bovine serum (CS-FBS) was prepared as described previously (35). Silica LK5D (0.25 mm × 20 cm × 20 cm) thin-layer chromatography (TLC) plates were purchased from Whatman International (Maidstone, UK). All radiochemicals were purchased from Du Pont-New England Nuclear (Boston, MA). Immunoblotting membranes were obtained from Millipore (Bedford, MA). The ECL Western blotting detection system and GammaBind® Plus Sepharose® were from Amersham Pharmacia Biotech (Piscataway, NJ). Protein A was purchased from RepliGen (Cambridge, MA). Anti-CCTα rabbit polyclonal antiserum was kindly provided by S. Jackowski (10). A rabbit polyclonal antibody to synthetic peptide corresponding to residues 164–176 (36) was generated by Covance Research Products (Richmond, CA). The random primed labeling kit used was Redprime II™ (Amersham, Pharmacia, Buckinghamshire, UK). The MLE-12 cell line was kindly provided by J. Whitsett (Cincinnati, OH) (37). Hite's medium was obtained from the University of Iowa Tissue Culture and Hybridoma Facility (Iowa City, IA).

Cell culture

Cells were maintained in Hite's medium with 10% fetal bovine serum (FBS) at 37°C in an atmosphere containing 5% CO_2. After reaching confluence, the cells were harvested with 0.25% trypsin–0.1% EDTA and plated onto 60-mm tissue culture dishes (~350,000 cells per dish). After incubation overnight, the medium was removed and the cells were rinsed twice with medium containing 0.1% fatty acid-free albumin, and then incubated with Hite's medium containing either 10% FCS, 10% CS-FBS, and 10% LPDS (2 mg/mL), or 10% LPDS in combination with VLDL (20–100 μg/mL), LDL (20–100 μg/mL), or high density lipoprotein (HDL, 20–500 μg/mL) for 72 h. Cells lysates were prepared by brief sonication in buffer A (150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM dithiothreitol [DTT], 0.025% sodium azide, 1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4) at 4°C. Cell cytosolic and microsomal subfractions were prepared by sequential centrifugation (38). Cell nuclei were prepared by nuclease treatment of cellular lysates as described (39).

Phosphatidylcholine and disaturated phosphatidylcholine analysis

Lipids were extracted from equal amounts of cellular protein according to the method of Bligh and Dyer (40). After drying under nitrogen gas, lipids were spotted on silica LK5D plates and resolved in chloroform–methanol–petroleum ether–acetic acid–boric acid 40:20:30:10:1.8 (v/v) (41). Samples that comigrated with PtdCho standard as detected by exposure to iodine vapor were scraped from the silica gel, and quantitatively assayed for phosphorus content (42). To assess PtdCho and disaturated phosphatidycholine (DSPtdCho) synthesis, cells cultured in Hite's medium containing either 10% FBS or 10% LPDS for 72 h

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 14, 2012

were pulsed with 1 μCi of [*methyl*-^{3}H]choline chloride during the final 2 h of incubation. Cellular lipids were extracted, PtdCho was resolved by TLC, and the lipid was reacted with osmium tetroxide before a run in the second dimension (41). Incorporation of label into PtdCho or DSPtdCho was quantitated by scintillation counting.

Enzyme assays

The activity of choline kinase was assayed as described (43). The reaction mixture (0.1-mL volume) contained 100 mM Tris-HCl buffer (pH 8.0), 10 mM magnesium acetate, [^{14}C]choline (specific activity, ~7,100 dpm/nmol), 10 mM ATP, and 50–100 μL of cell sample. After a 1-h incubation at 37°C, the reaction was terminated with 0.02 mL of cold 55% tricloroacetic acid. Twenty-microliter aliquots of the mixture were spotted on Whatman 3MM paper and choline metabolites were resolved by paper chromatography with ethanol–concentrated ammonia–isopropanol 65:35:20 (v/v/v). The spots that comigrated with the radiolabeled standard, cholinephosphate, were cut and used for scintillation counting.

The activity of CT was determined by measuring the rate of incorporation of phospho[*methyl*-^{14}C]choline into CDP-choline, using a charcoal extraction method (19). Assays were performed with and without the inclusion of a lipid activator, PtdCho–oleic acid, in the reaction mixture.

The activity of cholinephosphotransferase was assayed as described (44). Each reaction mixture contained 50 mM Tris-HCl buffer (pH 8.2), Tween 20 (0.1 mg/mL), 1 mM 1,2-dioleoylglycerol, 0.8 mM phosphatidylglycerol, 0.5 mM [^{14}C]CDP-choline (specific activity, 1,110 dpm/nmol), 5 mM dithiothreitol, 5 mM EDTA, 10 mM $MgCl_2$, and 50–100 μL of sample. The lipid substrate was prepared by combining appropriate amounts of 1,2-dioleoylglycerol (1 mM) and phosphatidylglycerol (0.8 mM) in a test tube, drying the mixture under nitrogen gas, and briefly sonicating it before addition to the assay mixture to achieve the final desired concentration. The reaction proceeded for 1 h at 37°C, and terminated with 4 mL of methanol–chloroform–water 2:1:7 (v/v/v). The PtdCho product was resolved by TLC with chloroform–methanol–ammonium hydroxide as solvent. The remainder of the assay was performed exactly as described (44).

Immunoblot analysis

For immunoblot analysis, equal amounts of protein from cell homogenates and from cytosolic, microsomal, and nuclear extracts were used. Each sample was adjusted to give a final solution of 60 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromphenol blue, and 5% 2-mercaptoethanol; heated at 100°C for 5 min; electrophoresed through a 10% SDS-polyacrylamide gel; and transferred to a polyvinylidene difluoride membrane. All studies were performed to analyze the CCTα isoform as this was the only isoform detected in MLE cells (data not shown). Probing for CCT was performed with the ECL Western blotting detection system. The dilution factor for anti-CCT antibody was 1:1,000.

Immunoprecipitation of CCT

CCT synthesis was assessed in control and LPDS-treated cells by labeling cells with [^{35}S]methionine (60 μCi/mL in methionine-deficient medium) during the last 4 h of culture at 37°C. After labeling, cells were scraped in radioimmunoprecipitation assay (RIPA) buffer (10 mM Na_2HPO_4, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothrietol, 1 mM PMSF, 20 μM leupeptin, 1% Triton X-100, 0.1% SDS, 20 mM L-methionine, and 2 mM L-cysteine; pH 7.4), sonicated briefly, and centrifuged at 15,000 *g* for 60 sec, and the supernatants precleared by treatment for 2 h at 4°C with Sepharose CL-4B and preimmune rabbit serum. Cleared supernatants, containing equal amounts of protein (500 μg), were incubated overnight at 4°C with 1 μg of rabbit anti-CCT antibody, which was previously bound to GammaBind® Plus Sepharose®. The next morning, the immunoprecipitates were washed with lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5 mM EGTA, 1 mM PMSF, 50 mM NaF, 1 mM $NaVO_4$, 1 mM PMSF, and 1 μM aprotinin; pH 7.6) and the pellets placed in SDS protein sample buffer, and heated to 95°C for 5 min. Soluble proteins were separated by 10% SDS-PAGE, and the gels were stained with Coomassie Blue, destained, and then dried for both autoradiography and liquid scintillation counting of excised 42K protein bands.

Detection of CCT mRNA

Total cellular RNA was isolated from cells by cesium chloride gradient centrifugation after lysis with guanidine thiocyanate (45). Total RNA (30 μg) containing ethidium bromide was separated electrophoretically on a 1% agarose gel containing 2.2 M formaldehyde with a circulating running buffer of 20 mM morpholinepropanesulfonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde. RNA was capillary blotted to a nitrocellulose membrane with a Turbo-blot apparatus, and hybridized at 42°C with a ^{32}P-labeled probe (7.5 × 10^6 cpm/15 mL), using standard Northern blot hybridization protocols (10). Radiolabeled probes for CCT were prepared by random prime labeling of cDNA. The probe for CCTα consisted of a 1.1-kb *Hin*dIII–*Bam*HI fragment of the rodent CCT cDNA inserted in pcDNA3. The blot was washed four times for 1 min at 22°C, and then at 42°C for 10 min, after hybridization with the CCT probe.

Nuclear run-on assay

Cells were cultured in FBS or LPDS for various times and rinsed in PBS, lysed (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40 [NP-40]), and centrifuged at 1,500 rpm for 5 min at 4°C, and the nuclear pellet was resuspended in glycerol buffer before storage at −70°C. Nuclear transcription was assessed by incubating nuclei in reaction buffer (10 mM Tris [pH 8.0], 0.3 M KCl, 5 mM $MgCl_2$) with 1 mM nucleotides (ATP, CTP, GTP) and 10 μL of [γ-^{32}P]uridine triphosphate (400 μCi/sample), and each sample was placed in an orbital shaker for 30 min at 30°C. After terminating the reaction with ribonuclease-free DNase I (1 mg/mL) and proteinase K (20 mg/mL), RNA was extracted with phenol–chloroform–isoamyl alcohol 25:24:1 (v/v/v). The RNA solution was placed in 10% trichloroacetic acid (TCA)–60 mM sodium pyrophosphate with 10 μL of tRNA (10 mg/mL) and cooled on ice for 30 min. The precipitate was filtered (0.45-μm pore size filter) and radiolabeled RNA was eluted. After precipitating and centrifuging for 30 min at 9,000 rpm, the pellet was resuspended in 1 mL containing TES [10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.4)], 10 mM EDTA, and 0.2% SDS. Samples were counted and equalized by counts per minute of ^{32}P-labeled RNA per mL and equalized samples hybridized at 65°C for 36 h to CT cDNA previously bound to nitrocellulose. Filters were washed 6 times in 1× saline–$NaPO_4$–EDTA (SSPE) with 1% SDS at 65°C, once in 0.1× SSPE with 1% SDS at 65°C, once in 2× SSPE with RNase A (1.25 mg/mL) at 37°C, and 3 times in 2× SSPE at room temperature. Filters were dried and exposed to autoradiographic film for detection of CT transcripts.

RESULTS

Phosphatidylcholine and disaturated phosphatidylcholine analysis

Incubation of MLE cells with LPDS significantly decreased the mass of PtdCho in MLE cells by nearly 30%

Downloaded from www.jlr.org by guest, on June 14, 2012

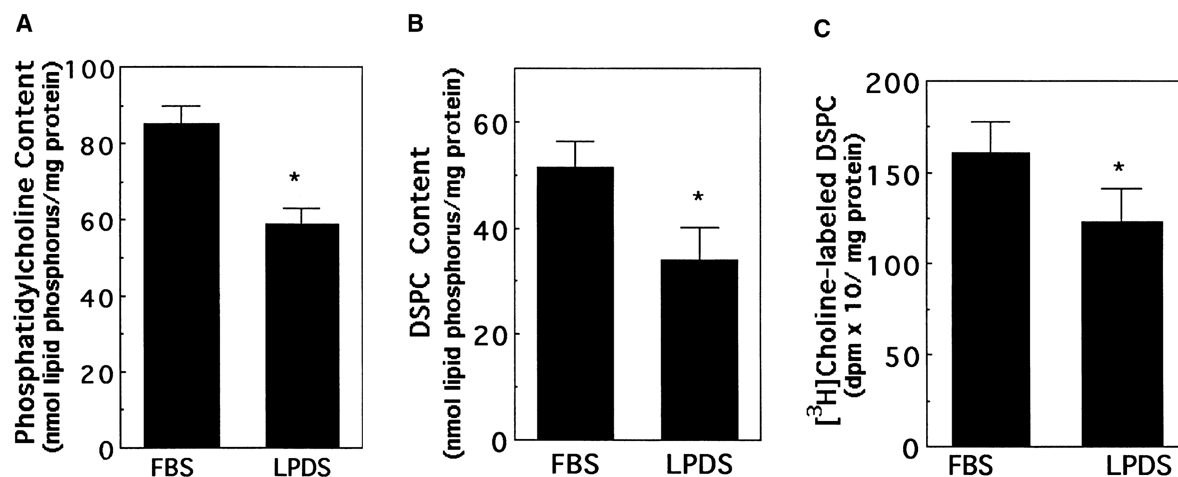

Fig. 1. Phosphatidylcholine (PtdCho), disaturated PtdCho (DSPtdCho), and choline incorporation into DSPtdCho in MLE cells grown in lipoprotein-deficient serum (LPDS). Cells were incubated for 72 h in Hite's medium supplemented with either 10% FBS or 10% LPDS. The mass of PtdCho (A) and DSPtdCho (B) was determined after lipid extraction from cell lysates, separation by TLC, and quantification by the phosphorus assay. (C) Cells were pulsed with 1 μCi of [^{3}H]choline for the final 2 h of incubation, and the radioactivity in cellular [^{3}H]DSPtdCho was determined. Values are means ± SEM from 3 independent experiments. * $P < 0.05$ LPDS versus FBS.

after 72 h of exposure (**Fig. 1A**). The magnitude of these effects is important because, as in primary alveolar type II epithelial cells, PtdCho accounts for nearly 60% of the total phospholipid in MLE cells (46). The major species of PtdCho within alveolar type II epithelial cells and MLE cells is DSPtdCho, an integral component of pulmonary surfactant that exhibits surface tension-lowering properties (47). DSPtdCho mass in MLE cells was also reduced by incubation with LPDS (Fig. 1B). We next examined whether the decrease in PtdCho mass was secondary to a decrease in PtdCho synthesis by measuring the incorporation of [*methyl*-^{3}H]choline into PtdCho and DSPtdCho. After 72 h of LPDS exposure, choline incorporation into PtdCho decreased by 21% relative to cells exposed to FBS. Choline incorporation into DSPtdCho was also reduced by approximately 24% compared with FBS-exposed cells (Fig. 1C). These results suggest that the overall decrease in PtdCho synthesis after LPDS treatment is reflected by a reduction in the amount of surfactant PC.

Enzymes assays

Lipoprotein deprivation significantly altered the activity of enzymes of the CDP-choline pathway, the principal pathway for the de novo synthesis of PtdCho (**Fig. 2**). Compared with cells cultured in FBS, culture of cells in LPDS for 72 h reduced the activity of choline kinase, the first committed enzyme of the CDP-choline pathway, by more than 40% (Fig. 2A). Further, an even greater reduction was observed in the activity of the terminal enzyme of the pathway, cholinephosphotransferase, which decreased by 64% in cells cultured with LPDS compared with FBS (Fig. 2). In contrast, the activity of CCT, the rate-regulatory

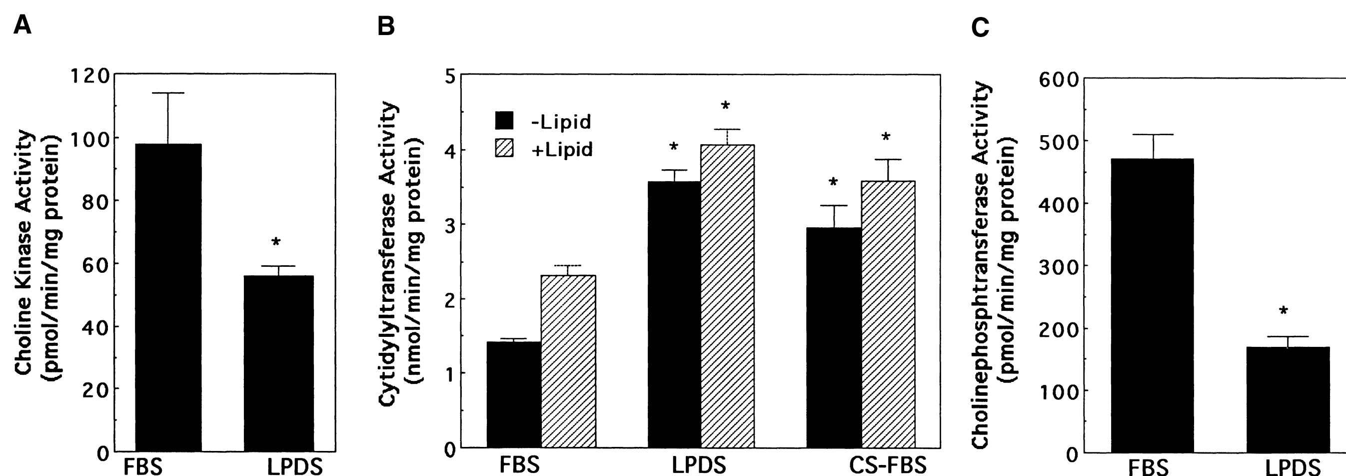

Fig. 2. Effect of LPDS on (A) choline kinase, (B) cytidylyltransferase, and (C) cholinephosphotransferase activities in MLE cell lysates. MLE cells were incubated for 72 h in Hite's medium containing 10% FBS or 10% LPDS. Cells were harvested and assayed for enzymes required for PtdCho biosynthesis. Assays for cytidylyltransferase activity were conducted in the presence (+Lipid) or absence (−Lipid) of PtdCho-oleic acid emulsions. Results are means ± SEM from 3 independent experiments. * $P < 0.05$ LPDS versus FBS.

Downloaded from www.jlr.org by guest, on June 14, 2012

enzyme within the CDP-choline pathway, was markedly enhanced in cells cultured with LPDS. LPDS stimulated CCT activity by 150% compared with cells cultured in medium containing 10% FBS (Fig. 2B). When cells were exposed to CS-FBS, CCT activity also increased 2-fold above control values. These increases in CCT activity after LPDS or CS-FBS treatment were observed whether or not the assay was performed with the inclusion of the lipid activator, PtdCho–oleic acid, in the reaction mixture. Collectively, these results indicate that lipoprotein deprivation differentially regulates the activity of enzymes required for PtdCho biosynthesis. LPDS markedly upregulates CCT activity and yet inhibits choline kinase and cholinephosphotransferase activities, resulting in an overall decrease in PtdCho biosynthesis within alveolar type II epithelial cells.

Immunoblot analysis

The observation that lipoprotein deprivation stimulated CCT activity in mammalian cells, using a functional assay, is remarkable because lipoprotein loading has also been shown to potently increase CCT activity (18, 19). Prior studies have shown that exposure of cells to LDL and VLDL can increase CCT activity by posttranslational mechanisms (18, 19). However, the kinetics of CCT stimulation after lipoprotein loading and lipid deprivation differ in that maximal effects of LPDS exposure on enzyme

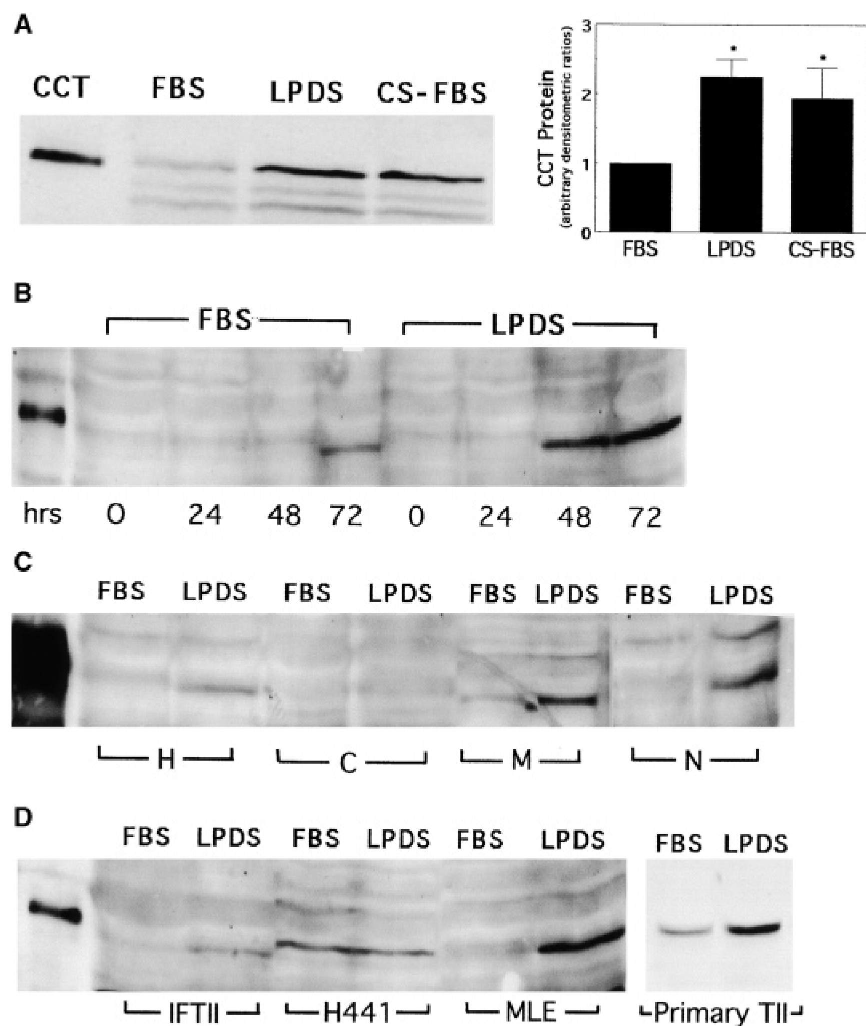

Fig. 3. Immunoblot analysis of CCT protein in MLE cells. (A) Cells were grown for 72 h in Hite's medium containing 10% FBS, 10% LPDS or 10% CS-FBS (carbon-stripped fetal bovine serum). Cell lysates (300 μg of protein) were separated by SDS-10% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with an anti-CCT rabbit polyclonal antibody. The far left lane of all immunoblots shows purified rat liver CCT. The top right bar graph reveals CCT protein levels quantified by densitometric analysis of autoradiograms with the FBS controls arbitrarily assigned a value of 1 (means ± SEM, n = 4). * $P < 0.05$ LPDS or CS-FBS versus FBS. (B) Cells were incubated in Hite's medium containing 10% FBS or 10% LPDS for periods ranging from 0 to 72 h. Cells were then harvested and analyzed for CCT content. (C) Cellular fractions (homogenate [H], cytosol [C], microsomal [M], nuclear [N]) were analyzed for CCT protein after cells were incubated for 72 h in Hite's medium containing 10% FBS or 10% LPDS. (D) Immunoblots for expression of CCT protein in rat immortalized fetal type II cells (IFTII), a human adenocarcinoma cell line (H441), MLE cells, and primary alveolar type II epithelial cells (Primary TII). Cells were grown for 72 h in Hite's medium containing 10% FBS or 10% LPDS.

Downloaded from www.jlr.org by guest, on June 14, 2012

activity are seen only after prolonged culture. Thus, we hypothesized that chronic lipoprotein deprivation increases CCT activity as a compensatory response involving a pretranslational mechanism. Immunoblot analysis was performed to determine if LPDS stimulation of CCT activity was secondary to an increase in the amount of enzyme. Culture of MLE cells with either LPDS or CS-FCS increased the levels of immunoreactive CCT 2-fold above control levels (**Fig. 3A**). An increase in steady state CCT protein was first detected in cells after 48 h of LPDS exposure and persisted at least to 72 h (Fig. 3B). The physiologically active forms of CCT are predominantly detected within the microsomal and nuclear subfractions of cells (8). Consistent with its effects in whole cells, immunoblot studies revealed that LPDS increased the amount of microsomal and nuclear-associated enzyme (Fig. 3C). However, only low levels of CCT protein were detected in the soluble fraction of MLE cells; these levels were not affected by LPDS treatment. To ascertain whether LPDS effects on CCT protein were specific to MLE cells, additional analyses were performed with a rat immortalized fetal alveolar pretype II epithelial cell line (48), primary alveolar type II epithelial cells, and a human adenocarcinoma cell line (H441) (49). Similar to results in MLE cells, incubation of the immortalized alveolar pretype II epithelial cell line with LPDS led to induction of CCTα protein (Fig. 3D). Studies conducted in primary alveolar type II epithelium also showed increases in immunoreactive CCT after 72 h of exposure to LPDS (Fig. 3D). In contrast, LPDS induction of CCT was not observed in H441 cells. These results indicate that LPDS stimulates CCT activity in alveolar type II epithelial cells by increasing the amount of CCT mass.

To determine if specific lipoproteins could alter CCT protein expression, MLE cells were cultured in LPDS in the presence of either HDL, VLDL, or LDL. Levels of CCT protein were then determined by immunoblotting (**Fig. 4**). LPDS induction of CCT protein was reduced to baseline (control) levels after inclusion of HDL (Fig. 4A), VLDL (Fig. 4B), or LDL (Fig. 4C) in the culture medium. The addition of LDL at 100 μg/mL suppressed CCT protein expression to almost undetectable levels (Fig. 4C). Compared with HDL or VLDL, exposure of cells to LDL appeared to be more potent in reducing CCT protein expression. These results suggest that CCT expression in cells is reversibly regulated by specific lipoproteins.

De novo synthesis of CCT

We next examined whether LPDS alters de novo synthesis of CCT. For synthesis studies, cells were pulsed with [^{35}S]methionine and CCT was then immunoprecipitated with isoform-specific antibody. Pulse times between 15 and 60 min were within the linear range with regard to incorporation of [^{35}S]methionine into total TCA-precipitable protein and into precipitable CCT in cells cultured with FBS (**Fig. 5A**). Similar rates for incorporation of [^{35}S] methionine into total TCA-precipitable protein were noted for cells pulsed for 15 to 60 min with either FBS or LPDS (Fig. 5A). However, after long-term (48–72 h) culture, greater levels of immunoprecipitatable radiolabeled CCT were observed in cells cultured with LPDS compared with cells exposed to FBS (Fig. 5B). Thus, these results indicate that lipoprotein deprivation does not result in an overall increase in total protein synthesis, but selectively increases the amount of newly synthesized CCT in MLE cells.

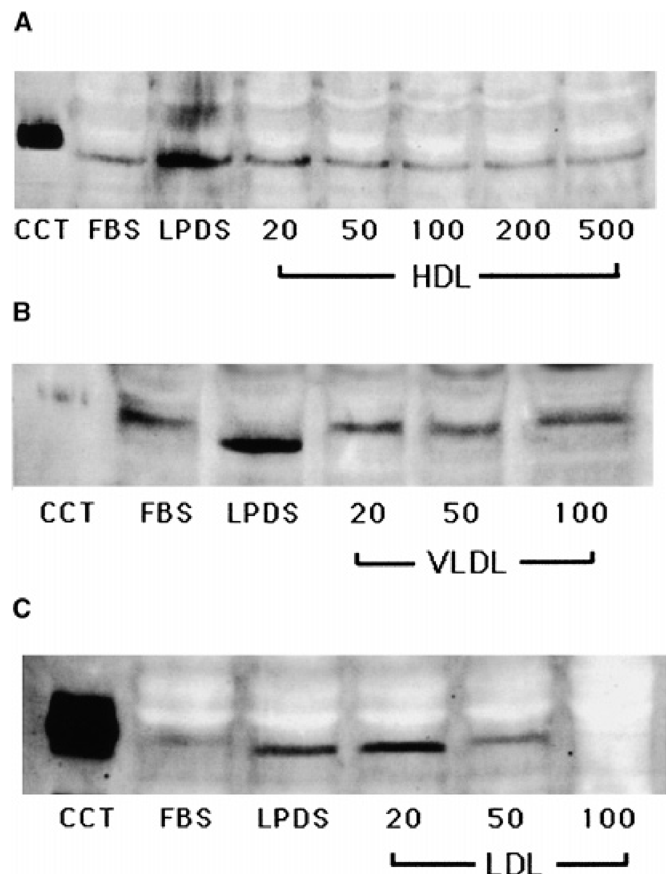

Fig. 4. Effect of lipoprotein supplementation on CCT protein in MLE cells. Cells were grown for 72 h in Hite's medium containing either 10% FBS, 10% LPDS, or 10% LPDS supplemented with individual lipoproteins. Cells were harvested and immunoblot analysis was conducted to detect CCT protein. Cells supplemented with (A) HDL (20 to 500 μg of protein per mL), (B) VLDL (20 to 100 μg of protein per mL), and (C) LDL (20 to 100 μg of protein per mL).

Detection of CCTα mRNA

We next conducted studies to determine if LPDS increased CCT protein by increasing the amount of CCT mRNA. By using probes prepared from 1.3 kb of rodent CCTα cDNA, Northern blotting was performed on total RNA isolated from control cells and cells exposed to LPDS for various times (**Fig. 6A**). As shown in Fig. 6, LPDS exposure modestly increased expression of the 5.0-kb CCTα transcript during 2 to 8 h of incubation and increased CCTα mRNA by more than 2-fold after 48 h. Northern analysis of poly(A) mRNA also confirmed that LPDS increased the levels of the CCT transcript (Fig. 6C). These results suggest a pretranslational mechanism for LPDS induction of CCT protein in alveolar type II epithelial cells.

Downloaded from www.jlr.org by guest, on June 14, 2012
ASBMB JOURNAL OF LIPID RESEARCH

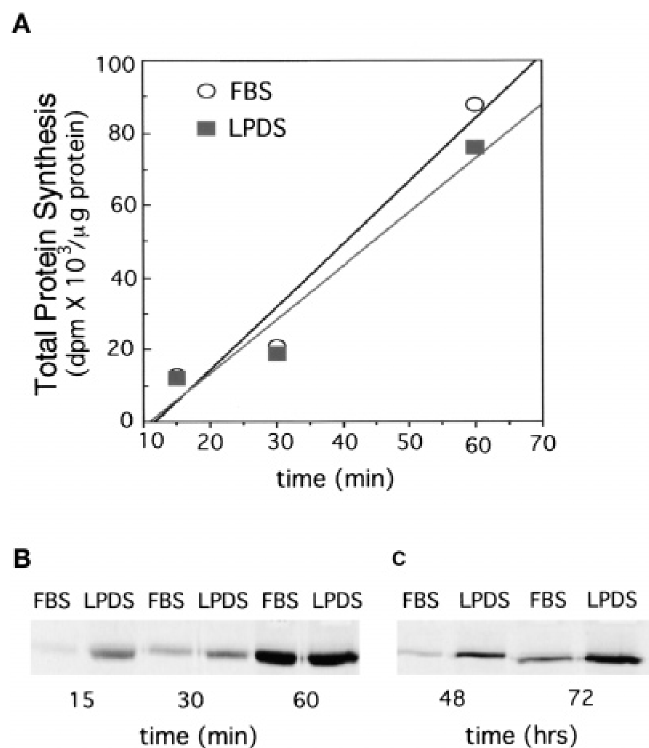

Fig. 5. The effect of LPDS on de novo synthesis of CCT protein in MLE cells. (A) Cells were grown for 72 h in Hite's medium containing 10% FBS or 10% LPDS, and then pulsed with [^{35}S]methionine (60 µCi/mL in methionine-deficient medium) for 15, 30, or 60 min. (A) Total protein synthesis determined from radioactivity found in 1 µg of TCA-precipitable protein. (B) CCT was immunoprecipitated from cell lysates (500 µg of protein) with 1 µg of anti-CCT rabbit polyclonal antibody. Immunoprecipitates were separated by SDS-10% polyacrylamide gel electrophoresis (PAGE) and gels dried for autoradiography. (C) Cells were grown for 48 or 72 h as described above and pulsed with [^{35}S]methionine (60 µCi/mL). CCT immunoprecipitates were subjected to SDS-PAGE and autoradiography.

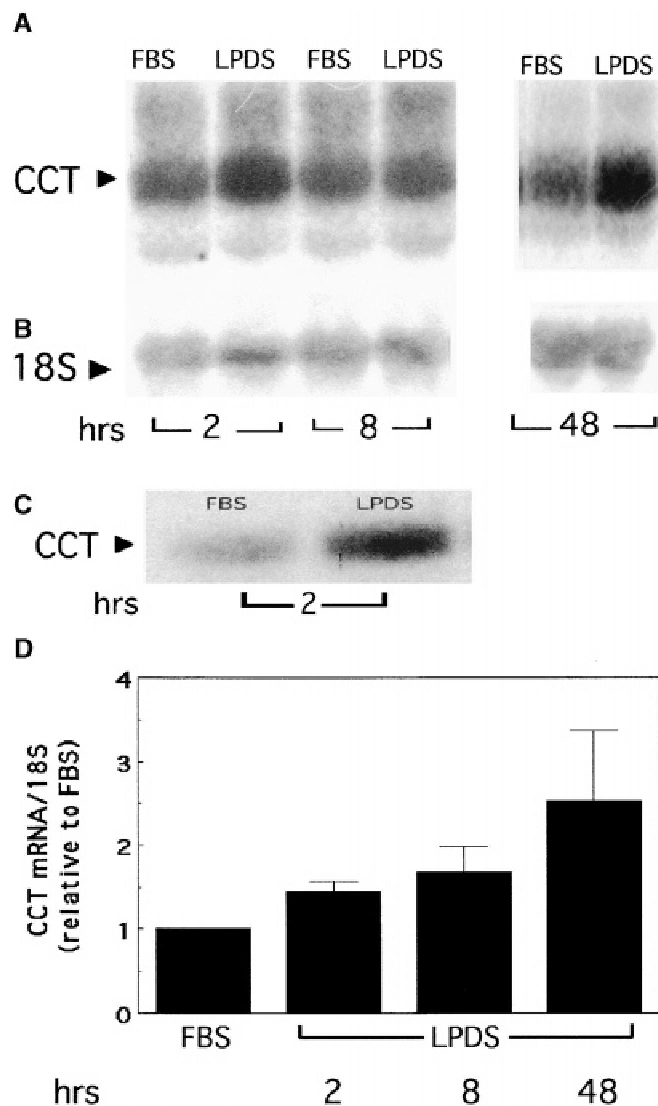

Fig. 6. Effect of LPDS on CCT mRNA in MLE cells. Cells were grown in Hite's medium with 10% FBS for 72 h before changing to fresh medium containing 10% FBS or 10% LPDS for 2 to 48 h. At the indicated times, total cellular RNA (50 µg) was isolated and the amount of (A) CCT mRNA or (B) 18S RNA was determined by Northern analysis as described in the text. Representative autoradiograms are shown. (C) CCT mRNA levels in FBS- and LPDS-exposed cells as determined by Northern analysis with 10 µg of poly(A)-rich RNA. (D) Densitometric analysis of CCT mRNA in cells cultured in medium containing 10% FBS or 10% LPDS for 2 to 48 h. The CCT mRNA/18S ratios for FBS were arbitrarily assigned a value of 1. The densitometric data are from 3 independent experiments. Values are shown as means ± SEM.

We next conducted studies to determine if LPDS increased steady state CCT mRNA by increasing the mRNA half-life (**Fig. 7**). Cells were cultured in Hite's medium containing 10% FBS or Hite's medium containing LPDS for various times in the presence of actinomycin D (5 µg/mL). After culture, cells were harvested for total RNA and levels of CCT mRNA were quantitated as described above. Levels of CCT mRNA decreased gradually in cells treated with either FBS or LPDS, and no significant differences in mRNA half-life were observed (FBS $t_{1/2} = 8.4 \pm 2.8$ h vs. LPDS $t_{1/2} = 6.9 \pm 1.5$ h, n = 4). These results suggest that lipoprotein deprivation increases CCT protein by stimulating gene transcription.

Nuclear run-on assay

To examine whether LPDS altered transcription of the CCT gene, we performed nuclear run-on assays after culturing cells in FBS or LPDS for various times. Compared with cells cultured in FBS, LPDS increased CCT gene transcription in MLE cells after 6 and 48 h of exposure (**Fig. 8**). These results indicate that lipoprotein deprivation increases CCT mRNA, at least in part, at the level of CCT transcription within type II cells.

DISCUSSION

The present results indicate that lipoprotein deprivation markedly increased the activity of the rate-regulatory enzyme, CCT. Unlike many studies showing activation of CCT by posttranslational mechanisms, we provide evi-

Downloaded from www.jlr.org by guest, on June 14, 2012
ASBMB JOURNAL OF LIPID RESEARCH JLR

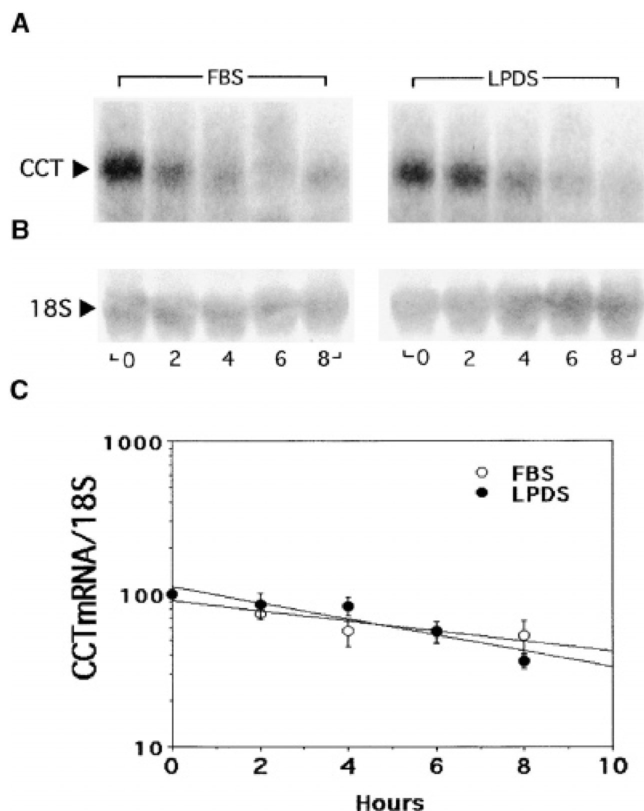

Fig. 7. The effect of LPDS on turnover of CCT mRNA in MLE cells. Cells were grown in Hite's medium containing 10% FBS for 72 h before changing to fresh medium with either 10% FBS or 10% LPDS containing actinomycin D (5 μg/mL) for 0 to 8 h. Northern analysis of total cellular RNA (50 μg) was performed to determine amounts of (A) CCT mRNA and (B) 18S RNA. (C) Densitometric analysis of autoradiograms shows CCT mRNA/18S OD ratios for cells grown in medium containing actinomycin D (5 μg/mL) with either 10% FBS or 10% LPDS. Values are expressed on a logarithmic scale and represent means ± SEM of 3 independent experiments.

dence that increases in CCT activity are most likely explained by increases in CCT gene transcription. The results of these studies might serve as a springboard to investigate sterol-dependent regulatory elements within the CCT gene that might function as part of an overall feedback response mechanism for PtdCho synthesis in the setting of chronic lipid deprivation.

The current results revealed that under conditions of long-term culture in lipoprotein-deficient medium, alveolar cells show reductions in PtdCho mass and an impaired ability to synthesize surfactant PtdCho. Lipoprotein deprivation significantly reduced the activities of choline kinase and cholinephosphotransferase, which together with reduced lipid substrate availability likely account for the decrease in PtdCho mass. There is limited information regarding the regulatory mechanisms for these enzymes. The reduction in choline kinase activity after LPDS treatment might be secondary to a reduction in substrate availability, either choline or ATP levels, for example, or reduced levels of enzyme mass. The purification of this enzyme should allow for generation of antibodies useful for determining enzyme content after LPDS treatment (50).

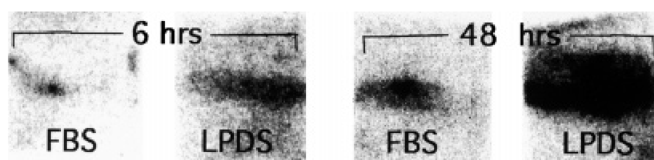

Fig. 8. The effect of of LPDS on CCT gene transcription in MLE cells. Cells were grown in Hite's medium containing 10% FBS for 72 h before changing to fresh medium with either 10% FBS or 10% LPDS for 6 or 48 h. After culture, cells were harvested and processed for CCT transcriptional activity as described in Materials and Methods. The data are representative of two independent experiments.

On the other hand, the mechanisms for LPDS-associated decrease in cholinephosphotransferase activity might be mediated by an increase in cellular argininosuccinate, as this product accumulates in the fasting state and is known to inhibit enzyme activity (51). In addition, it appears that polyunsaturated fatty acids stimulate cholinephosphotransferase activity in vivo (52). Therefore LPDS might decrease cellular levels of polyunsaturated fatty acids that are needed for optimal enzyme activation.

The fact that cells exposed to LPDS expressed a 3-fold increase in CCT activity concomitant with an increase in CCT protein suggests that these responses might represent a novel compensatory mechanism for alveolar epithelial cells exposed to prolonged periods of lipid deprivation. Ultracentrifugation and carbon-stripping of fetal bovine serum were performed to remove serum lipids. Both approaches resulted in modified serum preparations that when added to cell cultures effectively increased enzyme mass and stimulated protein synthesis as demonstrated by increased incorporation of [^{35}S]methionine into CCT. The effect of LPDS on increasing CCT mass was not restricted to MLE cells, but was also observed in a fetal type II cell line and in primary rat alveolar type II epithelial cells. Thus, both fetal and adult type II cells are sensitive to lipid deprivation, lending further support to the idea that these responses might be physiologically important in vivo.

Current understanding suggests that mechanisms for CCT activation include lipid regulation, membrane translocation, and reversible phosphorylation. When cells are chronically exposed to conditions of lipid deprivation, the availability of lipid activators should be reduced and thus regulation by stimulatory lipids seems unlikely. We observed a consistent increase in CCT mass in total cell lysates and in microsomal and nuclear subfractions of the cells; little protein was found in the cytosolic fraction. Thus, we cannot rule out the possibility that our antibody did not recognize cytosolic CCT and that enhanced activity was partly due to a physiologic shift from the cytosolic to the membrane fraction. In addition, multiple bands were observed on our immunoblots, suggesting the presence of both phosphorylated and dephosphorylated forms of CCT in cells exposed to LPDS. However, most evidence shows that lipoprotein loading, rather than lipid deprivation, promotes dephosphorylation of CCT and enhances enzyme activity in cells (18). Our observation that

Downloaded from www.jlr.org by guest, on June 14, 2012
ASBMB JOURNAL OF LIPID RESEARCH

a 3-fold increase in CCT activity was associated with a 2-fold increase in both enzyme mass and mRNA argues against dephosphorylation or membrane translocation as the primary mechanism for CCT activation after LPDS treatment. Further, because the magnitude of the increase in enzyme activity exceeded the increase in CCT mass after LPDS exposure, we conclude that overall specific CCT activity was relatively increased in these studies.

In contrast to prior work, an increase in transcription of the CCT gene appears to provide the best explanation for our finding that CCT protein and mRNA were increased in cells chronically exposed to lipid deprivation. The magnitude of increase in CCT transcript levels after lipid deprivation was similar to increases in CCT mRNA previously observed after partial hepatectomy (24). Other studies showing induction of CCT mRNA during lung development and in response to growth factors appear to be describing the result of an increase in CCT mRNA stability (23–25). However, our observation that CCT mRNA levels were increased after lipid deprivation cannot be explained by enhanced mRNA stability as CCT mRNA half-life was found to be similar for cells cultured in either lipid-replete or lipid-deprived medium when studied in the presence of a transcriptional inhibitor. Nuclear run-on assays provided confirmation that lipoprotein deprivation increased transcription of the CCT gene (Fig. 8). In our preliminary studies, transient transfections of MLE cells with a reporter construct containing the proximal 5′ flanking region of the mouse CCT promoter exhibit an increase in reporter activity in response to lipoprotein deprivation (R. K. Mallampalli et al., unpublished observations).

Several genes involved in lipid metabolism are known to be regulated in a transcriptional manner (29, 53). Some of these genes are induced in response to lipid deprivation (29). Chief among these include the HMG-CoA reductase and fatty acid synthase genes, which are both upregulated via activation of the transcription factors SREBP-1 and SREBP-2 in response to cholesterol deprivation (29, 53). Studies have identified several regulatory elements involved in activation and repression of CCT transcription (27, 54). SREBP consensus elements are found within the CCT gene, and the CCT promoter region is thought to contain binding elements for other transcriptional proteins, such as Sp1, Ap1, and C/EBP (27). Future studies investigating the role of SREBP in upregulating the CCT gene after sterol deprivation are attractive, and if demonstrated, would provide a unifying control feedback mechanism for transcriptional regulation of genes involved in cholesterol, fatty acid, and PtdCho synthesis. However, the recognition sites that interact with the C/EBP family of transcription factors also deserve further study because these proteins appear to be involved in surfactant metabolism (55).

R.K.M. is an Established Investigator of the American Heart Association. This study was supported by a Merit Review Award from the Office of Research and Development, Department of Veteran's Affairs, NIH Grant RO1HL55584, and a Career Investigator Award from the American Lung Association (to R.K.M.).

Manuscript received 28 September 1999 and in revised form 21 March 2000.

REFERENCES

1. Kent, C. 1995. Eukaryotic phospholipid biosynthesis. *Annu. Rev. Biochem.* **64:** 315–343.
2. Cornell, R., G. L. Grove, G. H. Rothblat, and A. F. Horwitz. 1977. Lipid requirement for cell cycling. The effect of selective inhibition of lipid synthesis. *Exp. Cell Res.* **109:** 299–307.
3. Grunze, M., B. Forst, and B. Deuticke. 1980. Dual effect of membrane cholesterol on simple and mediated transport processes in human erythrocytes. *Biochim. Biophys. Acta.* **600:** 860–869.
4. Mallampalli, R. K., S. N. Mathur, L. J. Warnock, R. G. Salome, G. W. Hunninghake, and F. J. Field. 1996. Betamethasone modulation of sphingomyelin hydrolysis up-regulates CTP:cholinephosphate cytidylyltransferase activity in adult rat lung. *Biochem. J.* **318:** 333–341.
5. Kalmar, G. B., R. J. Kay, A. Lachance, R. Aebersold, and R. B. Cornell. 1990. Cloning and expression of rat liver CTP: phosphocholine cytidylyltransferase: an amphipathic protein that controls phosphatidylcholine synthesis. *Proc. Natl. Acad. Sci. USA.* **87:** 6029–6033.
6. Rutherford, M. S., C. O. Rock, N. A. Jenkins, D. J. Gilbert, T. G. Tessner, N. G. Copeland, and S. Jackowski. 1993. The gene for murine CTP:phosphocholine cytidylyltransferase (Ctpct) is located on mouse chromosome 16. *Genomics.* **18:** 698–701.
7. Kalmar, G. B., R. J. Kay, A. C. LaChance, and R. B. Cornell. 1994. Primary structure and expression of a human CTP:phosphocholine cytidylyltransferase. *Biochim. Biophys. Acta.* **1219:** 328–334.
8. Kent, C. 1997. CTP:phosphocholine cytidylyltransferase. *Biochim. Biophys. Acta.* **1348:** 79–90.
9. Slomiany, A., E. Grzelinska, C. Kasinathan, K. Yamaki, D. Palecz, B. A. Slomiany, and B. L. Slomiany. 1992. Biogenesis of endoplasmic reticulum transport vesicles transferring gastric apomucin from ER to Golgi. *Exp. Cell Res.* **201:** 321–329.
10. Lykidis, A., K. G. Murti, and S. Jackowski. 1998. Cloning and characterization of a second human CTP:phosphocholine cytidylyltransferase. *J. Biol. Chem.* **273:** 14022–14029. [Published erratum appears in *J. Biol. Chem.* 1998; **273:** 19357.]
11. Lykidis, A., I. Baburina, and S. Jackowski. 1999. Distribution of CTP:phosphocholine cytidylyltransferase (CCT) isoforms—identification of a new CCT beta splice variant. *J. Biol. Chem.* **274:** 26992–27001.
12. Weinhold, P. A., M. E. Rounsifer, S. E. Williams, P. G. Brubaker, and D. A. Feldman. 1984. CTP:phosphorylcholine cytidylyltransferase in rat lung. The effect of free fatty acids on the translocation of activity between microsomes and cytosol. *J. Biol. Chem.* **259:** 10315–10321.
13. Feldman, D. A., M. E. Rounsifer, and P. A. Weinhold. 1985. The stimulation and binding of CTP: phosphorylcholine cytidylyltransferase by phosphatidylcholine-oleic acid vesicles. *Biochim. Biophys. Acta.* **833:** 429–437.
14. Cornell, R. B. 1991. Regulation of CTP:phosphocholine cytidylyltransferase by lipids. 2. Surface curvature, acyl chain length, and lipid-phase dependence for activation. *Biochemistry.* **30:** 5881–5888. [Published erratum appears in *Biochemistry.* 1991; **30:** 9810.]
15. Cornell, R., and D. E. Vance. 1987. Translocation of CTP:phosphocholine cytidylyltransferase from cytosol to membranes in HeLa cells: stimulation by fatty acid, fatty alcohol, mono- and diacylglycerol. *Biochim. Biophys. Acta.* **919:** 26–36.
16. Feldman, D. A., C. R. Kovac, P. L. Dranginis, and P. A. Weinhold. 1978. The role of phosphatidylglycerol in the activation of CTP:phosphocholine cytidylyltransferase from rat lung. *J. Biol. Chem.* **253:** 4980–4986.
17. Pelech, S. L., P. H. Pritchard, D. N. Brindley, and D. E. Vance. 1983. Fatty acids promote translocation of CTP:phosphocholine cytidylyltransferase to the endoplasmic reticulum and stimulate rat hepatic phosphatidylcholine synthesis. *J. Biol. Chem.* **258:** 6782–6788.
18. Shiratori, Y., M. Houweling, X. Zha, and I. Tabas. 1995. Stimulation of CTP:phosphocholine cytidylyltransferase by free cholesterol loading of macrophages involves signaling through protein dephosphorylation. *J. Biol. Chem.* **270:** 29894–29903.
19. Mallampalli, R. K., R. G. Salome, S. L. Bowen, and D. A. Chappell. 1997. Very low density lipoproteins stimulate surfactant lipid synthesis in vitro. *J. Clin. Invest.* **99:** 2020–2029.
20. Sohal, P. S., and R. B. Cornell. 1990. Sphingosine inhibits the activity of rat liver CTP:phosphocholine cytidylyltransferase. *J. Biol. Chem.* **265:** 11746–11750.

Downloaded from www.jlr.org by guest, on June 14, 2012

21. Boggs, K. P., C. O. Rock, and S. Jackowski. 1995. Lysophosphatidylcholine and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine inhibit the CDP-choline pathway of phosphatidylcholine synthesis at the CTP:phosphocholine cytidylyltransferase step. *J. Biol. Chem.* **270:** 7757–7764.
22. Yang, W., and S. Jackowski. 1995. Lipid activation of CTP:phosphocholine cytidylyltransferase is regulated by the phosphorylated carboxyl-terminal domain. *J. Biol. Chem.* **270:** 16503–16506.
23. Tessner, T. G., C. O. Rock, G. B. Kalmar, R. B. Cornell, and S. Jackowski. 1991. Colony-stimulating factor 1 regulates CTP:phosphocholine cytidylyltransferase mRNA levels. *J. Biol. Chem.* **266:** 16261–16264.
24. Houweling, M., L. B. Tijburg, W. J. Vaartjes, J. J. Batenburg, G. B. Kalmar, R. B. Cornell, and L. M. Van Golde. 1993. Evidence that CTP:choline-phosphate cytidylyltransferase is regulated at a pretranslational level in rat liver after partial hepatectomy. *Eur. J. Biochem.* **214:** 927–933.
25. Hogan, M., L. J. Zimmermann, J. Wang, M. Kuliszewski, J. Liu, and M. Post. 1994. Increased expression of CTP:phosphocholine cytidylyltransferase in maturing type II cells. *Am. J. Physiol.* **267:** L25–L32.
26. Hogan, M., M. Kuliszewski, W. Lee, and M. Post. 1996. Regulation of phosphatidylcholine synthesis in maturing type II cells: increased mRNA stability of CTP:phosphocholine cytidylyltransferase. *Biochem. J.* **314:** 799–803.
27. Bakovic, M., K. Waite, W. Tang, I. Tabas, and D. E. Vance. 1999. Transcriptional activation of the murine CTP:phosphocholine cytidylyltransferase gene (Ctpct): combined action of upstream stimulatory and inhibitory cis-acting elements. *Biochim. Biophys. Acta.* **1438:** 147–165.
28. Walkey, C. J., G. B. Kalmar, and R. B. Cornell. 1994. Overexpression of rat liver CTP:phosphocholine cytidylyltransferase accelerates phosphatidylcholine synthesis and degradation. *J. Biol. Chem.* **269:** 5742–5749.
29. Brown, M. S., and J. L. Goldstein. 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell.* **89:** 331–340.
30. Cornell, R. B., and H. Goldfine. 1983. The coordination of sterol and phospholipid synthesis in cultured myogenic cells. Effect of cholesterol synthesis inhibition on the synthesis of phosphatidylcholine. *Biochim. Biophys. Acta.* **750:** 504–520.
31. Storey, M. K., D. M. Byers, H. W. Cook, and N. D. Ridgway. 1997. Decreased phosphatidylcholine biosynthesis and abnormal distribution of CTP:phosphocholine cytidylyltransferase in cholesterol auxotrophic Chinese hamster ovary cells. *J. Lipid Res.* **38:** 711–722.
32. Cornell, R. B., and A. F. Horwitz. 1980. Apparent coordination of the biosynthesis of lipids in cultured cells: its relationship to the regulation of the membrane sterol:phospholipid ratio and cell cycling. *J. Cell Biol.* **86:** 810–819.
33. Rooney, S. A. 1989. Fatty acid biosynthesis in developing fetal lung. *Am. J. Physiol.* **257:** L195–L201.
34. Chappell, D. A., G. L. Fry, M. A. Waknitz, L. E. Muhonen, and M. W. Pladet. 1993. Low density lipoprotein receptors bind and mediate cellular catabolism of normal very low density lipoproteins in vitro. *J. Biol. Chem.* **268:** 25487–25493.
35. Viscardi, R. M., S. Ullsperger, and M. C. McKenna. 1991. Carbon stripping extracts serum free fatty acids: implications for media supplementation of cultured type II pneumocytes. *Lab. Invest.* **65:** 250–257.
36. Jamil, H., A. K. Utal, and D. E. Vance. 1992. Evidence that cyclic AMP-induced inhibition of phosphatidylcholine biosynthesis is caused by a decrease in cellular diacylglycerol levels in cultured rat hepatocytes. *J. Biol. Chem.* **267:** 1752–1760.
37. Wikenheiser, K. A., D. K. Vorbroker, W. R. Rice, J. C. Clark, C. J. Bachurski, H. K. Oie, and J. A. Whitsett. 1993. Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. *Proc. Natl. Acad. Sci. USA.* **90:** 11029–11033.
38. Mallampalli, R. K., R. G. Salome, and G. W. Hunninghake. 1993. Lung CTP:choline-phosphate cytidylyltransferase: activation of cytosolic species by unsaturated fatty acid. *Am. J. Physiol.* **265:** L158–L163.
39. Morand, J. N., and C. Kent. 1986. A one-step technique for the subcellular fractionation of total cell homogenates. *Anal. Biochem.* **159:** 157–162.
40. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
41. Gilfillan, A. M., A. J. Chu, D. A. Smart, and S. A. Rooney. 1983. Single plate separation of lung phospholipids including disaturated phosphatidylcholine. *J. Lipid Res.* **24:** 1651–1656.
42. Chalvardjian, A., and E. Rudnicki. 1970. Determination of lipid phosphorus in the nM range. *Anal. Biochem.* **36:** 225–226.
43. Gross, I., D. W. Dynia, S. A. Rooney, D. A. Smart, J. B. Warshaw, J. F. Sissom, and S. B. Hoath. 1986. Influence of epidermal growth factor on fetal rat lung development in vitro. *Pediatr. Res.* **20:** 473–477.
44. Miller, J. C., and P. A. Weinhold. 1981. Cholinephosphotransferase in rat lung. The in vitro synthesis of dipalmitoylphosphatidylcholine from dipalmitoylglycerol. *J. Biol. Chem.* **256:** 12662–12665.
45. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* **18:** 5294–5299.
46. Viscardi, R. M., K. Strauss, and J. D. Hasday. 1997. Oleic acid stimulates rapid translocation of cholinephosphate cytidylyltransferase in type II cells. *Biochim. Biophys. Acta.* **1349:** 157–170.
47. Rooney, S. A. 1985. The surfactant system and lung phospholipid biochemistry. *Am. Rev. Respir. Dis.* **131:** 439–460.
48. Mallampalli, R. K., C. S. Floerchinger, and G. W. Hunninghake. 1992. Isolation and immortalization of rat pre-type II cell lines. *In Vitro Cell. Dev. Biol.* **28A:** 181–187.
49. O'Reilly, M. A., A. F. Gazdar, R. E. Morris, and J. A. Whitsett. 1988. Differential effects of glucocorticoid on expression of surfactant proteins in a human lung adenocarcinoma cell line. *Biochim. Biophys. Acta.* **970:** 194–204.
50. Kim, K. H., D. R. Voelker, M. T. Flocco, and G. M. Carman. 1998. Expression, purification, and characterization of choline kinase, product of the CKI gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273:** 6844–6852.
51. O, K. M., and P. C. Choy. 1993. Effects of fasting on phosphatidylcholine biosynthesis in hamster liver: regulation of cholinephosphotransferase activity by endogenous argininosuccinate. *Biochem. J.* **289:** 727–733.
52. Glenny, H. P., M. Bowley, S. L. Burditt, J. Cooling, P. H. Pritchard, R. G. Sturton, and D. N. Brindley. 1978. The effect of dietary carbohydrate and fat on the activities of some enzymes responsible for glycerolipid synthesis in rat liver. *Biochem. J.* **174:** 535–541.
53. Bennett, M. K., J. M. Lopez, H. B. Sanchez, and T. F. Osborne. 1995. Sterol regulation of fatty acid synthase promoter. Coordinate feedback regulation of two major lipid pathways. *J. Biol. Chem.* **270:** 25578–25583.
54. Tang, W., G. A. Keesler, and I. Tabas. 1997. The structure of the gene for murine CTP:phosphocholine cytidylyltransferase, Ctpct. Relationship of exon structure to functional domains and identification of transcriptional start sites and potential upstream regulatory elements. *J. Biol. Chem.* **272:** 13146–13151.
55. Li, F., E. Rosenberg, C. I. Smith, K. Notarfrancesco, S. R. Reisher, H. Shuman, and S. I. Feinstein. 1995. Correlation of expression of transcription factor C/EBP alpha and surfactant protein genes in lung cells. *Am. J. Physiol.* **269:** L241–L247.

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 14, 2012