Regulation of the CDP-choline pathway by sterol regulatory element binding proteins involves transcriptional and post-transcriptional mechanisms

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The synthesis of phosphatidylcholine (PtdCho) by the CDP-choline pathway is under the control of the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase (CCT). Sterol regulatory element binding proteins (SREBPs) have been proposed to regulate CCT at the transcriptional level, or via the synthesis of lipid activators or substrates of the CDP-choline pathway. To assess the contributions of these two mechanisms, we examined CCTα expression and PtdCho synthesis by the CDP-choline pathway in cholesterol and fatty acid auxotrophic CHO M19 cells inducibly expressing constitutively active nuclear forms of SREBP1a or SREBP2. Induction of either SREBP resulted in increased expression of mRNAs for sterol-regulated genes, elevated fatty acid and cholesterol synthesis ($>$ 10–50-fold) and increased PtdCho synthesis (2-fold). CCTα mRNA was increased 2-fold by enforced expression of SREBP1a or SREBP2. The resultant increase in CCTα protein and activity (2-fold) was restricted primarily to the soluble fraction of cells, and increased CCTα activity *in vivo* was not detected. Inhibition of the synthesis of fatty acids or their CoA esters by cerulenin or triacsin C respectively following SREBP induction effectively blocked the accompanying elevation in PtdCho synthesis. Thus PtdCho synthesis was driven by increased synthesis of fatty acids or a product thereof. These data show that transcriptional activation of CCTα is modest relative to that of other SREBP-regulated genes, and that stimulation of PtdCho synthesis by SREBPs in CHO cells is due primarily to increased fatty acid synthesis.

Key words: fatty acids, CHO cells, cholesterol, CTP:phosphocholine cytidylyltransferase, phosphatidylcholine.

INTRODUCTION

The CDP-choline pathway is the predominant route for phosphatidylcholine (PtdCho) synthesis in all mammalian tissues except the liver, which has the capacity to methylate phosphatidylethanolamine (PtdEtn) to form PtdCho (reviewed in [1,2]). A majority of studies have identified CTP:phosphocholine cytidylyltransferase (CCT) as the rate-limiting and regulated enzyme in the CDP-choline pathway. The human CCTα and CCTβ genes encode three isoforms: the ubiquitously expressed CCTα isoform, and CCTβ1 and its splice variant CCTβ2, which display restricted tissue distributions [3,4]. The CCT enzyme is composed of a core catalytic region, followed by a helical membrane binding domain and a C-terminal phosphorylation domain. CCTα, unlike the CCTβ isofoms, has an N-terminal nuclear localization signal that targets the protein to the nucleus in many cell types [5,6]. The regulation of CCT activity involves a complex interplay between the tissue-specific expression of CCTα and CCTβ isoforms [3,4], subcellular localization [7], transcriptional regulation [8,9] and post-transcriptional events involving reversible membrane interaction and phosphorylation [10]. These various levels of regulation are co-ordinated to maintain PtdCho synthesis required for membranes, lipoprotein synthesis, cell division and cell signalling.

The CCTα gene has a classical 'housekeeping' promoter that lacks a TATA or CAAT box. Characterization of the CCTα promoter has revealed the presence of multiple Sp1 elements that, in the context of adjacent elements in the -148 to -9 region [11], regulate transcription [9,12]. Based on the activation of CCTα reporter constructs by co-transfections with combinations of Sp1, Sp2 and Sp3, it was proposed that CCTα transcription is controlled by the relative levels of these three factors [12]. However, it has yet to be established whether Sp1, Sp2 or Sp3 is responsible for the observed changes in CCTα mRNA expression during hepatocarcinogensis [13], hepatectomy [14], the cell cycle [8] or growth factor treatment [15].

The murine CCTα promoter also contains a single sterol response element (SRE) between positions -156 and -147 that is immediately adjacent to an AP-1 (activator protein-1) and an Sp1 site [9,16]. SREs are present in the promoters of numerous sterol-regulated genes involved in sterol synthesis and uptake, as well as fatty acid biosynthesis and desaturation [17–19]. The SRE is a consensus binding site for the membrane-tethered sterol regulatory element binding protein 1 (SREBP1) and SREBP2. SREBPs undergo two-step proteolytic processing after sterol-regulated SREBP-cleavage-activating protein (SCAP)-dependent transport to the Golgi apparatus [20,21]. This results in the production of a mature soluble transcription factor that translocates to the nucleus. SREBP1 (which includes the 1a and 1c isofoms) and SREBP2 have primary, but overlapping, roles in the regulation of fatty acid and cholesterol metabolism respectively [22]. Due to their global effects on the synthesis of membrane components and precursors, SREBPs also have the potential to regulate phospholipid synthesis at the substrate level via fatty acid production, as well as by direct transcriptional regulation of phospholipid biosynthetic enzymes.

Abbreviations used: CCT, CTP:phosphocholine cytidylyltransferase; CPT, choline phosphotransferase; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; FPS, farnesyl pyrophosphate synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMG, 3-hydroxy-3-methylglutaryl; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; SRD, sterol-regulation defective; SRE, sterol response element; SREBP, sterol regulatory element binding protein; SCAP, SREBP-cleavage-activating protein.

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PtdCho synthesis by the CDP-choline pathway is a potential example of this type of dual regulation by SREBPs. Expression of the mRNA for the rate-limiting enzyme of the pathway, CCTα, is increased in response to sterol depletion of CHO cells, human macrophage THP-1 cells and murine type II alveolar cells [16,23,24]. Reporter studies using the proximal CCTα promoter confirmed that the SRE mediated the response to altered sterol levels and transfected SREBPs. It is noteworthy that sterol depletion of cells expressing a CCTα promoter construct resulted in a 1.7-fold increase in reporter gene expression, compared with an 8-fold regulation of reporter expression driven by the low-density lipoprotein (LDL) receptor promoter under the same conditions [23]. This suggests that CCTα mRNA is regulated by SREBPs, but that the absolute magnitude of this regulation is minor relative to that of other known sterol-regulated genes. In murine type II alveolar cells, increased CCTα mRNA in response to growth in lipoprotein-deficient medium was accompanied by a 2-fold increase in CCT protein expression. Paradoxically, increased CCT expression was accompanied by a significant decrease in both PtdCho synthesis and mass [24].

We have also addressed the issue of the regulation of PtdCho synthesis by SREBPs using sterol-regulation-defective (SRD) cells with defined defects in SREBP expression or processing. PtdCho synthesis was inhibited in SRD6 cells with defective SREBP processing, while oxysterol-resistant CHO cells with constitutive SREBP processing due to expression of the SCAP D443N mutant (SRD4 cells or SCAP D443N-transfected CHO cells) displayed elevated PtdCho synthesis [25,26]. In all of these cells, CCTα mRNA levels were not altered, and in SRD6 cells CCTα protein was actually increased even though SREBP processing was defective. Restoration of PtdCho synthesis in SRD6 cells by addition of exogenous oleate [26], or impairment of PtdCho synthesis in SRD4 cells by inhibition of fatty acid synthesis [25], led us to conclude that the SREBP pathway regulates PtdCho synthesis primarily through effects on fatty acid levels. Although these previous studies have indicated that SREBPs regulate the CDP-choline pathway, the relative contributions of CCT induction and fatty acid availability to overall PtdCho synthesis rates have not been accurately assessed.

To address the relative contributions of transcriptional compared with substrate effects on PtdCho synthesis, we have employed CHO M19 cells, which have negligible mature nuclear SREBP due to a deletion of the Site-2 protease, and low levels of cholesterol and fatty acid synthesis, but which inducibly express mature SREBP1a and SREBP2. These cells were made to inducibly express truncated nuclear versions of SREBP1a and SREBP2 (designated N-BP-1a and N-BP-2 cells respectively) under the control of the ecdysone receptor for the purpose of studying the relative contributions of the two SREBPs to cholesterol and fatty acid synthesis [17]. Here we have used this cell model to monitor the effects of the acute induction of SREBP-regulated genes on all facets of the CDP-choline pathway. Results of these studies show that induction of mature SREBP1a or SREBP2 increased PtdCho synthesis and the expression of CCTα mRNA and protein. However, the absolute induction of CCTα mRNA and protein was minor (approx. 2-fold), CCT activity was increased primarily in the soluble fraction of cells, and there was no evidence of increased CCT activity in intact cells by pulse–chase studies. Instead, increased PtdCho synthesis in N-BP cells following SREBP induction was coupled to elevated synthesis of a fatty acid-derived compound. This suggests that SREBPs regulate the CDP-choline pathway mainly through effects on endogenous fatty acid synthesis, and to a lesser extent by transcriptional induction of CCTα.

EXPERIMENTAL

Materials

Tissue culture media, fetal calf serum and G418 were from Life Technologies. Zeocin and muristerone A were from Invitrogen. Lipoprotein-deficient serum (LPDS) was prepared by centrifugation at $d = 1.21$ g/ml as previously described [27]. Cerulenin was from Sigma-Aldrich, and triacsin C was purchased form Biomol. Cerulenin and triacsin C were prepared as 45 mM and 4.9 mM stocks solutions respectively in DMSO. [*methyl*-^{3}H]Choline (75 Ci/mmol), phospho[*methyl*-^{14}C]choline (50 mCi/mmol), [1-^{14}C]acetate (54 mCi/mmol), CDP-[*methyl*-^{14}C]choline,[^{3}H]serine (23 Ci/mmol), [1,2-^{14}C]ethanolamine (109 mCi/mmol), [α-^{32}P]-dCTP (300 Ci/mmol) and [^{32}P]phosphate (9000 Ci/mmol) were from New England Nuclear/Mandel. Anti-FLAG M2 monoclonal antibody was from Upstate Biotechnology. *Escherichia coli* diacylglycerol (DAG) kinase was from Calbiochem. Labelled sterols and phospholipids were separated on silica gel 60 TLC plates from EM Science. Water-soluble choline metabolites were resolved by TLC on silica gel G plates from Fisher Scientific.

Cell culture

CHO M19 cells inducibly expressing nuclear SREBP1a or SREBP2 under the control of the ecdysone receptor (Invitrogen) (provided by Michael Brown, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.), hereafter referred to as N-BP-1a and N-BP-2 cells respectively, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % (v/v) fetal calf serum, 15 μM cholesterol, 1 mM sodium mevalonate, 20 μM oleate, 500 μg/ml G418 and 500 μg/ml zeocin. For experiments, cells were subcultured in the same medium but without G418 or zeocin. After 48 h, cells were switched to DMEM containing 5 % (v/v) LPDS for 24 h. Cells were then induced to express nuclear SREBPs by addition of muristerone A in DMEM/5 % (v/v) LPDS (refer to Figure legends for specific details).

RNA analysis

Total RNA was isolated from cells by the guanidine isothiocyanate/phenol/chloroform extraction method [28]. S1 nuclease protection assays for quantification of mRNAs for the LDL receptor, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, farnesyl pyrophosphate synthase (FPS), HMG-CoA reductase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as described previously [29]. Synthesis of the S1 DNA probe for CCTα was as described in [26]. An M13 template for synthesis of the rat fatty acid synthase S1 probe was provided by Timothy Osborne (University of California, Irvine, CA, U.S.A.) [30]. A ^{32}P-labelled fatty acid synthase S1 probe was hybridized with total RNA (25 μg) for 10 min at 80 °C, followed by 16 h at 40 °C. S1 nuclease-resistant products were resolved in 6 % (w/v) polyacrylamide/7 M urea gels, dried and exposed to Kodak XAR film at −70 °C for 3–18 h. CCTα mRNA and protein were quantified by scanning of films and analysed using the NIH Image software package.

Analysis of labelled sterols, fatty acids and phospholipids

Following incubation of N-BP cells with [^{3}H]choline (refer to Figure legends for specific details), medium was removed, cells were rinsed once with cold PBS (10 mM phosphate and

150 mM NaCl) and cells were harvested in methanol/water (5:4, v/v). Water-soluble choline-labelled metabolites and [^{3}H]PtdCho were isolated and resolved by TLC as previously described [26]. [^{3}H]Serine-labelled phosphatidylserine (PtdSer), PtdEtn and sphingomyelin were extracted from cells as described above. Lipids were separated by TLC on silica gel 60 plates in chloroform/methanol/water (65:25:4, by vol.) and identified by fluorography and co-migration with authentic standards. Lipids were scraped from TLC plates into vials and radioactivity was measured by liquid scintillation counting. Incorporation of [^{14}C]ethanolamine into PtdEtn was determined in a similar manner, except that the TLC step was omitted, since [^{14}C]PtdEtn accounted for $> 99\,\%$ of the radioactivity in total lipid extracts. Cholesterol and fatty acid synthesis was measured by [^{14}C]acetate incorporation into saponified total cell extracts as previously described [25,31]. Isotope incorporation into phospholipids, fatty acids and sterols was normalized to total cell protein [32].

Enzyme assays

For assays of CCT activity, cells were fractionated into soluble and membrane components by a modification of the digitonin permeabilization method [6,33]. Briefly, cells on 60 mm dishes were permeabilized on ice for 1 min in 10 mM Tris/HCl (pH 7.4), 0.25 mM sucrose, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and 0.05 mg/ml digitonin. This soluble cell fraction was removed and centrifuged at 20 000 ***g*** for 15 min to remove debris. The remaining cell ghosts were harvested in 0.5 ml of permeabilization buffer without digitonin and homogenized by 10 passages through a 23 gauge needle (designated the membrane fraction). CCT activity in soluble and membrane fractions was assayed in the presence of PtdCho/oleate vesicles (1:1, mol/mol) by measuring conversion of phospho[^{14}C]choline into CDP-[^{14}C]choline [34].

For choline kinase and choline phosphotransferase (CPT) assays, cells were harvested in 20 mM Tris/HCl (pH 7.4), 0.1 mM PMSF, 1 mM EDTA and 5 mM dithiothreitol, homogenized by 15 passages through a 23-gauge needle and sonicated for 5 s. Homogenates were subject to centrifugation at 200 000 ***g*** for 30 min, the soluble fraction was collected and the membrane pellet was resuspended in the homogenization buffer. Choline kinase was assayed in the soluble fraction by measuring the formation of phospho[^{3}H]choline from [^{3}H]choline [35], while CPT activity was assayed in the membrane/particulate fraction as described in [36].

CCTα immunoblotting

Total cell homogenates or soluble/membrane fractions prepared by digitonin permeabilization were separated by SDS/PAGE and transferred to nitrocellulose membranes. CCTα was detected by incubation of nitrocellulose membranes with a polyclonal antibody directed against the C-terminal phosphorylation domain (provided by Martin Post, University of Toronto, Ontario, Canada). To ensure equal loading and transfer of proteins, nitrocellulose filters were stained with Ponceau S and immunoblotted for a non-sterol-regulated protein (oxysterol binding protein) [37]. This was followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and detection by the enhanced chemiluminescence method (Amersham Biosciences).

RESULTS

Induction of SREBPs increases PtdCho synthesis

CHO M19 cells are sterol and fatty acid auxotrophs, due to complete deletion of the Site-2 protease that releases the mature nuclear form of SREBPs from endoplasmic reticulum membranes [38,39]. Enforced expression of the mature nuclear forms of SREBP1a and SREBP2 in CHO M19 cells using the ecdysone receptor system was used to delineate the relative effectiveness of these transcription factors in activating genes involved in cholesterol and fatty acid synthesis [17]. In the present study, we have used this cell model to investigate the mechanism of regulation of the CDP-choline pathway for PtdCho biosynthesis by SREBP1a and SREBP2. When the expression of nuclear SREBP1a or SREBP2 was induced with an ecdysone receptor agonist (muristerone A), there was a dose-dependent increase in the synthesis of fatty acids (Figure 1A) and cholesterol (Figure 1B) that reached a maximum at 2 μM muristerone A. It is important to note that while cholesterol synthesis was virtually absent from uninduced N-BP-1a and N-BP-2 cells (approx. 1–2 % of maximally induced values), residual fatty acid synthesis in uninduced cells represented approx. 10 % of maximally induced values. Under these induction conditions, PtdCho synthesis (measured by [^{3}H]choline incorporation) was stimulated to a maximum of 2-fold at 2 μM muristerone A in both cell lines (Figure 1C). Consistent with the observed increases in lipid and sterol synthesis, expression of FLAG-tagged nuclear SREBP1a and SREBP2 increased in a dose-dependent manner, and reached a maximum at 2 μM muristerone A. Synthesis of cholesterol, fatty acids and PtdCho was maximal after 18–24 h induction (results not shown); thus all further experiments were performed after a 24 h induction with 2 μM muristerone A.

To test whether the synthesis of other phospholipids was affected by SREBP induction, N-BP cells were cultured in the presence or absence of muristerone A, and the synthesis of PtdEtn (using [^{14}C]ethanolamine) by the CDP-ethanolamine pathway (Figure 2A), or of PtdSer and PtdEtn (using [^{3}H]serine) by the base-exchange/decarboxylation pathways (Figure 2B), was determined. In both instances, induction of SREBPs had no effect on, or caused only a slight increase in, the synthesis of phospholipids by these two routes. Synthesis of sphingomyelin (Figure 2B) was also unaffected by acute SREBP induction. Thus enforced expression of SREBP1a and SREBP2 increased the synthesis of cholesterol, fatty acids and PtdCho, but not that of PtdSer, PtdEtn or sphingomyelin, in a co-ordinated manner.

To ascertain which step in the CDP-choline pathway was stimulated by SREBPs, the distribution of soluble [^{3}H]choline-labelled intermediates of the CDP-choline pathway was analysed following induction of SREBP expression in N-BP cells for 24 h (Figure 3). As expected, SREBP induction resulted in a 2–2.5-fold increase in the incorporation of [^{3}H]choline into PtdCho. Phosphocholine, the substrate for the rate-limiting enzyme CCT, constituted the major pool of radioactive precursor in the pathway. However, phosphocholine pools in both N-BP cell lines were not altered by SREBP induction, arguing against significant activation of CCT. The [^{3}H]choline pool in cells, as well as levels of the PtdCho degradation product glycerophosphocholine, were also not significantly affected by SREBP induction. CDP-[^{3}H]choline levels were significantly increased upon SREBP1a expression. However, this intermediate constitutes only a minor portion of total [^{3}H]choline-labelled metabolites and is a substrate for CPT, which is usually not considered rate-limiting for PtdCho synthesis.

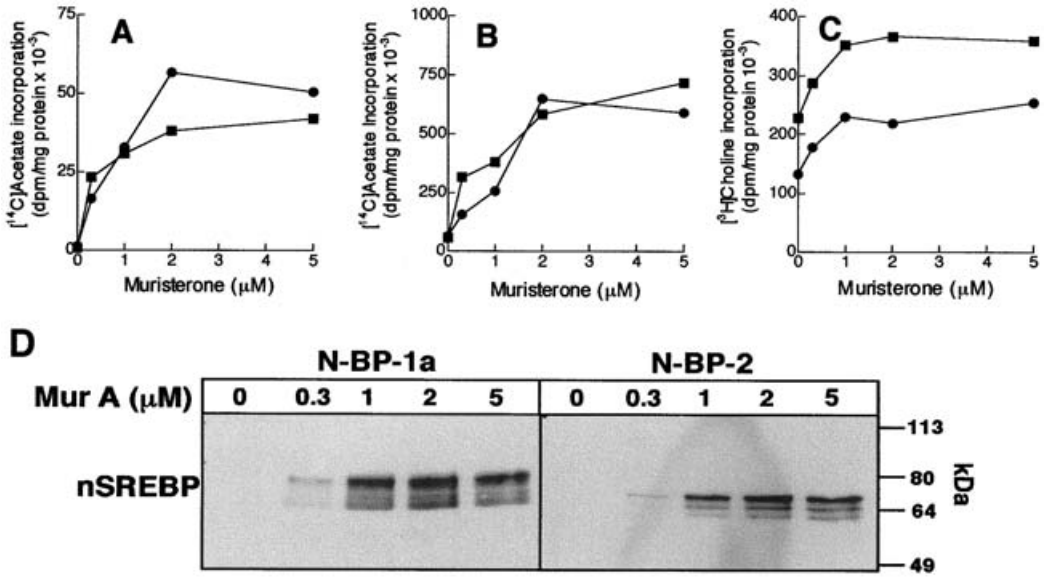

Figure 1 SREBP induction increases sterol, fatty acid and PtdCho synthesis

CHO NB-P-1a and N-BP-2 cells were cultured in DMEM containing 5 % (v/v) LPDS for 24 h prior to induction with the indicated concentrations of muristerone A (Mur A) in the same medium for 24 h. N-BP-1a (●) or N-BP-2 (■) cells were then pulse-labelled with [^{14}C]acetate (5 μCi/ml for 3 h) to measure cholesterol (**A**) and fatty acid (**B**) synthesis, or with [^{3}H]choline (1 μCi/ml for 2 h) to measure PtdCho synthesis (**C**), as described in the Experimental section. (**D**) Expression of truncated nuclear forms of SREBP1a and SREBP2 in N-BP cells was measured by immunoblotting of total cell extracts (25 μg of protein) with an anti-FLAG antibody. Results are from a single experiment that was repeated two other times with a similar outcome.

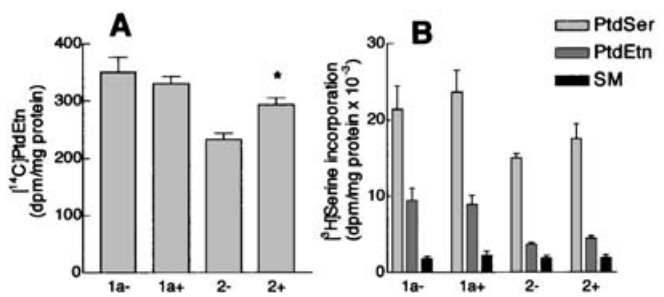

Figure 2 Lack of effect of SREBP induction on synthesis of other phospholipids

N-BP-1a or N-BP-2 cells were cultured and induced as described in the legend to Figure 1. After induction with 2 μM muristerone A (+) or solvent control (−) for 24 h, cells were pulse-labelled with [^{14}C]ethanolamine (2 μCi/ml for 2 h) or [^{3}H]serine (15 μCi/ml for 2 h) to measure the synthesis of PtdEtn (**A**) or PtdEtn, PtdSer and sphingomyelin (SM) (**B**) respectively. Results are means and S.E.M. for three experiments; $^{*}P < 0.05$ compared with non-induced cells.

Regulation of CCTα by SREBP1a and SREBP2

Although the [^{3}H]choline labelling experiments shown in Figure 3 did not indicate a substantial increase in CCT activity, previous studies demonstrating increased CCTα promoter activity and expression in response to sterol depletion and transfected SREBPs prompted further investigation. Initially, CCTα mRNA was quantified in muristerone A-induced N-BP-1a and N-BP-2 cells and compared with mRNA levels for several known sterol-regulated genes (Figure 4). As expected, mRNAs for the LDL receptor, HMG-CoA synthase, FPS and fatty acid synthase were virtually absent from uninduced N-BP cells, but increased dramatically following induction with muristerone A. In contrast, CCTα mRNA was detected in uninduced cells, and there were 2.6 ± 0.4-fold and 2.0 ± 0.3-fold increases ($n = 3$ mean $\pm$ S.E.M.) in expression relative to uninduced controls following enforced expression of SREBP1a and SREBP2 respectively (Figure 4A). This was similar to the pattern with HMG-CoA reductase mRNA, which also displayed limited expression in the absence of SREBP induction. Treatment of N-BP cells with increasing concentrations of muristerone A also revealed basal expression of CCTα mRNA and a maximum 2–3-fold induction at 2–5 μM muristerone A (Figure 4B).

To establish whether increased CCTα mRNA resulted in a corresponding increases in protein and enzyme activity, total-cell homogenates or digitonin-soluble and membrane fractions were immunoblotted with an anti-CCTα polyclonal antibody (Figures 5A and 5B). Induction with increasing concentrations of muristerone A resulted in a dose-dependent increase in CCTα protein expression (Figure 5A). At 2 μM muristerone, this corresponded to a 1.8 ± 0.3-fold and a 2.1 ± 0.2-fold increase (relative to uninduced controls) in CCTα protein expression in N-BP-1a and N-BP-2 cells respectively. The expression of oxysterol binding protein, a low-abundance oxysterol receptor that displays sterol- and SREBP-independent expression, served as a load control for this experiment. Expression of total CCTα protein as an indicator of enzyme activity can be misleading, since the active form of this enzyme is localized to membranes, primarily the nuclear envelope in CHO cells [5,6]. To assess changes in CCTα localization following SREBP induction, N-BP cells were separated into soluble and membrane fractions by digitonin permeabilization, and immunoblotted (Figure 5B) and assayed for CCTα activity (Figure 5C). Digitonin is effective in releasing CCTα from the nucleus of cells [33]; thus 'soluble CCTα' refers to enzyme in the nucleoplasm. In uninduced N-BP cells, CCTα protein was found primarily in the membrane fraction (Figure 5B). Expression of SREBPs increased the amount of CCTα in the soluble fraction, but had little effect on enzyme mass in the membrane fraction. Enzyme assays of these fractions confirmed that increased CCTα activity was restricted primarily to the soluble fraction in N-BP cells. However, N-BP-1a cells did display a 40 % increase in CCTα activity in the membrane fraction. Using indirect immunofluorescence and

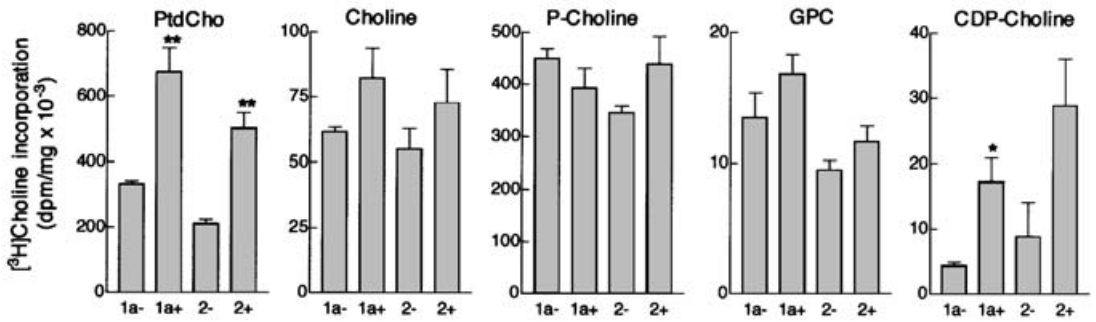

Figure 3 Distribution of choline metabolites in N-BP cells

N-BP-1a and N-BP-2 cells were cultured as described in the legend to Figure 1, and induced (+) or not (−) with 2 μM muristerone A. After 24 h, cells were pulse-labelled with 1 μCi/ml [^{3}H]choline for 2 h, and incorporation into PtdCho, choline, phosphocholine (P-Choline), glycerophosphocholine (GPC) and CDP-choline was measured as described in the Experimental section. Results are means and S.E.M. for six experiments; $^*P < 0.05$, $^{**}P < 0.01$ compared with uninduced cells.

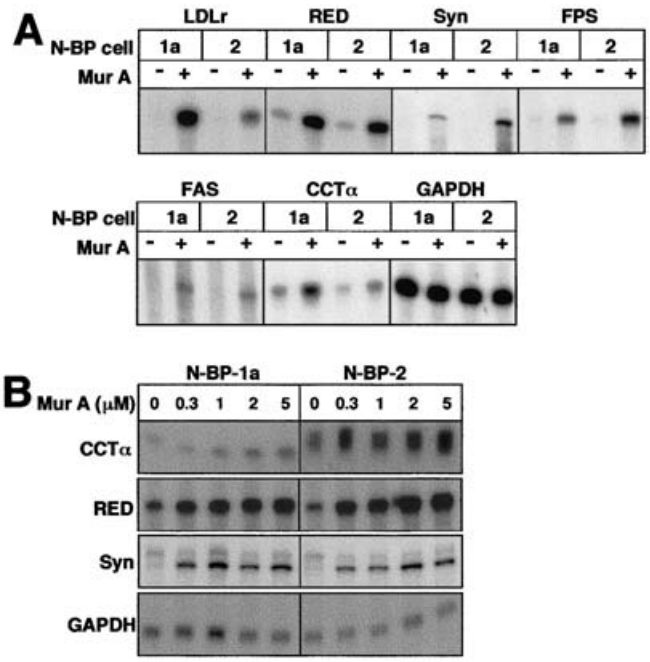

Figure 4 Comparison of CCTα mRNA expression with that of other SREBP-regulated genes

N-BP cells were cultured as described in the legend to Figure 1. (**A**) Cells were induced with muristerone A (Mur A; 2 μM) for 24 h, total RNA was harvested and mRNAs for the LDL receptor (LDLr), HMG-CoA reductase (RED), HMG-CoA synthase (Syn), FPS, fatty acid synthase (FAS), CCTα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by S1 nuclease protection assays. (**B**) N-BP cells were induced with the indicated concentrations of muristerone A (0–5 μM) for 24 h, and mRNAs for CCTα, HMG-CoA reductase, HMG-CoA synthase and GAPDH were measured. Representative experiments are shown that were repeated two other times with similar results.

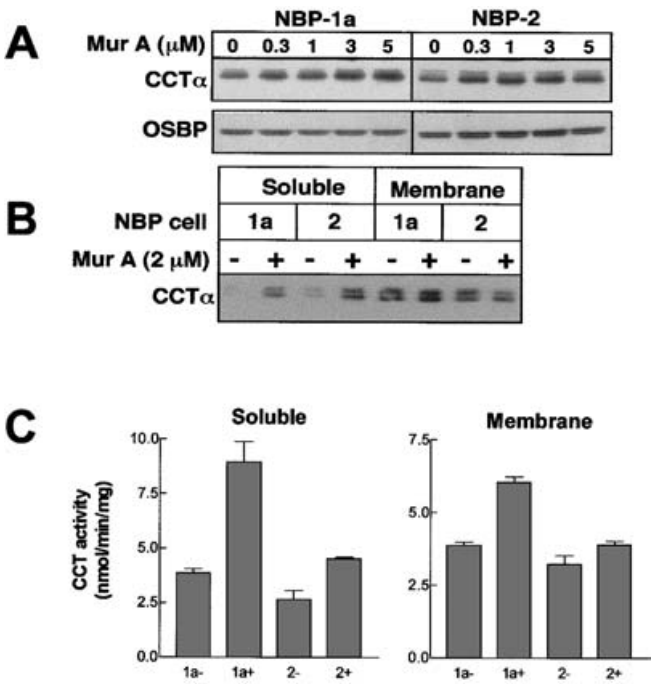

Figure 5 Expression of CCTα protein and activity in N-BP cells

Cells were cultured as described in the legend to Figure 1. (**A**) SREBP expression was induced by the addition of the indicated concentrations of muristerone A (Mur A; 0–5 μM). After 24 h, Triton X-100-soluble total cell extracts were prepared and immunoblotted (25 μg in each lane) for CCTα and oxysterol binding protein (OSBP). (**B**) Following induction with muristerone A (2 μM) for 24 h, digitonin permeabilization was used to prepare soluble and total membrane fractions. CCTα protein was detected in each fraction (25 μg) by immunoblotting. (**C**) Digitonin-released soluble and membrane fractions from induced (+) and uninduced (−) N-BP-1a and N-BP-2 cells were assayed for CCTα activity in the presence of PtdCho/oleate vesicles as described in the Experimental section. For experiments shown in (**B**) and (**C**), the recovery of protein in soluble and membrane fractions was similar for N-BP-1a and N-BP-2 cells, and was not altered by muristerone A induction. The ratio of total protein recovered in soluble and membrane fractions was approx. 1:2 for all treatments. Representative experiments are shown that were reproduced one or two other times with similar results.

confocal microscopy, we were also unable to detect any significant increase in CCTα levels in the nuclear envelope upon SREBP induction in N-BP cells (results not shown).

The initial step in the CDP-choline pathway is catalysed by choline kinase, and the final step to produce PtdCho is catalysed by CPT. To determine if induction of SREBP1a or SREBP2 affected these two steps in the pathway, choline kinase and CPT activities were assayed in the soluble and membrane fractions respectively from induced and uninduced N-BP cells. As shown in Table 1, induction of SREBP expression by muristerone A did not affect CPT activity. While choline kinase activity was also not significantly affected by SREBP expression, it is notable that both N-BP cell lines displayed a 20–25 % increase in enzyme activity.

It is conceivable that the modest increases in CCTα expression observed upon SREBP1a and SREBP2 induction could translate

Table 1 Choline kinase and CPT activity in N-BP-1a and N-BP-2 cells

Choline kinase and CPT activities were assayed in the soluble and total membrane fractions respectively of control or induced (2 μM muristerone; +MurA) N-BP-1a and N-BP-2 cells. Results are means ± S.D. of four separate experiments.

	Activity (μmol/min per mg of protein)	
Cell line	Choline kinase	CPT
N-BP-1a	0.42 ± 0.13	0.77 ± 0.16
N-BP-1a + MurA	0.53 ± 0.07	0.71 ± 0.14
N-BP-2	0.52 ± 0.07	0.71 ± 0.08
N-BP-2 + MurA	0.62 ± 0.07	0.69 ± 0.10

into elevated activity in intact cells, but that the continuous-pulse protocol used in experiments shown in Figure 3 would not detect such subtle changes. As an alternative, a [^{3}H]choline pulse–chase regime was use to monitor loss of radioactivity from the phosphocholine pool in control and induced N-BP cells. Results from these experiments showed that phospho[^{3}H]choline levels were similar in N-BP cells at the end of the chase period (0 time) and decayed at the same rate regardless of whether cells were induced or not (results not shown). This further confirmed that apparent increases in CCTα expression following SREBP induction did not translate into discernible increases in the conversion of phosphocholine into PtdCho *in vivo*.

SREBP induction of PtdCho synthesis is coupled to fatty acid production

Previously we showed that elevated PtdCho synthesis and turnover in SRD4 cells could be attenuated by suppression of fatty acid synthesis with cerulenin [25]. N-BP cells offer an excellent system to further examine how endogenous fatty acids synthesis regulated by the SREBP pathway exerts control over the CDP-choline pathway, because of the range of fatty acid synthetic rates that are achieved and the ability to independently block fatty acid synthesis during SREBP induction. Moreover, results presented thus far suggest that SREBP regulation of PtdCho synthesis is not due solely to increased CCTα expression. To ascertain if SREBP induction of fatty acid synthesis was responsible for elevated PtdCho synthesis, N-BP cells were treated or not with muristerone A prior to inhibition of fatty acid synthesis (measured by [^{14}C]acetate incorporation) with cerulenin for 4 h (Figures 6A and 6B). In uninduced N-BP-1a and N-BP-2 cells, cerulenin inhibited residual fatty acid synthesis by 60–70 % (Figure 6A). Cerulenin treatment following SREBP induction in N-BP cells inhibited fatty acid synthesis by > 80 %, effectively lowering fatty acid synthetic rates to those observed in uninduced cells. There was no appreciable effect of cerulenin on the incorporation of [^{14}C]acetate into cholesterol (results not shown). Cerulenin treatment of induced or uninduced N-BP-1a and N-BP-2 cells resulted in > 50 % inhibition of [^{3}H]choline incorporation into PtdCho (Figure 6B). Thus inhibition of residual fatty acid synthesis in uninduced cells was effective in further decreasing PtdCho synthesis, and cerulenin treatment of induced cells significantly lowered PtdCho synthesis to levels observed in uninduced cells. In all cases, cerulenin did not affect phospho[^{3}H]choline levels, suggesting that reduced CCTα activity was not the primary cause of inhibition of PtdCho synthesis. Next we tested whether conversion of fatty acids into CoA esters was required for stimulation of

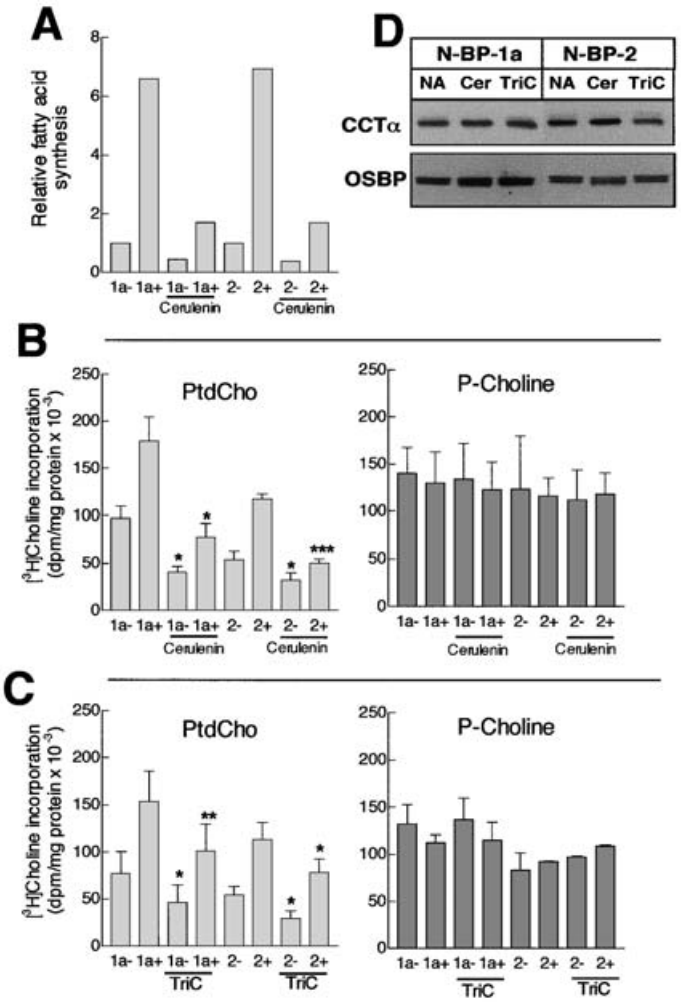

Figure 6 Inhibition of the synthesis of fatty acids or CoA esters attenuates SREBP induction of PtdCho synthesis

NB-P cells were induced with 2 μM muristerone (+) or solvent control (−) for 24 h. (**A**) After the induction period, cells were treated with 45 μM cerulenin or solvent control for 4 h. During the last 1 h of this treatment, cells were pulse-labelled with [^{14}C]acetate, and incorporation into fatty acids was measured and expressed relative to that in untreated/uninduced cells. Results are the means of two experiments. (**B**) After the induction period, cells were treated with cerulenin or solvent control for 4 h, and incorporation of [^{3}H]choline (1 μCi/ml) into PtdCho and phosphocholine (P-choline) was measured by pulse-labelling for the final 1 h. Results are means and S.E.M. for three experiments. (**C**) After the induction period, cells were treated with 9.6 μM triacsin C (TriC) or solvent control for 4 h, and PtdCho synthesis was measured as described in (**B**). Results are means and S.E.M. for three experiments. (**D**) Expression of CCTα in cerulenin- and triacsin C-treated, muristerone-induced N-BP cells was assessed by immunoblotting of total cell homogenates (25 μg). Expression of oxysterol binding protein (OSBP) was used as a load control. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared with corresponding untreated induced or uninduced cells.

PtdCho synthesis by treating cells with triacsin C (Figure 6C), an inhibitor of fatty acyl-CoA synthetases 1 and 4 [40]. These triacsin C-sensitive acyl-CoA synthetases are primarily involved in the production of fatty acyl-CoAs for *de novo* triacylglycerol and phospholipid synthesis [41]. Treatment of N-BP-1a and N-BP-2 cells with triacsin C significantly decreased PtdCho synthesis to a similar extent under both induced and uninduced conditions (Figure 6C). The extent of inhibition was similar to that observed with cerulenin, and there was also no effect on phospho[^{3}H]choline levels. Triacsin C altered total fatty acid and cholesterol synthesis by < 10 % for all conditions shown in

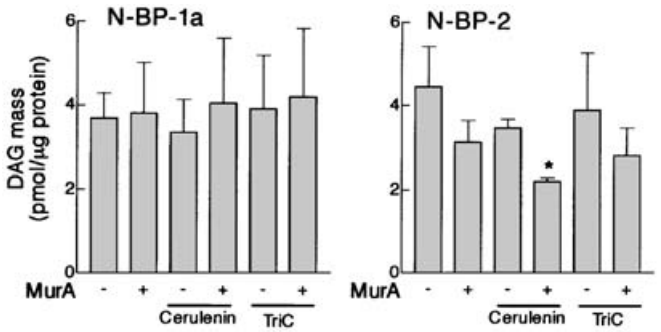

Figure 7 DAG levels do not correlate with altered PtdCho synthesis in N-BP cells

NB-P cells were induced with 2 μM muristerone A (MurA; +) or solvent control (−) for 24 h, treated with cerulenin (45 μM) or triacsin C (TriC; 9.6 μM) for 4 h, harvested and total lipids extracted as described in the Experimental section. DAG mass in total lipid extracts was measured by the DAG kinase method [45]. Results are means and S.E.M. for three experiments; $^*P < 0.05$ compared with uninduced cells.

Figure 6(C) (results not shown). In muristerone A-induced N-BP-1a and N-BP-2 cells, treatment with either cerulenin or triacsin C did not alter CCTα protein expression (Figure 6D), demonstrating that blocking PtdCho synthesis with these drugs did not involve direct effects on CCTα expression.

The results in Figure 6 suggest that fatty acids, fatty acyl-CoAs or a fatty acid-derived product are responsible for the up-regulation of PtdCho synthesis following SREBP induction. We determined whether DAG could be the required activator by measuring its mass in N-BP cells under induced and uninduced conditions, and in the presence and absence of cerulenin or triacsin C (Figure 7). SREBP1a induction had no effect on DAG mass in N-BP-1a cells; nor did inhibition of synthesis of fatty acids or their CoA esters with cerulenin or triacsin C. In N-BP-2 cells, induction of SREBP2 in either the presence or the absence of cerulenin or triacsin C reduced DAG levels by 20–30%. Thus DAG mass was not significantly affected by changes in fatty acid synthesis, and did not correlate with changes in rates of PtdCho synthesis.

DISCUSSION

SREBP transcription factors are global regulators of sterol and fatty acid synthesis that have been implicated in the maintenance of lipid homoeostasis, as well as in physiological responses to insulin action [42] and in adipocyte differentiation [43]. Due to their pivotal role in controlling cholesterol and fatty acid levels, SREBPs may regulate the synthesis of other complex lipids, thus providing an integrated network to control membrane composition and the function of membrane-associated proteins. We have used a fatty acid and cholesterol auxotrophic cell line inducibly expressing SREBP1a or SREBP2 to demonstrate that the CDP-choline pathway for PtdCho synthesis is regulated co-ordinately with sterol and fatty acid synthesis via transcriptional and post-transcriptional mechanisms. This involves a co-ordinated increase in both the rate-limiting enzyme and in the supply of fatty acid substrate for PtdCho synthesis. However, activation of CCTα expression by SREBP was minor, and enzyme activity was not increased in intact N-BP cells. This was consistent with our previous observation of altered PtdCho synthesis in SRD cells without an apparent change in CCTα expression. The present results support the concept that the supply of endogenous fatty acids is the dominant mechanism whereby SREBPs regulate the CDP-choline pathway in CHO cells.

The mechanism of action of SREBPs on the CDP-choline pathway was addressed in the present study by careful correlation of CCTα mRNA, protein and activity levels with rates of PtdCho synthesis following acute induction of SREBP expression. Previous studies using reporter gene constructs clearly showed that the SRE in the CCTα promoter conferred sensitivity to sterols and was strongly activated by SREBP1a and SREBP2 [16,23]. However, resultant effects on PtdCho synthesis have not been definitively proven. For example, cultured alveolar cells with increased CCTα expression due to growth in delipidated medium had reduced PtdCho synthesis [24], but mice refed with a low-fat/high-carbohydrate diet to induce CCTα showed increased surfactant and lung PtdCho synthesis [16]. Using CHO SRD cells, we demonstrated that SREBPs regulated PtdCho synthesis and CCTα at a post-transcriptional level, and PtdCho synthesis was either increased or decreased without corresponding changes in CCTα mRNA or protein expression [25,26]. The reasons for these apparently contradictory results could stem from differences in experimental approach or cell lines. Unlike our past studies that relied on SRD mutants or stably transfected cells to influence SREBP activity, the inducible N-BP cells allow for assessment of changes in lipid synthesis following acute induction of nuclear forms of SREBP1a and SREBP2. This precludes any long-term adaptive response that the cells might make in the constitutive expression of mature SREBPs. With this system, we observed an approx. 2-fold increase in CCTα mRNA levels in response to either SREBP1a or SREBP2 induction, which was significantly less than that for other SREBP-regulated genes. CCTα was also expressed in the absence of SREBPs, indicating that other elements/factors drive basal transcription and that SREBPs are not essential for expression.

Increased CCTα mRNA levels were translated into a modest increase in CCTα protein levels (approx. 2-fold) that was found primarily in the inactive soluble fraction of digitonin-permeabilized cells. However, it should be noted that induced N-BP-1a cells, and to a lesser extent N-BP-2 cells, had increased CCTα activity in the 'active' membrane fraction (Figure 5C). The presence of the majority of induced CCTα in the 'inactive pool' was consistent with our finding that *in vivo* CCT activity was not increased, as determined by phospho[^{3}H]choline pool size and rate of decay from this pool during pulse–chase experiments. This suggests that SREBP can induce CCTα expression, but that appropriate post-transcriptional signals are then required to activate the enzyme. It was notable that the high rate of endogenous fatty acid synthesis in induced N-BP cells was not sufficient to cause significant translocation of the expanded pool of soluble CCTα protein to membranes. Thus, unlike addition of exogenous oleate to CHO cells, which promotes CCTα activation via translocation to membranes [25,44], increased endogenous fatty acid synthesis had more subtle effects on CCTα and the CDP-choline pathway. Although we cannot completely exclude the possibility that phospho[^{3}H]choline pools are not indicative of CCTα activity *in vivo* or that the 'soluble' CCTα released in our assay was partially active, our results show that induction of CCTα by SREBP makes a minor contribution to increased PtdCho synthesis.

To establish that endogenous fatty acid synthesis and the CDP-choline pathway are coupled, we selectively inhibited the synthesis and CoA-activation of fatty acids using cerulenin and triacsin C respectively. There are several important conclusions from these experiments: (1) increased fatty acid synthesis and CoA activation in response to SREBP induction drives PtdCho synthesis, (2) basal fatty acid synthesis in the absence of

SREBPs was sufficient to support PtdCho synthesis, and (3) these effects were independent of CCTα expression or *in vivo* activity. Precisely how fatty acid and PtdCho synthesis are coupled has yet to be determined. Inhibition by triacsin C suggests that activation of the CDP-choline pathway requires the synthesis of a complex lipid. Based on a lack of correlation of DAG mass with fatty acid synthesis rates or of effects of inhibitors on PtdCho synthesis, we can conclude either that DAG is not the fatty acid-derived activator or that changes in a regulatory/substrate DAG pool were masked by a larger metabolically inert pool. To explain the effects of SREBP induction on PtdCho synthesis, we propose a model that combines transcriptional and post-transcriptional/substrate mechanisms. SREBP1a and SREBP2 increase the transcription of the rate-limiting enzyme in the pathway, CCTα, to a modest extent. This alone is not sufficient to increase PtdCho synthesis, as indicated by the low PtdCho synthesis in the presence of inhibitors of fatty acid synthesis/activation. At the same time, SREBPs also increase the expression of key fatty acid biosynthetic and modifying enzymes, leading to increased synthesis of unsaturated fatty acids [17]. Fatty acids then serve to activate CCTα or to provide substrate for the pathway. Fatty acid synthetic rates in N-BP-1a and N-BP-2 cells were increased by 7–10-fold upon SREBP induction (Figure 1), but PtdCho synthesis was increased by only 2-fold. In this regard, limited up-regulation of CCTα by SREBPs could prevent excessive PtdCho synthesis that would otherwise divert fatty acids and DAG from other essential cellular functions, but at the same time maintains PtdCho synthesis in the presence of increased cellular sterol synthesis.

Collectively, the present study provides a complete picture of the influence of acute induction of nuclear forms of SREBP1a and SREBP2 on the CDP-choline pathway, and demonstrates how SREBPs regulate the synthesis of PtdCho at several levels. This has important implications for our understanding of how cells co-ordinately regulate the synthesis of membrane components, as well as of lipids and cholesterol required for other processes such as lipoprotein or bile secretion. Initial results suggest that regulation of PtdCho synthesis by SREBPs could be unique, since synthesis of phospholipids by other pathways was not affected by SREBP induction (Figure 2).

Robert Zwicker and Gladys Keddy provided excellent technical assistance in the culture and maintenance of cell lines. We thank our colleagues Dr David Byers, Dr Harold Cook and Dr Christopher McMaster for providing helpful comments during the course of this work. This work was supported by program grant PG-11476 and a Scientist Award from the Canadian Institutes of Health Research (to N. D. R.), and a graduate student trainee award from Cancer Research and Education Nova Scotia (to T. A. L.).

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Received 12 February 2003/21 March 2003; accepted 26 March 2003
Published as BJ Immediate Publication 26 March 2003, DOI 10.1042/BJ20030252