Transcriptional repression of the CTP:phosphocholine cytidyltransferase gene by sphingosine

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We examined the effects of the bioactive lipid, sphingosine, on the expression of the rate-limiting enzyme involved in surfactant phosphatidylcholine synthesis, CCT α (CTP:phosphocholine cytidyltransferase α). Sphingosine decreased phosphatidylcholine synthesis by inhibiting CCT activity in primary alveolar type II epithelia. Sphingosine decreased CCT α protein and mRNA levels by approx. 50 % compared with control. The bioactive lipid did not alter CCT α mRNA stability, but significantly inhibited its transcriptional rate. In murine lung epithelia, sphingosine selectively reduced CCT α promoter-reporter activity when transfected with a 2 kb CCT α promoter/luciferase gene construct. Sphingo-

sine also decreased transgene expression in murine type II epithelia isolated from CCT α promoter-reporter transgenic mice harbouring this 2 kb proximal 5'-flanking sequence. Deletional analysis revealed that sphingosine responsiveness was mapped to a negative regulatory element contained within 814 bp upstream of the coding region. The results indicate that bioactive sphingolipid metabolites suppress surfactant lipid synthesis by inhibiting gene transcription of a key surfactant biosynthetic enzyme.

Key words: cytidyltransferase, MLE-12, phosphatidylcholine, sphingolipid.

INTRODUCTION

Pulmonary alveolar surfactant is a lipoproteinaceous surface-active fluid synthesized within alveolar type II epithelial cells that contains mainly DSPtdCho (disaturated phosphatidylcholine) and key hydrophobic proteins. Surfactant deficiency contributes to the pathogenesis of ARDS (acute respiratory distress syndrome), a disorder characterized by protein-rich alveolar oedema, gas-exchange abnormalities and ventilatory impairment. Surfactant replacement therapy for ARDS patients is promising, but not totally effective [1]. This may be attributed to limitations of delivered therapy, including ineffective intra-alveolar administration, insufficient surface activity and intra-alveolar degradation. Exogenous surfactant therapy also has to overcome the effects of various 'inhibitory factors' that are elaborated in ARDS. These inhibitory factors can directly oppose a natural surfactant's ability to lower surface tension and/or alter surfactant DSPtdCho synthesis [2].

Sphingolipids have emerged as a novel class of bioactive regulatory molecules derived primarily from the hydrolysis of the parent sphingolipid, sphingomyelin. Bioactive sphingolipids are generated by sphingomyelin hydrolysis in response to mediators associated with ARDS such as TNF α (tumour necrosis factor α), Fas/Apo ligand and ionizing radiation [3]. For example, TNF α triggers sphingomyelin hydrolysis generating ceramide, which rapidly deacylates to sphingosine [4]. Ceramide and sphingosine, along with their phosphorylated derivatives, appear to have diverse roles as second messengers involved in regulating cell proliferation, differentiation and apoptosis [5]. In the lung, sphingolipids produce porcine pulmonary oedema and lactosylceramides are increased in ARDS [6,7]. Studies in our laboratory and by

others demonstrate that TNF α increases lung ceramide, which in turn potentially increases alveolar surface tension and inhibits DSPtdCho synthesis [8–12]. Together, these results suggest that sphingolipid products are abundantly generated during alveolar inflammation that may be responsible for altered biophysical properties or decreased surfactant pool size by inhibiting PtdCho biosynthesis.

The biosynthesis of surfactant DSPtdCho is tightly regulated within the CDP-choline (cytidine diphosphocholine) pathway. A key enzyme in this pathway is CCT (CTP:phosphocholine cytidyltransferase; EC 2.7.7.15), an enzyme that catalyses a very slow reaction converting cholinephosphate into CDP-choline in the presence of CTP [13]. The primary structure of CCT (termed CCT α) in mammals consists of four functional domains, including an N-terminal nuclear-localization motif, a catalytic core, an α -helical lipid-binding domain and a C-terminal phosphorylation domain [14]. CCT α is localized to several intracellular sites, but in alveolar epithelia it appears to be predominantly detected within the cytosol and endoplasmic reticulum [13,15]. In addition to CCT α , two splice variants encoded by a different gene (CCT β 1 and CCT β 2) have been identified in humans ([16,17], but see [16a]). These isoforms differ in the extent of their C-terminal phosphorylation but exhibit some similar regulatory features.

CCT activity in cells is largely regulated by the availability of lipids. Enzyme activity is significantly stimulated by exogenous unsaturated fatty acids, diacylglycerol and anionic phospholipids [13]. Other lipids such as lysophosphatidylcholine, ceramide and sphingosine are potent inhibitors of CCT function [12,18]. Sphingosine and ceramide are reversible inhibitors of purified CCT, whereas glucosylceramide stimulates activity [10,12,19,20]. CCT is also a phosphoenzyme and the degree by which lipids

Abbreviations used: AP-1, activator protein 1; ARDS, acute respiratory distress syndrome; CCT α , CTP:phosphocholine cytidyltransferase α ; CDP-choline, cytidine diphosphocholine; CK, choline kinase; CMV, cytomegalovirus; PtdCho, phosphatidylcholine; DSPtdCho, disaturated PtdCho; FBS, fetal bovine serum; LDH, lactate dehydrogenase; NRE, negative regulatory element; RSV, rous sarcoma virus; SPP, sphingosine 1-phosphate; TK, thymidine kinase; TNF α , tumour necrosis factor α .

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alter CCT activity is influenced by its phosphorylation status [21].

CCT post-translational control has been extensively studied but its transcriptional regulation remains largely unknown. The genomic organization of murine CCT α has been determined: it spans 40 kb, has two transcription start sites and the 5'-flanking region lacks TATA or CAAT boxes but contains GC-rich regions typical of promoters found in housekeeping genes [22]. Our recent studies and those of others have determined that the core promoter is localized to a relatively small DNA sequence (approx. 200 bp), exhibits robust activity and is stimulated by the presence of a putative sterol-regulated enhancer located between -156 and -147 nt [23,24]. Transcriptional analysis indicates that the proximal 5'-flanking sequence contains consensus-binding elements for several ubiquitous transcription factors including Sp1 (specificity protein 1), nuclear factor κ B and AP-1 (activator protein 1) [23,25,26]. Sphingolipids modulate nuclear factor κ B and AP-1 DNA-binding activity thereby regulating the transcriptional rate of some genes [27–30]. These observations coupled with inhibitory effects of bioactive sphingolipids on surfactant function allowed us to hypothesize that sphingosine might also decrease DSPtdCho synthesis by inhibiting CCT α gene transcription within alveolar epithelia.

MATERIALS AND METHODS

Materials

Actinomycin D and TRI Reagent were from Sigma (St. Louis, MO, U.S.A.). Sphingolipids were obtained from Metraya (Pleasant Gap, PA, U.S.A.). Silica LK5D (0.25 mm $\times$ 20 cm $\times$ 20 cm) TLC plates were purchased from Whatman International (Maidstone, Kent, U.K.). All radiochemicals were purchased from DuPont New England Nuclear Chemicals (Boston, MA, U.S.A.). Immunoblotting membranes were obtained from Millipore (Bedford, MA, U.S.A.). The ECL[®] Western-blotting detection system was from Amersham Biosciences (Piscataway, NJ, U.S.A.). Anti-CCT α rabbit polyclonal antiserum to synthetic peptide was generated by Covance Research Products (Richmond, CA, U.S.A.). The random primed labelling kit used was Redprime II[™] (Bucks., U.K.). The pBK-CMV plasmid (where CMV stands for cytomegalovirus) was purchased from Stratagene (La Jolla, CA, U.S.A.). The pREP4 and pCR-TOPO4 plasmids and *Escherichia coli* Top10 competent cells were obtained from Invitrogen (Carlsbad, CA, U.S.A.) and the FuGENE6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN, U.S.A.). The MLE-12 cell line was obtained from A.T.C.C. (Manassas, VA, U.S.A.). The GeneClean2 kit was obtained from Bio101 (Carlsbad, CA, U.S.A.). The luciferase assay system was obtained from Promega (Madison, WI, U.S.A.) and the Galactolight plus[™] kit was from TROPIX (St. Louis, MO, U.S.A.). The Advantage cDNA polymerase was from Clontech Laboratories (Palo Alto, CA, U.S.A.). All DNA sequencing was performed by the University of Iowa DNA core facility. Luciferase and β -galactosidase activities were determined using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, U.S.A.). Hite's medium was obtained from the University of Iowa Tissue Culture and Hybridoma Facility (Iowa City, IA, U.S.A.). The α -ENaC promoter (+1388/+55) coupled with luciferase has been described in [31].

Cell culture

Adult rat primary and murine alveolar type II epithelial cells were isolated and characterized as described in [32,33]. The purity of

type II cells from each species was >95% in our laboratory as determined by tannic acid staining. Cells were cultured in Dulbecco's minimum essential medium for up to 18 h with or without sphingolipids (1–10 μ M). MLE cells were maintained in Hite's medium with 10% (v/v) FBS (fetal bovine serum) at 37 °C in 5% CO₂ atmosphere. After reaching confluence, the cells were harvested using 0.25% trypsin with 0.1% EDTA and plated on to 60 mm tissue culture dishes (approx. 350 000 cells/dish). After overnight incubation, the medium was removed and the cells were rinsed twice in serum-free medium, then incubated with Hite's medium alone (control medium) or in combination with various amounts of individual sphingolipids (1–10 μ M) for up to 30 h. Cell viability as determined by LDH (lactate dehydrogenase) release and cell proliferation as assessed by [³H]thymidine uptake were assayed as described in [8]. Cell lysates were prepared by brief sonication in buffer A (150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM dithiothreitol, 0.025% sodium azide and 1 mM PMSF, pH 7.4) at 4 °C. Cell nuclei were prepared by nuclease treatment of cellular lysates as described in [34].

PtdCho and DSPtdCho analysis

Cells cultured in Hite's medium containing either control medium or medium containing individual sphingolipids (1–10 μ M) for up to 30 h were pulsed with 1 μ Ci [*Me*-³H]choline chloride during the final 4 h of incubation. Total cellular lipids were extracted, separated using TLC and incorporation of the label into PtdCho and DSPtdCho was quantified as described in [35].

Enzyme activities

CCT, CK (choline kinase) and cholinephosphotransferase activities were determined as described in [35].

Immunoblot analysis

Equal amounts of protein from cell lysates were used for an SDS/PAGE (10% gel) and transferred on to a nitrocellulose membrane as described in [35]. CCT α and β -actin were probed using the ECL[®] Western-blotting detection system with a dilution factor for all antibodies at 1:1000.

Detection of CCT α mRNA

Total cellular RNA was isolated from cells by using TRI Reagent. Total RNA (30 μ g) was separated electrophoretically using a 1% agarose gel, capillary-blotted to a nitrocellulose membrane and hybridized as described in [35]. The CCT α probe was prepared by random primed labelling of cDNA fragments isolated from agarose gels using a GeneClean kit. The probe for rodent CCT α cDNA was a 1.1 kb *Hind*III–*Bam*HI fragment inserted in pcDNA3.

Detection of murine type II cell CCT α transcripts using real-time PCR analysis

After RNA isolation, Taqman reverse transcription reagents (Applied Biosystems) were used to generate cDNA from cellular RNA [36]. Real-time PCR was then performed on cDNA using the Applied Biosystems 7700 real-time PCR instrument and the SYBR Green PCR master mix. CCT α mRNA detection primers were 5'-CCTGGAAATGTTTGGTCCAGA-3' and 3' primer, 5'-CTCTGCTTGGGACTGATGG-3'. Taqman rodent 18 S was used

as the internal control using the following primers: 5'-TAAGTC-CCTGCCCTTTGTACACA-3' and 3'-primer, 5'-GATCCGAGG-GCCTCACTAAAC-3'.

Nuclear run-on assay

Nuclear pellets were isolated as described previously [37]. Nuclear transcription was assessed under optimal conditions in reactions containing nuclei with radiolabelled substrates. Reactions were terminated, RNA extracted and processed as described in [37]. Samples were counted and equalized by c.p.m. of ^{32}P -labelled RNA/ml and hybridized to CCT α or 18 S cDNA previously bound to nitrocellulose [37]. Filters were rinsed, dried and exposed to autoradiographic film for detection of CCT α and 18 S transcripts.

Plasmid construction of pGL3-CCT, pGL3-CK, pGL3-CMV, pGL3-RSV and pGL3-TK

The murine CCT α core promoter fragment (– 1867/+ 71) was generated by PCR using the following primers: 5'-TTGTGT-GTTTTACCCCTTATG-3' (left) and 5'-TCAACTCTCCAG-GCTCCGGT-3' (right) as described in [24]. This fragment was then directionally cloned into pGL3 basic. Various CCT α deletion constructs were also generated by restriction digestion or by separate PCR cloning. All cloned fragments included the proximal 5'-flanking region, two adjacent transcription start sites and up to 71 nt of the first exon corresponding to the 5'-untranslated region of the mouse CCT α transcript [24]. These constructs were also coupled with luciferase and then used for transient-transfection studies.

The rat CK promoter (– 1530/+ 90) (GenBank[®] accession number D37884 [38]) was amplified using primers 5'-GAAAC-TTGGGCTGTCCTGAA-3' and 5'-TCACCGGCTTCACTGACC-3'. PCR contained 1.5 mM MgCl₂, 200 μM dNTP, 0.4 μM of each primer and 1.0 unit of high-fidelity *Taq* DNA polymerase in a 50 μl of mixture. PCR was performed using high-fidelity *Taq* DNA polymerase and the following profile: 94 °C for 2 min, then 94 °C for 30 s, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s and 68 °C for 2 min plus a final extension at 68 °C for 2 min. The amplified 1620 bp fragment was cloned into pCR4-TOPO (Invitrogen) and then subcloned into *NotI*–*SpeI* sites of pGL3 basic.

Commercially available plasmids pBK-CMV and pREP4 vectors were used as templates for PCR amplification of the CMV, RSV (roux sarcoma virus) and TK (thymidine kinase) promoters. Primers used for amplification of CMV, RSV and TK promoters were 5'-CMV primer, ACTCGAGCTAGCGGATCTGACGGTTC; 3'-CMV primer, AGATCTCGGGGTCATTAGTTCA-TAGG; 5'-RSV primer, ACTCGAACTCTCAGTACAATCT-GCTC; 3'-RSV primer, AGATCTGGCGTTTATTGTATCGAGC; 5'-TK primer, ACTCGAGTTTGCTGGCGGTGTCC; and 3'-TK primer, AGATCTGCAGGGTCGCTCGGTGTTC. An *XhoI* site was added to the end of all 5'-primers, whereas a *BglIII* site was added to the end of all 3'-primers to allow for convenient cloning into pGL3-basic.

PCR was performed using Advantage cDNA polymerase and the following profile: 94 °C for 2 min, then 94 °C for 30 s and 68 °C for 3 min for a total of 18 cycles. The resulting PCR products (CMV, RSV and TK fragments) were ligated into the pCR-TOPO4 vector before transformation into *E. coli* competent cells. The CMV (590 bp), RSV (571 bp) and TK (163 bp) fragments were then directionally subcloned into the pGL3-basic vector using *BglIII* and *XhoI* sites. All promoter sequences and orientation were confirmed by DNA sequencing.

Construction of the CCT α promoter– β -galactosidase cDNA cassette

Cloning procedures were performed on a modified vector made from pCR2.1 (Invitrogen) and pCI (Promega) (pStec-TgDNA2) kindly provided by D. Stec (University of Iowa). The *E. coli* lacZ gene was obtained from a pBS401 vector. After amplifying the 1938 bp fragment of the CCT α promoter (– 1867/+ 71), it was cloned into the *SacI* site of the modified vector. The *E. coli* LacZ reporter gene (3.2 kb) was then cloned into the *SacI* site. After the cloning procedure, DNA sequences and orientation were verified by DNA sequencing.

Generation and screening of CCT α promoter-reporter murine transgenics

Transgenic mice were generated at the University of Iowa Transgenic Animal Facility, where microinjections of the cDNA cassette into oocytes were performed to generate transgenic founders. Transgenic offsprings were identified by PCR analysis using tail DNA with β -galactosidase primers. To detect transgene integration and copy number, genomic DNA (20 μg) from mice was digested with *NheI*, resolved by agarose-gel electrophoresis and transferred on to nylon membranes (Zetaprobe GT; Bio-Rad Laboratories, Hercules, CA, U.S.A.). An 899 bp genomic DNA fragment of the murine CCT α gene that corresponded to the first exon and adjacent sequence from 5'-flanking region and the first intron was used as a hybridization probe. Briefly, the DNA fragment was labelled by random primer extension with Klenow DNA polymerase and [α - ^{32}P]dCTP (Decaprime II DNA labeling kit; Ambion, Austin, TX, U.S.A.) and then hybridized with the transferred membrane as described previously [31].

Immunohistochemical detection of β -galactosidase

Adult mice were killed and their lungs were perfused with 30 ml of PBS to remove blood. The lungs were instilled with 4 % (w/v) paraformaldehyde in phosphate buffer (pH 7.4) and then immersed in this fixative for 6 h at 25 °C. The fixed lungs were dehydrated, embedded in paraffin at 60 °C, and cut into 8 μm sections using a microtome. Immunostaining was performed with a Vector MOM Immunodetection kit (Vector Laboratories, Burlingame, CA, U.S.A.) according to the manufacturer's instructions. Briefly, sections were deparaffinized, hydrated, and incubated with 3 % H₂O₂ to reduce endogenous peroxidase activity. Sections were then incubated with either no primary antibody (negative control) or with monoclonal antibodies against β -galactosidase (CCT α transgenics) or against β -actin (positive control) at a 1:200 dilution. Sections were washed and incubated with a biotinylated secondary antibody followed by incubation with a preformed avidin–horseradish peroxidase complex. Diaminobenzidine was used as the peroxidase substrate and sections were counterstained with haematoxylin.

Transient transfection and reporter gene assays

Transfections were conducted in 0.5 % Hite's medium with Eugene 6 and 0.75 μg of test plasmid for 90 min. Immediately after transfections, cells were exposed to a medium containing individual sphingolipids for 3 h. Cell lysates were prepared for analysis of luciferase and β -galactosidase activities as determined by luminometer readings after these values were normalized for transfection efficiency by co-transfection with CMV- β -gal (0.25 μg). β -Galactosidase activities in cells and murine tissues isolated from CCT α promoter-reporter transgenic mice were measured after preheating samples at 48 °C for 55 min to inactivate endogenous β -galactosidase activity.

Figure 1 Sphingosine down-regulates parameters of PtdCho synthesis

Primary rat alveolar type II epithelial cells (**A**) and MLE cells (**B**) were incubated for 18 h in serum-free medium, supplemented with or without various amounts of sphingosine. Cells were pulsed with 2 μ Ci [³H]choline for the final 4 h of incubation, and the radioactivity in cellular [³H]PtdCho was determined after lipid extraction from cell lysates, separation using TLC and quantification by scintillation counting. CCT activity was assayed in primary type II (**C**) or MLE (**D**) cells cultured under conditions described in (**A**). (**E**) MLE cells were cultured in serum-free medium alone or with 10 μ M sphingosine for various time periods and CCT activity was assayed. (**F**) LDH release in supernatants was assayed in MLE cells exposed to serum-free medium (control) alone or with sphingosine (S), SPP, C₂ ceramide (C₂) or ceramide 1-phosphate (C-1P) at 10 μ M for 30 h. (**G**) MLE cells were cultured as in (**B**) but labelled with 2.5 μ Ci [³H]thymidine for the final 4 h of culture before harvesting of cells after 18 h to determine cellular incorporation of the label. In each panel, * $P \leq 0.05$ for 10 μ M sphingosine versus control or 1 μ M sphingosine by ANOVA. In (**B**), ** $P < 0.01$ versus control. Results are means $\pm$ S.E.M. for three independent experiments.

Quantitative analysis of sphingosine

Sphingosine was extracted from cell lysates and *o*-phthalaldehyde derivatives were prepared for quantitative analysis by HPLC as described in [26]. *o*-Phthalaldehyde derivatives were separated on a Beckman Ultrasphere C-18 column and derivatives were detected using a Thermoseparation Products Spectra System FL3000 fluorescence detector at 340 nm excitation and 454 nm emission wavelengths.

RESULTS

Sphingosine inhibits PtdCho synthesis

Incubation of primary rat alveolar type II cells with sphingosine significantly decreased incorporation of [*Me*-³H]choline into PtdCho and DSPtdCho. After 18 h of lipid exposure, choline incorporation into PtdCho decreased by 11 and 72 % at the 5 and 10 μ M concentrations respectively relative to control cells (Figure 1A). Sphingosine in separate studies also decreased choline incorporation into DSPtdCho, the major surface-active phospholipid species of alveolar surfactant, from 14747 ± 209 (control) to 7325 ± 1966 d.p.m./mg of protein (sphingosine 10 μ M; $n = 3$, $P = 0.07$). In MLE cells, sphingosine decreased choline incorporation into PtdCho in a dose-dependent manner (Figure 1B). Sphingosine also decreased choline incorporation into PtdCho after 30 h of exposure in these cells [93139 ± 4964 (control) versus 65511 ± 4783 d.p.m./mg of protein (sphingosine), $P = 0.01$, $n = 6$].

Sphingosine decreased PtdCho synthesis in lung epithelia by significantly inhibiting the activity of CCT, the rate-regulatory enzyme within the CDP-choline pathway. There was generally an overall trend towards a decrease in CCT activity with increasing concentrations of sphingosine after 24 h of exposure in primary alveolar cells (Figure 1C). The lipid also produced a 34 and 40 % decrease in CCT activity in MLE cells at the 5 and 10 μ M concentrations respectively (Figure 1D). Under similar conditions, 10 μ M sphingosine for 24 h did not alter activities for CK, the first committed enzyme of the CDP-choline pathway [15.6 ± 3.5 (control) versus 21.6 ± 2.5 pmol $\cdot$ min⁻¹ $\cdot$ μ g⁻¹ (sphingosine)] or of cholinephosphotransferase, the terminal enzyme of the pathway [2.4 ± 0.2 (control) versus 2.3 ± 0.2 pmol $\cdot$ min⁻¹ $\cdot$ μ g⁻¹ (sphingosine)]. Thus sphingosine decreases the synthesis of surfactant phospholipid by selectively inhibiting activity of the rate-regulatory enzyme required for PtdCho biosynthesis.

To determine if sphingosine exerts early biochemical inhibition of CCT activity, analysis was conducted where MLE cells were exposed to 10 μ M sphingosine and enzyme activity was assayed up to 30 min after lipid exposure (Figure 1E). The sphingolipid did not significantly alter CCT activity over this time frame even for a concentration of up to 50 μ M (results not shown). Further studies were performed to determine whether various sphingolipids produced cytotoxicity or altered cell growth. Various sphingolipids tested did not significantly alter cellular viability as determined by LDH release after 30 h of cellular exposure to 10 μ M of individual lipids (Figure 1F). Sphingosine also did not modulate cell growth as assessed by [³H]thymidine incorporation into cells (Figure 1G).

Figure 2 Sphingosine decreases CCTα protein levels in lung epithelia

Immunoreactive CCTα and β-actin levels were determined in (A) primary rat alveolar type II epithelial cells and (B) MLE cells. Cells were incubated for 18 h (A) or 8 h (B) with or without various concentrations of sphingosine (1–10 μM). Cell lysates (100 μg of protein) were separated by SDS/PAGE (10% gel), transferred on to a nitrocellulose membrane and probed with an anti-CCTα rabbit polyclonal antibody (above) or polyclonal antibody to β-actin (below). The far-left lane of (A, B) immunoblots corresponds to purified rat liver CCTα. (C) Densitometric analysis of immunoblots showing the average percentage decrease ± S.E.M. in CCTα levels in primary cells versus control using arbitrary densitometric values at the 10 μM sphingosine concentration. Results are means ± S.E.M. for four independent experiments.

Sphingosine decreases CCTα protein

To investigate if sphingosine inhibition of CCT activity was associated with decreased levels of enzyme, we performed immunoblotting experiments (Figure 2). Culture of primary alveolar cells for 24 h with the sphingolipid resulted in substantially decreased immunoreactive CCTα content compared with control at the 10 μM concentration (Figure 2A). Studies were also conducted in MLE cells, which express higher levels of steady-state CCTα protein. Sphingosine significantly decreased CCTα mass at the 5 and 10 μM concentration (Figure 2B); densitometric analysis of autoradiograms revealed that the lipid decreased CCTα levels by 49 ± 12% ($n = 4$) versus control (Figure 2C). Thus inhibitory effects of sphingosine on PtdCho biosynthesis are secondary to decreased levels of CCTα enzyme.

Sphingosine decreases steady-state CCTα mRNA

Northern blotting revealed that sphingosine decreased CCTα protein by decreasing steady-state CCTα mRNA levels in MLE cells. As shown in Figure 3(A), sphingosine exposure substantially decreased the expression of the 5.0 kb CCTα transcript within 6 h of incubation. Densitometric analysis of autoradiograms revealed that, on average, an approx. 57–62% decrease in steady-state CCTα mRNA level was observed after sphingolipid treatment at the 5 and 10 μM concentrations compared with control (Figure 3B). These findings were corroborated using quantitative PCR and suggest that sphingosine regulates CCTα at the pretranslational level in lung epithelial cells.

We next conducted studies to determine if sphingosine decreased steady-state CCTα mRNA by decreasing mRNA half-life (Figure 3C). Cells were cultured in Hite's medium containing 10% FBS before switching to serum-free medium with or without sphingosine for various time periods in the presence of actinomycin D (5 μg/ml). After culture, cells were harvested for total RNA and levels of CCTα mRNA were quantified as described above. Levels of CCTα mRNA decreased gradually in control cells or after sphingosine treatment, but no significant differences in mRNA half-life were observed between the groups (control, $t_{1/2} = 2.1 \pm 0.08$ h versus sphingosine, $t_{1/2} = 2.7 \pm 0.48$ h, $n = 3$). These results suggest that sphingosine decreases CCTα protein by inhibiting mRNA synthesis.

Figure 3 Sphingosine decreases steady-state CCTα mRNA in lung epithelia

(A) MLE cells were grown for 6 h in serum-free medium with or without sphingosine (1–10 μM). Total cellular RNA (30 μg) was isolated, separated on 1% agarose gels and the amount of CCTα mRNA or 18 S RNA was determined by Northern-blot analysis. (B) Densitometric analysis of autoradiograms was performed using arbitrary ratios of CCTα/18 S to quantify effects of sphingosine on CCTα mRNA ($n = 4$, * $P < 0.05$ versus control). (C) The half-life of CCTα mRNA was estimated in MLE cells. Cells were grown in Hite's medium containing 10% FBS for 72 h before changing to serum-free medium alone (control) or in combination with 5 μM sphingosine containing 5 μg/ml actinomycin D for 0–6 h. Northern-blot analysis of total cellular RNA (50 μg) was performed to determine the amount of CCTα mRNA and 18 S RNA. Densitometric analysis of autoradiograms shows CCTα mRNA/18 S absorbance ratios at various time points. Values are expressed on a logarithmic scale and expressed as means ± S.E.M. for three independent experiments.

Sphingosine inhibits CCTα mRNA synthesis

To confirm inhibitory effects of sphingosine on CCTα transcription, we performed nuclear run-on assays after culturing cells with or without the sphingolipid (5 μM). Compared with cells cultured in the medium alone, sphingosine decreased CCTα mRNA synthesis (Figure 4A) after 1.5 and 3 h of exposure without altering 18 S rRNA in MLE cells (Figure 4B). Overall, the bioactive lipid decreased CCTα transcript levels by approx. 55% versus control (Figure 4C). These results indicate that the bioactive sphingolipid decreases CCTα mRNA by reducing CCTα gene transcription within lung epithelia.

Sphingosine inhibits CCTα gene transcription

We investigated the molecular basis for sphingosine inhibitory effects on CCTα gene transcription using transient transfectional analysis where an approx. 2 kb fragment of the proximal 5'-flanking region of the CCTα gene was coupled with the luciferase

Figure 4 Spingosine inhibits CCT α mRNA synthesis

MLE cells were grown in Hite's medium containing 10% FBS for 72 h before changing to serum-free medium alone (control, C) or in combination with 5 μ M spingosine (S) for 1.5 or 3 h. After culture, cells were harvested and processed for CCT α (A) and 18 S (B) transcriptional activity as described in the Materials and methods section. (C) Densitometric analysis of autoradiograms showing the average percentage decrease $\pm$ S.E.M. in CCT α mRNA synthesis after spingosine versus control using arbitrary densitometric values. Results are means $\pm$ S.E.M. for three independent experiments.

reporter. Cells were transfected with the construct and subsequently exposed to the medium with or without spingosine (1–10 μ M) for 3 h. Lysates were then analysed immediately for reporter gene expression. Spingosine produced a dose-dependent decrease in CCT α promoter activity (Figure 5A). Ceramide (C_2 ceramide) and ceramide 1-phosphate also decreased CCT α promoter-reporter activity, but overall effects were of lesser magnitude compared with spingosine (Figure 5B). In contrast, SPP (sphingosine 1-phosphate) tended to increase CCT α promoter activity relative to control. To determine if effects of spingosine were specific to the CCT α promoter, we examined spingosine effects on other promoters of mammalian and viral origin (Figure 5C). Spingosine did not significantly alter promoter activities for the RSV, TK, α -EnaC or CK genes, but potently inhibited CMV promoter activity compared with control ($P < 0.05$).

Since ceramides also inhibited CCT α promoter activity, we next investigated whether these N-acylated sphingoid derivatives were converted into spingosine. Primary rat type II cells or MLE cells were incubated with various amounts of C_2 ceramide and the mass of spingosine was measured using HPLC (Figure 6). Indeed, exogenous ceramide was converted into spingosine in a dose-dependent manner within alveolar cells, suggesting that effects of ceramides on CCT α gene expression might be mediated through catalytic conversion into spingosine.

Additional studies were performed to identify a potential silencer or NRE (negative regulatory element) for spingosine within the 5'-flanking region of the CCT α gene (Figure 7). Deletional analysis revealed that promoter-reporter constructs –1867/+71 and –983/+71 exhibited significant reductions in luciferase activity in response to spingosine, but the region harbouring the core promoter, –169/+71, did not. Thus spingosine down-regulates CCT α gene transcription by regulating a *cis*-acting NRE contained within 814 bp of the CCT α promoter.

To address if effects of spingosine were also observed in an *in vivo* model, we generated a CCT α – β -galactosidase fusion gene that harboured an approx. 2 kb promoter; this construct was injected into mouse oocytes to produce transgenic founders that were bred to establish ultimately lines of CCT α -promoter-reporter mice. Initial screening by tail clip DNA analysis using PCR revealed the generation of three transgene positive lines (Figure 8A). An offspring from one of these lines (F₁ CCTp595) was tested for transgene integration and copy number by Southern blotting which confirmed PCR results. These results demonstrated that the signal intensity of the transgene matched the native gene signal, indicating that the transgene was integrated as two copies in the

Figure 5 Spingosine inhibits CCT α gene transcription

Transfections were conducted in 0.5% Hite's medium with Fugene 6 and 0.75 μ g of test plasmid for 90 min using a luciferase reporter vector containing approx. 2 kb (–1867/+71) of the CCT α promoter cloned into PGL3 Basic. (A) After transfection, cells were exposed to either increasing concentrations of spingosine or (B) spingosine (S), SPP, C_2 ceramide (C_2) or ceramide 1-phosphate (C-1P) all at 10 μ M for 3 h. (C) Cells were transfected with various viral and mammalian promoters, all cloned into an identical background within an empty plasmid (PGL3 Basic) and subsequently exposed to serum-free medium (control) or 10 μ M spingosine as above. The promoters tested include CCT α , CK, RSV, TK, α -epithelial sodium channel (ENaC) and the CMV immediate early gene promoter. In all panels, luciferase and β -galactosidase activities in cellular extracts were determined by luminometer readings. (A) $**P < 0.05$ (10 μ M) versus (0) dose and $*P < 0.05$ (7.5 μ M) versus 1 μ M dose by ANOVA; (B) $**P < 0.05$ C-1P versus (0) and $*P < 0.01$ either C or S versus (0) or SPP by ANOVA; (C) $*P < 0.05$ spingosine treated versus control plasmids for CCT α or CMV by the *t* test.

Figure 6 Lung epithelia convert exogenous ceramide into spingosine

MLE cells were cultured for 3 h in a medium with 2% FBS alone or in the presence of FBS with increasing amounts of C_2 ceramide. Cells were then harvested for the determination of spingosine content using HPLC. Results are means $\pm$ S.E.M. for three independent experiments. $*P < 0.05$ versus control by ANOVA. Inset: primary rat alveolar type II cells were cultured with 10 μ M ceramide for 3 h and analysed in a similar fashion. $*P < 0.05$ versus control by *t* test.

Figure 7 Deletional analysis of the CCT α promoter

Transfections were conducted in 0.5% Hite's medium with Eugene 6 and 0.75 μ g of test plasmid for 90 min using one of five different luciferase reporter vectors containing fragments spanning -1867/+71 to -62/+71 nt of the CCT α promoter cloned into PGL3 Basic. After transfection, cells were exposed to control medium or sphingosine as in Figure 5(A). Luciferase and β -galactosidase (β -gal) activities in cellular extracts were determined by luminometer readings. Results are from $n=3$ independent experiments. * $P < 0.05$ sphingosine-treated versus control plasmids by the t test.

founder genome (Figure 8B). Analysis of β -galactosidase expression revealed a high-level activity within the whole lung (Figure 8C). Immunohistochemical analysis of lungs from transgene-positive founders revealed that when compared with transgene-negative control littermates, reporter gene expression was detected within alveolar epithelium (Figure 8C, middle, arrowheads).

We next isolated murine alveolar cells from these mice that were observed to exhibit a high degree of purity as determined by tannic acid staining for lamellar bodies, a marker of type II cells (> 95%) (Figure 9A); these cells were subsequently cultured in the presence or absence of sphingosine to assess CCT α and β -galactosidase reporter gene expression. Similar to MLE cells and rat primary type II cells, CCT α promoter-reporter murine type II cells exposed to sphingosine exhibited a significant reduction in endogenous enzyme (CCT α) activity and β -galactosidase activity (Figure 9B). Thus sphingosine inhibited CCT α promoter-reporter activity *in vitro* in a transiently transfected type II alveolar epithelial cell line and in primary cultures of type II cells, where the promoter-reporter is stably integrated into the genome.

Figure 8 Characterization of a CCT α -promoter-reporter murine transgenic

(A) Detection of the β -galactosidase reporter in adult transgenic founder lines harbouring an approx. 2 kb CCT α promoter. Tail-clip analysis of genomic DNA from three independent founder lines tested positive (lanes 1, 6 and 7) for the reporter gene. Lane 11 is a molecular-mass ladder, and lanes 12 and 13 are positive control lanes. (B) Left panel: schematic representation of the murine CCT α gene and probe used for Southern-blot analysis. X, XbaI; N, NheI; A, AclI. This probe was designed to detect the native CCT α gene and the β -galactosidase transgene simultaneously allowing estimation of transgene copy number using the native gene signal intensity as a reference. Right panel: Southern-blot analysis of NheI-digested genomic DNA from four progeny of a founder line from different litters demonstrating the presence of the transgene in three of four littermates. (C) Pulmonary transgene expression. Left panel: lung homogenates isolated from transgene-positive founders (F₁) and wild-type (Wt) (negative control) littermates from one line. Relative expression of β -galactosidase activity was assayed from three mice in each group. Results are expressed as relative light units (RLU)/ μ g of protein. Minimal activity was detected in control organs. Right panels: immunohistochemical localization of β -galactosidase. Lung tissue from transgenic mice was fixed in paraformaldehyde, dehydrated, embedded in paraffin and microsectioned. Sections were incubated with either no primary antibody (negative control) or immunostained with monoclonal antibodies against β -galactosidase (CCT α transgenics) or against β -actin (positive control). Sections were washed and incubated with a biotinylated secondary antibody followed by a preformed avidin-horseradish peroxidase complex using diaminobenzidine as a substrate resulting in the deposition of a brownish red precipitate for detection. Sections were counterstained with haematoxylin. Arrowheads (middle panel) represent positive staining in alveolar epithelium.

Figure 9 Sphingosine inhibits gene transcription in a CCT α -promoter-reporter murine transgenic

(A) Tannic acid staining in primary alveolar cells isolated from CCT α promoter-reporter transgenics. Results reveal the detection of multiple intracellular inclusion (lamellar) bodies characteristic of type II epithelia. (B) CCT (upper panel) and β -galactosidase (lower panel) activities were assayed in primary alveolar type II cells isolated from CCT α promoter-reporter transgenics. Cells were cultured for 18 h with or without 10 μ M sphingosine. In (B), the results represent $n = 3$ separate studies. * $P < 0.05$ versus control.

DISCUSSION

These results provide evidence that a key biomolecule involved in cell signalling, sphingosine, directs transcriptional repression of the CCT α gene. To our knowledge, down-regulation of PtdCho synthesis via CCT α gene transcriptional repression has not been previously demonstrated. We show that sphingosine inhibits a gene's transcriptional rate resulting in decreased enzyme levels in murine lung epithelia. Evidence supporting sphingosine-mediated repression of CCT α transcription was demonstrated both *in vivo* using primary alveolar epithelia isolated from murine CCT α promoter-reporter transgenics and by transfectional analysis using CCT α -truncated promoter constructs in a murine lung cell line. The pathophysiological relevance of these results is that surfactant PtdCho levels decrease and bioactive sphingolipids accumulate in broncho-alveolar lavage samples of patients or within lung tissues of animal models with acute lung injury [6,9]. The present results elucidate the molecular basis for some of these observations by demonstrating that sphingosine might be a key biomolecule suppressing PtdCho production by inhibiting gene transcription of a key regulatory enzyme involved in surfactant metabolism.

Sphingosine-induced decrease in CCT α protein expression might also be attributed to concurrent activation of proteolytic cleavage of the enzyme as shown by our previous studies and also by others [35,36,39]. We were not able to block the decrease in CCT α protein mass triggered by sphingosine using a caspase inhibitor (results not shown). Furthermore, we observed a fairly consistent pattern of sphingosine-induced down-regulation of CCT α protein expression by approx. 50% in lung epithelia that roughly corresponded to reduced CCT α transcriptional activity. The results with nuclear run-on experiments, mRNA half-life studies and reporter-gene analysis conclusively identified a role for sphingosine or possibly one of its intracellular metabolites, in inhibiting CCT α gene transcription without destabilizing its mRNA. Thus the reduction of sphingosine expression could be attributed entirely to diminished CCT α transcriptional activity resulting in reduced enzyme mRNA and immunoreactive protein content.

Several studies have amply demonstrated post-translational mechanisms for CCT α control but transcriptional regulation of the enzyme had been previously unexplored [13]. Two prior studies identified CCT α gene regulation by the Sp family of transcription factors and a dual transcriptional modulator, transcriptional enhancer factor-4 [25,40]. A recent study linked Sp transcription factors with p42/44 mitogen-activated protein kinases resulting in CCT α transcriptional activation [41]. The present studies suggest the existence of one or more distinct NRE upstream of the CCT α Sp sites that requires further investigation; these elements are transactivated by sphingosine in a dose-dependent and promoter-selective manner. The observation that sphingosine inhibited CCT α and CMV promoter activities without eliciting a significant response within TK, CK, α ENac or RSV genes suggests that these promoters do not harbour the *cis*-acting sphingosine NRE within their promoters or that they lack additional promoter-specific factors needed to alter aspects of promoter structure or transcriptional initiation. Other related bioactive sphingolipids were observed to exhibit varying degrees of inhibition of CCT α promoter-reporter activity. In this regard, ceramide has been shown to inhibit CCT α activity and significantly reduced CCT α promoter-reporter activity in our previous studies [11,19]. Our studies suggest that the ceramide effect may be direct or due to conversion into sphingosine via a ceramidase (Figure 6).

The nuclear events that transduce sphingosine's signalling in cells require further investigation. It is possible that a metabolite(s) of sphingosine inhibits CCT α gene transcription. In some systems, sphingosine is rapidly converted into SPP via sphingosine kinase; sphingosine and several related sphingolipids stimulate cell proliferation, partly by increasing AP-1 DNA binding [28,29,42]. To date, sphingosine-mediated AP-1 transactivation of genes appears due to conversion into SPP as sphingosine's effects are abolished by sphingosine kinase inhibition [42,43]. Although several AP-1 consensus sites exist within the proximal 5'-flanking region of the CCT α gene, it is unlikely that SPP generated from sphingosine inhibits CCT α expression in alveolar epithelia since SPP tended to increase modestly the promoter activity (Figure 5B). Moreover, preliminary metabolic labelling studies using [3 H]sphingosine revealed that very little SPP is generated from sphingosine in MLE cells and most of the label is retained within sphingosine and ceramides between 15 min and 24 h of analysis (results not shown). Thus on the basis of transient transfectional analysis, the present studies support a unique role for sphingosine in gene repression. However, our results do not exclude the possibility that endogenous ceramides also mediate CCT α transcriptional inhibition.

Since transient expression assays examine protein-DNA interactions in the context of disorganized accessible chromatin within

plasmid DNA, findings from such experiments may not accurately recapitulate gene regulation within chromosomal DNA. We therefore complemented our transfection studies with cells isolated from CCT α promoter-reporter mice. Mice harbouring the region (–1867/+71) of the CCT α promoter exhibited a transcriptional phenotype characterized by high-level β -galactosidase expression in lung and within alveolar epithelia. This was anticipated as robust expression of CCT α in alveolar cells is described and necessary for surfactant PtdCho biosynthesis [44–46]. Perhaps, more importantly, when tested for sensitivity to sphingosine, alveolar cells isolated from these transgenic mice displayed a co-ordinate reduction in CCT α and β -galactosidase activities, consistent with our transient transfection analysis. Thus the β -galactosidase reporter appears to mirror faithfully the endogenous gene in response to sphingosine using this *in vivo* system. Collectively, these results indicate that CCT α – β -galactosidase transgenics may be a useful model to study transcriptional modulators of CCT α expression.

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