

Betamethasone modulation of sphingomyelin hydrolysis up-regulates CTP:cholinephosphate cytidyltransferase activity in adult rat lung

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Glucocorticoids appear to play an integral role in stimulating surfactant synthesis by activating the rate-regulatory enzyme for phosphatidylcholine synthesis, CTP:cholinephosphate cytidyltransferase (CT). The activity of liver CT, *in vitro*, has been shown to be inhibited by the sphingomyelin hydrolysis product, sphingosine. In order to investigate the mechanisms by which glucocorticoids alter CT activity, *in vivo*, we administered betamethasone (1 mg/kg intraperitoneally) sequentially to adult male rats for 5 days. Betamethasone increased CT activity 2-fold relative to control in whole lung. The hormone also increased membrane-bound activity, but did not affect cytosolic enzyme activity. Betamethasone modestly increased CT mRNA as determined by the reverse-transcription PCR and Southern analysis of PCR products, but did not alter the levels of immunoreactive enzyme in lung membranes as demonstrated by Western blotting. The hormone did, however, produce a nearly 3-fold increase in membrane-associated sphingomyelin, and co-ordinately a sub-

stantial decrease in the levels of sphingosine in lung membranes. Sphingosine, but not sphinganine, was a competitive, reversible inhibitor of lung CT with respect to the enzyme activator, phosphatidylglycerol. Betamethasone decreased the activities of the sphingomyelin hydrolases: acid sphingomyelinase by 33% and of alkaline ceramidase by 21%. The hormone also inhibited the generation of sphingosine from lysosphingomyelin in lung membranes. There was no significant effect of the hormone on serine palmitoyltransferase activity, the first committed enzyme for sphingolipid biosynthesis. Further, administration of L-cycloserine, an inhibitor of sphingosine formation, was shown to stimulate CT activity by 74% and increase disaturated phosphatidylcholine in alveolar lavage by 52% relative to control. These observations suggest that glucocorticoids up-regulate surfactant synthesis at the level of a key regulatory enzyme by significantly altering the availability of inhibitory metabolites resulting from sphingomyelin hydrolysis.

INTRODUCTION

Glucocorticoids have a key role in promoting lung maturation and stimulating surfactant synthesis in the fetal lung [1,2]. These agents are also currently in use for treatment of a variety of adult lung disorders, many of which have recently been associated with a functional deficiency of surfactant [3–8]. However, compared with the fetal lung, the effects of glucocorticoids on parameters of surfactant synthesis in the adult lung has been relatively ignored. Young and Silbajoris demonstrated in adult rats that the long-term administration of glucocorticoids, *in vivo*, produced a 4-fold increase in the levels of disaturated phosphatidylcholine (DSPC) in alveolar lavage [9]. DSPC is the most distinctive lipid of alveolar surfactant, which, in part, provides the surface-tension-lowering effects of this material. Other studies have revealed significant effects of glucocorticoids on the morphological and physiological parameters of surfactant phospholipid synthesis in the adult system, although the biochemical basis for these observations has not been fully elucidated [10–12].

Prior studies in fetal lung have demonstrated that glucocorticoids increase surfactant phospholipid synthesis, in part, by increasing the activity of the enzyme CTP:cholinephosphate cytidyltransferase (CT, EC 2.7.7.15, [13–15]). This enzyme catalyses the conversion of choline phosphate into cytidine

diphosphate choline, the rate-limiting step for the *de novo* synthesis of phosphatidylcholine [16,17]. Although a number of physiological forms of CT have been described, it is generally agreed that the membrane-bound form of CT in mammalian tissues is active; however, activity is also detected in the cytosolic tissue fraction. In fetal lung, the cytosolic enzyme is in a predominantly inactive low-molecular-mass form, whereas in adult lung the cytosolic enzyme exists as an active high-molecular-mass multimer [18]. Several control mechanisms for CT have been proposed, including activation by reversible phosphorylation [19], by membrane translocation [16], by calcium [20], by enhanced gene expression [21], and by regulation by a variety of neutral and anionic lipids [14,18,22,23].

Sphingolipids have diverse biological roles including inhibition of cell differentiation, regulation of membrane stability, and signal transduction [24]. The sphingolipid degradation product, sphingosine, is generated via the sphingomyelin hydrolysis pathway. The initial step in this pathway involves hydrolysis of sphingomyelin, generating ceramide. The ceramide can then undergo deacylation to sphingosine or phosphorylation to ceramide 1-phosphate by the action of ceramide kinase [25]. The observation that sphingoid bases, such as sphingosine, are potent inhibitors of protein kinase C has stimulated considerable interest in understanding the role of such metabolites in con-

Abbreviations used: CT, CTP:cholinephosphate cytidyltransferase; RT-PCR, reverse-transcription PCR; DSPC, disaturated phosphatidylcholine; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MMLVRT, Moloney Murine Leukaemia Virus reverse-transcriptase; DTT, dithiothreitol; i.p., intraperitoneally; lyso-SP, lysosphingomyelin; ER, endoplasmic reticulum.

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trolling cell function. Recently, Sohal and Cornell reported that sphingosine, *in vitro*, inhibited CT activity isolated from rat liver [26]. In addition, other studies demonstrated that the lung contained the highest levels of sphingolipid bases [27], as well as among the highest levels of activities for some enzymes involved in sphingolipid metabolism [28,29]. These observations, together with prior studies demonstrating that glucocorticoids elevate sphingomyelin content in the lung [30,31], led us to evaluate whether endogenous sphingolipid bases, in response to glucocorticoids, regulate surfactant synthesis.

In the present study we present evidence indicating that glucocorticoids decrease the levels of free sphingosine in the lung by inhibiting sphingomyelin hydrolysis. The sphingolipid base was a potent, reversible, inhibitor of lung CT activity *in vitro*. Further, administration of L-cycloserine, an agent which depletes sphingosine in tissues, mimics the effects of glucocorticoids by increasing lung CT activity and alveolar surfactant content. Thus, these studies provide the first physiological link between sphingolipid metabolites and surfactant synthesis.

MATERIALS AND METHODS

Materials

Betamethasone, phospholipid standards, sphingomyelin, sphingosine, ceramides (Type III), pyridoxal 5'-phosphate, palmitoyl-CoA, L-serine and cycloserine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The sphinganine and sphingosylphosphorylcholine were obtained from Metraya, Inc. (Pleasant Gap, PA, U.S.A.). N-Acetylsphingosine was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). 3-Ketosphinganine was synthesized as described previously [32]. All solvents were of Optima grade (Fisher Chemical Co.). Silica LK5D (250 mm × 20 cm × 20 cm) chromatography plates were purchased from Whatman International (Maidstone, U.K.). The chemiluminescence reagents for immunodetection of CT were purchased from Amersham (Arlington Heights, IL, U.S.A.). The RNA-STAT reagents for RNA isolation were purchased from TEL-TEST 'B' Inc. (Friendswood, TX, U.S.A.). The mouse-specific primers for detection of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA by reverse-transcriptase PCR (RT-PCR) were obtained from Clontech Laboratories Inc. (Palo Alto, CA, U.S.A.). The Moloney Murine Leukaemia Virus reverse-transcriptase enzyme (MMLVRT) and all remaining PCR reaction components were purchased from Perkin-Elmer Corp. (Norwalk, CT, U.S.A.). The [*methyl*-¹⁴C]phosphorylcholine (50 mCi/mmol), [*choline methyl*-¹⁴C]sphingomyelin (54.5 mCi/mmol), [³H]acetic anhydride (50 mCi/mmol), L-[³H]serine (21.7 Ci/mmol) and autoradiographic enhancer spray (ENHANCE) were purchased from DuPont New England Nuclear Chemicals (Boston, MA, U.S.A.). The [³H]ceramide (114.4 μCi/μM, [33]) used for the ceramidase assay was a gift from Dr. Phillip Wertz (University of Iowa). The cytidyltransferase antibody to synthetic peptide was generously provided by Dr. Dennis Vance (University of Alberta, Edmonton, Canada, [34]). The cDNA for rat liver CT was kindly provided by Dr. Rosemary Cornell (Simon Fraser University, Burnaby, BC, Canada, [35]).

Animals and tissue preparation

Male Sprague-Dawley rats (Sasco Inc., Omaha, NE, U.S.A.) weighing 200–250 g were administered an intraperitoneal (i.p.) dose of either betamethasone (1.0 mg/kg [14]), or normal saline (0.5 ml) daily between 09:00 a.m. and 11:00 a.m. for five consecutive days. Each experimental and control group consisted of

four animals. On day 5, the animals were deeply anaesthetized with pentobarbital (50 mg/kg) and the lungs were surgically removed from the chest. The hilum and proximal airways were dissected free and removed from the tissue samples. Equal amounts of pooled lungs (4.38 ± 0.1 g) from control or betamethasone-treated groups were blot-dried and kept at 4 °C prior to homogenization. The cytosolic and microsomal tissue fractions were isolated following homogenization of lung tissues in Buffer A {150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2.0 mM dithiothreitol (DTT), 0.025 % sodium azide, 1.0 mM PMSF, pH 7.0 [14]} and sequential centrifugation at 10000 g for 10 min and then 125000 g for 60 min [14]. Some preparations were stored under nitrogen at –80 °C for enzyme and lipid analysis. Animals undergoing alveolar lavage were also anaesthetized as described above. The chests were opened, the trachea cannulized, the inferior vena cava severed, and the right ventricle perfused with normal saline (prewarmed at 37 °C). Bronchoalveolar lavage was performed by instilling 5 ml of normal saline in four aliquots with excellent returns. The aliquots were pooled and the lavage fluid was first subjected to centrifugation at 300 g for 10 min to remove cells, and spun again at 100000 g for 2 h. Lipid analysis was then performed on the crude surfactant pellet [36].

Enzyme assays

CT activity was determined by measuring the rate of incorporation of [*methyl*-¹⁴C]choline phosphate into CDP-choline using a charcoal extraction method [23]. Each reaction mixture contained 1.6 mM [*methyl*-¹⁴C]choline phosphate, 3.0 mM CTP, 12 mM magnesium acetate, 50 mM imidazole, 150 mM KCl and 2 mM EDTA, pH 7.0, in a total assay volume of 100 μl. The reaction was initiated by the addition of 20 μl of enzyme protein, allowed to proceed for 1 h at 37 °C, and was terminated by the addition of 10 % trichloroacetic acid. The reaction was linear with respect to time and the amount of enzyme protein added to the assay mixture. The recovery of the product, CDP-choline, was approx. 71 %. Unless stated otherwise, all assays were performed without the inclusion of a lipid activator in the reaction mixture.

Ceramidase activity was assayed using a modification of the procedure as described by Sugita et al. [37]. Each reaction mixture contained 50 μl of either 40 mM sodium acetate (pH 6) or 40 mM Tris/HCl (pH 9), 25 μl containing 62 nmol of [³H]ceramide (specific radioactivity 403 c.p.m./nmol), 0.1 mg of Tween 20, 0.25 mg of Triton X-100, and 0.4 mg of sodium cholate. The final assay volume was 600 μl. Each reaction proceeded for 2 h at 37 °C in an ultrasonic bath and contained up to 4 mg of protein in the assay mixture. Following incubation, the reaction was terminated on ice with the addition of 3 ml of chloroform/methanol (2:1, v/v), carrier palmitic acid (100 μg), and 0.6 ml of normal saline. After centrifugation, the organic layer was dried under nitrogen, spotted on TLC plates, and the fatty acid cleavage product was resolved from unreacted ceramide substrate in hexane/diethyl ether/acetic acid (70:30:1, by vol.). The areas on the plates corresponding to oleic acid standard were scraped from the gel and taken for scintillation counting. The ceramidase activity in the lung was linear using from 1 to 4 mg of added protein and the reaction expressed linearity with time up to 2 h. The recovery of the cleavage product using this method was 72 %.

Sphingomyelinase activity was assayed as described previously [38]. Each assay (0.2 ml volume) contained 25 μmol of Tris/glycine buffer (pH 7.4), 2.5 pmol of MgCl₂, 50 nmol of [*choline methyl*-¹⁴C]sphingomyelin (specific radioactivity 400 c.p.m./nmol), 0.5 mg of human serum albumin, 0.1 mg of cutscum and

50–100 μg of protein. After a 1 h incubation at 37 °C, the reaction was terminated with 1 ml of cold 10% trichloroacetic acid. Following the addition of BSA (100 μg), the mixture was centrifuged and a 1 ml aliquot of the supernatant was extracted with an equal volume of anhydrous ether at 4 °C. An aliquot of the aqueous phase was taken for scintillation counting. Lung sphingomyelinase activity was linear from 50 to 1000 μg of added protein and the reaction was linear with time up to 2 h. The recovery of the cleavage product, phosphocholine, was 77%.

Serine palmitoyltransferase activity was assayed in microsomes as described by Merrill et al. [28] and Chen et al. [39]. Each reaction mixture (300 μl total) contained 100 mM Hepes (pH 8.3), 5 mM DTT, 2.5 mM EDTA and 50 μM pyridoxal phosphate. After incubation of the mixture for 10 min at 37 °C, 150 μM palmitoyl-CoA, 1 mM L-[^3H]serine (specific radioactivity ~ 13333 c.p.m./nmol), and 300–500 μg of enzyme source were added. The reaction proceeded for an additional 35 min and was terminated with the addition of 0.2 ml of 0.5 M NH_4OH on ice. The aqueous phase was removed after the addition of 1.5 ml of chloroform/methanol (1:2, v/v), 2 ml of 0.5 M NH_4OH , and 50 μg of sphinganine as a carrier followed by centrifugation. The organic phase was rinsed twice with 2 ml of water, dried under a nitrogen stream, and the radioactivity determined by scintillation counting. Analysis by TLC using chloroform/methanol/2 M NH_4OH (40:10:0.6, by vol.) revealed that > 90% of the radiolabelled product co-migrated with the 3-ketosphinganine standard. The reaction was linear up to 45 min and from 100 to 500 μg of added protein.

Immunodetection of CT

Proteins were quantified using the Bradford assay [40]. Microsomal proteins were loaded and separated on SDS/10% -PAGE (50 μg /lane). The separated proteins were transferred from the gel to a nitrocellulose membrane at 4 °C over 5–7 h [14]. The membranes were then blocked for 4 h in Blotto at 25 °C, and subsequently exposed to rabbit anti-CT (1:500) overnight. The membranes were rinsed four times in Blotto, and exposed to goat anti-rabbit horseradish peroxidase-conjugated antibody (1:1000) for 1 h at 25 °C. The membrane was washed twice in Blotto, twice in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TTBS), and twice in TBS for 10 min each. The membrane was then exposed to the chemiluminescence reagents for 1 min and exposed to autoradiographic film (10 min) in order to detect immunoreactive proteins.

RT-PCR for CT mRNA

Total RNA was isolated by a single-step isolation procedure using an RNA-STAT[®] isolation solution based on the single-step method of RNA isolation described by Chomczynski and Sacchi [41]. A sample (2 μg) of total RNA was treated with DNase 1 to remove DNA contaminants, followed by incubation with 25 mM EDTA to chelate excess magnesium ions. The samples were then subjected to RT-PCR in both the presence and absence of the recombinant MMLVRT enzyme in a PCR 9600 Thermocycler. The reaction proceeded in a total volume of 50 μl . Samples were incubated at 37 °C for 60 min, followed by 3 min at 94 °C. Aliquots (10 μl) of the resulting RT-PCR products were incorporated into PCR reactions. Initially, we used these methods to detect the housekeeping gene, G3PDH, as an internal standard to assess the quality of the RNA, to correct for loading artifact of the RNA gels, and to ensure that there was no DNA contamination of our RNA preparations. The PCR was com-

posed of 30 cycles: 94 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min, followed by 72 °C for 7 min. The primer sequences for CT-PCR were as follows: RM CT 1 5'-AGTGCAGCGCTGTGCAGTC-3' and RM CT 2 5'-ACAGGTCACAGCCTTGAC-3'. These sequences correspond to +199 to +218 and +1160 to +1179 respectively of rat CT cDNA sequence [35] producing a 980 bp fragment; the mouse-specific primers used for G3PDH resulted in a 982 bp fragment (corresponding to 5' +51 to +75, 3' +1010 to +1033 of mouse G3PDH cDNA sequence). PCR products were separated by agarose electrophoresis and then analysed after Southern blotting and probing with random-primed ^{32}P -radiolabelled PCR cDNA control products for G3PDH and CT respectively. Membranes were subjected to autoradiography at -70 °C.

Lipid analysis

Phospholipid analysis was performed on lavage fluid and lung membranes. Phospholipids in lung membranes were extracted using the Bligh–Dyer method [42], separated in chloroform/methanol/petroleum ether/acetic acid/boric acid (40:20:30:10:1.8, by vol.) on LK5D silica plates, and measured quantitatively using the phosphorus assay [14,43]. The levels of DSPC in alveolar lavage were measured as described previously using osmium tetroxide [43]. Sphingosine was assayed using an acylation method recently described by Ohta et al. [44]. Lipids from equal amounts of protein from control and hormone-treated tissues were extracted by adding chloroform/methanol (1:2, v/v) to the preparations. Each experiment was run with known amounts of sphingosine standards. After mixing, phases were separated by the addition of 2 ml each of chloroform and 1 M NaCl. The lower chloroform phase was mixed with 3 ml of 0.2 M NaOH in methanol. After a 1 h incubation, the phases were separated with 3 ml each of chloroform and 1 M NaCl. The lower organic phase was rinsed three times with 3 ml of chloroform/methanol/water (3:48:47, by vol.) and the organic phase was dried under a nitrogen stream. The samples were then acylated by dissolving the lipid residue in 40 μl of 0.008 M NaOH in redistilled methanol/100 mM solution of [^3H]acetic anhydride (1:1, v/v) by sonication. The samples were acylated for 1 h at 37 °C. The excess acetic anhydride was removed by adding 0.2 ml of 0.2 M NaOH in methanol. After a 1 h incubation, the radiolabelled C_2 ceramide product was extracted in 0.78 ml of methanol, 0.98 ml of chloroform, and 0.9 ml of 1 M NaCl. The lower organic phase was rinsed again twice with 1 ml of chloroform/methanol/water (3:48:47, by vol.) and the organic phase was dried under nitrogen, resuspended in 40 μl of chloroform/methanol (4:1, v/v), and spotted on TLC plates. The plates were developed in chloroform/methanol/7 M NH_4OH /water (80:20:0.5:0.5, by vol.). The plates were sprayed with an autoradiographic enhancer spray and exposed to film for typically 48 h. In order to quantify the levels of lung sphingosine, we processed known amounts of sphingosine standards through the above procedure. The films were analysed densitometrically and compared with densitometric values of these known standards. In some studies, we confirmed our results using this method with a sphingosine assay as described by Van Veldhoven et al. [45].

Effect of sphingolipid bases on lung CT activity *in vitro*

Cytosolic preparations of lung were used to chromatographically partially purify CT as described previously [23]. Stock solutions of phosphatidylglycerol (10 mg/ml), sphingosine (10 mg/ml) and sphinganine (5 mg/ml) were prepared by dissolving the in-

dividual lipids in chloroform. An aliquot of the stock lipid was dried under a nitrogen stream and resuspended in an aliquot of buffer (50 mM imidazole, 150 mM KCl and 2 mM EDTA [14]) by brief sonication at 4 °C using a 250 W (Tekmar) pulse sonicator to achieve a final stock concentration of 200 μ M. Cytidylyltransferase activity was then assayed in the presence of 0–100 μ M of lipid as described previously. All lipid preparations were made fresh prior to performing the enzyme assay.

RESULTS

CT activity

The administration of betamethasone to adult rats produced a significant increase in CT activity in whole lung and in the microsomal tissue fraction. Consistent with prior reports, adult lung cytosol contained the bulk of the activity [18]. When specific activity was expressed as pmol/min per mg of tissue protein, enzyme activity increased by 43% in lung homogenate and by 29% in the microsomes after hormone treatment. However, these changes may not accurately reflect the effects of the hormone on a per cell basis, since the majority of protein detected in the adult lung represents a contribution from the extracellular compartment [46,47]. In addition, glucocorticoids have been shown to have inhibitory effects on cell replication and DNA synthesis [48]. Thus, when these data were corrected for DNA content, a much greater stimulatory effect of betamethasone was observed in the adult lung (Table 1). Enzyme activity increased in lung homogenate from 809 ± 94 pmol/min per mg of DNA (control) to 1599 ± 243 pmol/min per mg of DNA (betamethasone), a 98% increase in activity. The activity of the membrane-associated enzyme also increased from 819 ± 74 pmol/min per mg of DNA to 1364 ± 232 pmol/min per mg of DNA after hormone treatment (a 67% increase), when we expressed microsomal CT activity on a per cell basis using DNA values derived from our tissue homogenates. Betamethasone did not significantly alter cytosolic CT activity.

Immunodetection of CT, and RT-PCR and Southern analysis for CT mRNA

In contrast to its effects on enzyme activity, betamethasone did not alter the levels of immunoreactive CT in lung microsomes (Figure 1) or in whole lung (results not shown). CT mRNA in adult lung was undetectable by Northern blot analysis, therefore we measured CT message using RT-PCR and Southern blotting.

Table 1 The effect of betamethasone on the subcellular distribution of CT in adult rat lung

Adult male rats were administered betamethasone (1 mg/kg per day i.p.) or diluent for 5 days. The lung tissues were isolated and assayed for CT activity. All enzyme activities were determined in the absence of lipid activator in the assay mixture. The results are expressed as the mean \pm S.E.M. The data are representative of four separate experiments; each experiment consisted of eight rats. The values in parentheses represent enzyme activity as pmol/min per mg of DNA. Statistical analysis was performed using Student's *t*-test.

Subcellular fraction	Activity (pmol/min per mg of protein)		<i>P</i> value
	Control	Betamethasone	
Homogenate	56.3 ± 6.3 (809 ± 94)	80.5 ± 10.7 (1599 ± 243)	< 0.005
Cytosol	369.2 ± 34.9	358.1 ± 29.3	NS
Microsomes	51.8 ± 2.0	66.7 ± 6.1	< 0.05

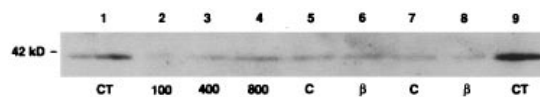


Figure 1 Immunodetection of CT in adult lung

Adult rats were administered betamethasone (1 mg/kg for 5 days) and the levels of immunoreactive protein were determined in lung membrane preparations by Western blotting. Lanes 1 and 9, purified CT from rat liver (10 μ g); lanes 2–4, 100 μ g, 400 μ g and 800 μ g of protein from crude rat lung membranes extracts; lanes 5–8 were loaded with equal amounts of membrane protein (400 μ g) isolated from either control (C) or betamethasone (β)-treated membrane extracts. The results are representative of three separate experiments.

We did not observe bands when RT-PCR and Southern blot analysis were performed without the reverse-transcription step, indicating that our amplified RT-PCR products were not due to DNA contamination of our RNA preparations. We also amplified cDNA from our reverse-transcription reaction using primers for the housekeeping gene, G3PDH, as an internal standard. RT-PCR and Southern blot analysis revealed that betamethasone increased the amount of a predicted 980 bp CT product approx. 1.6-fold when we corrected for loading artifact using G3PDH (Figure 2).

Lipid analysis

Betamethasone stimulation of membrane-bound CT activity was associated with an increase in the amount of specific microsome-associated phospholipids. The hormone modestly increased the levels of phosphatidylcholine by 22% ($P = 0.09$) and phosphatidylinositol by 41% ($P < 0.05$) compared with control values (Table 2). In contrast, betamethasone produced the greatest increase in the amount of membrane-associated sphingomyelin, which increased nearly 3-fold relative to control ($P < 0.005$). These effects of betamethasone on membrane phospholipids were also associated with an increase in the amount of DSPC in alveolar lavage by 2-fold compared with control values (results not shown).

Since the glucocorticoid produced a marked increase in the mass of sphingomyelin, suggesting that the hormone might also alter the levels of sphingomyelin hydrolysis products, we assayed

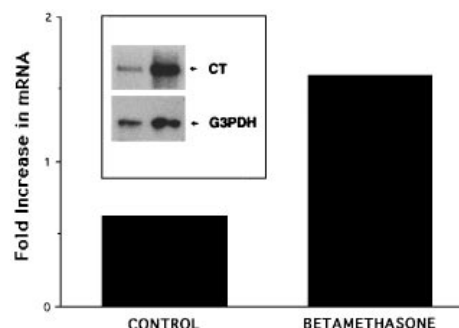


Figure 2 Detection of CT mRNA by RT-PCR and Southern blotting

Total RNA was isolated from control and betamethasone-treated adult lung preparations. Reverse-transcription (RT) and the PCR were performed using 1 μ g of total RNA. Following 30 cycles of PCR, Southern blotting revealed the presence of the CT product [980 bp (insert)]; a 982 bp product was also detected for G3PDH. Below: densitometric analysis of the autoradiograms was performed expressing the levels of CT as a ratio of CT/G3PDH as derived from arbitrary densitometric values.

Table 2 Effect of betamethasone on the phospholipid composition of microsomes in adult rat lung

Adult rat membranes were isolated after animals were treated with betamethasone (1 mg/kg per day \times 5 days) or diluent. Total lipids were extracted from 800 μ g of membrane protein and individual phospholipids were separated using TLC. The phospholipids were eluted from the gel and quantified using the phosphorus assay. The phospholipids assayed include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM). The data are expressed as nmol of phospholipid phosphorus (P)/mg of microsomal protein. The results represent the mean \pm S.E.M. of four separate experiments. Statistical analysis was performed using Student's *t*-test.

Phospholipid	Content (nmol of lipid P/mg of protein)	
	Control	Betamethasone
PC	29.3 \pm 2.0	35.7 \pm 2.0
PE	20.7 \pm 0.9	16.3 \pm 1.3
PG	7.0 \pm 1.5	10.0 \pm 1.7
PI	7.3 \pm 0.3	10.3 \pm 0.3*
PS	9.7 \pm 0.9	5.0 \pm 0.6
SM	13.3 \pm 0.9	35.7 \pm 3.5*

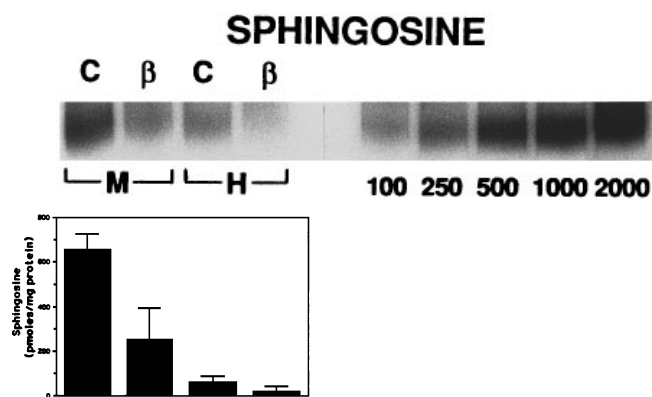
* $P < 0.05$ versus control.

Table 3 Effect of betamethasone on acid and neutral sphingomyelinase and acid and alkaline ceramidase activities in adult rat lung

Adult male rats were administered betamethasone (1 mg/kg per day i.p.) or diluent for 5 days. The lung tissues were isolated and assayed for sphingomyelinase and ceramidase activities. All enzyme activities are expressed as nmol/h per mg of protein. The results are expressed as the mean \pm S.E.M. The data are representative of three separate experiments; each experiment consisted of eight rats. Statistical analysis was performed using Student's *t*-test.

Enzyme	Activity (nmol/h per mg of protein)	
	Homogenate	Microsomes
Sphingomyelinase, pH 4.0		
Control	57.53 \pm 6.5	16.64 \pm 1.0
Betamethasone	38.39 \pm 4.3*	13.69 \pm 1.2
Sphingomyelinase, pH 7.4		
Control	12.01 \pm 0.48	12.20 \pm 0.73
Betamethasone	11.42 \pm 0.18	11.46 \pm 0.97
Ceramidase, pH 6.0		
Control	0.65 \pm 0.06	0.44 \pm 0.03
Betamethasone	0.52 \pm 0.05	0.35 \pm 0.04
Ceramidase, pH 9.0		
Control	1.05 \pm 0.05	0.92 \pm 0.04
Betamethasone	0.83 \pm 0.05**	0.68 \pm 0.04***

* $P < 0.05$; ** $P < 0.005$; *** $P = 0.001$.

**Figure 3** Effect of betamethasone on lung sphingosine content

Sphingolipids from 1 mg of lung membrane (M) and homogenate (H) preparations were extracted and acylated with [3 H]acetic anhydride as described [44]. The [3 H] C_2 -ceramide product was resolved using TLC and subjected to autoradiography. Above right: various amounts of sphingosine standards (100–2000 pmol) were acylated and chromatographed as described above. The N-acylated sphingosine product was also assayed in control (C) and betamethasone (β)-treated lung preparations. Below: densitometric analysis of the autoradiograms was performed in order to quantify the levels of endogenous lung sphingosine in control and hormone-treated lung extracts. The results are representative of four separate experiments.

the levels of endogenous free sphingosine in the lung (Figure 3). The levels of sphingosine in whole lung rat tissue were approx. 67 pmol/mg of protein; however, the levels of sphingosine in whole lung homogenates were almost undetectable after betamethasone treatment (Figure 3). In addition, betamethasone decreased the amount of sphingosine in lung microsomes from 659 \pm 67 pmol/mg of protein (control) to 252 \pm 143 pmol/mg of protein (betamethasone) (a 61% decrease, Figure 3). In results not shown, these findings were confirmed by a second method involving purification of sphingolipids using ion-exchange chromatography, acylation using hexanoic anhydride, and quan-

titative phosphorylation to N- C_6 -[32 P]sphingosine phosphate [45]. These results suggest that betamethasone might increase CT activity by decreasing the amount of an endogenous sphingolipid inhibitor for the enzyme.

Effect of betamethasone on sphingomyelin hydrolases

We next assayed the levels of lung sphingomyelinase and ceramidase activities in order to determine if the inhibitory effects of glucocorticoids on lung sphingosine formation were due to inhibition of sphingomyelin hydrolysis. The optimal conditions for time and enzyme concentrations were used to assay enzyme activities. The activity of lung sphingomyelinase demonstrated pH dependence, as activity of the enzyme was highest at an acid pH optima (results not shown).

Betamethasone produced a significant decrease in acid sphingomyelinase activity in whole lung by 33% ($P < 0.05$ versus control, Table 3). The hormone also decreased acid sphingomyelinase activity in the membrane fraction by 18%, although these effects did not reach statistical significance ($P = 0.09$). There was no significant effect of betamethasone on neutral sphingomyelinase activity (Table 3). In addition to acid sphingomyelinase, betamethasone also consistently decreased lung ceramidase activity (Table 3). Lung ceramidase activity was observed to exhibit acid and alkaline pH optima (results not shown). The hormone significantly inhibited alkaline ceramidase activity by 21% and 26% in lung homogenate and membranes respectively. Betamethasone also tended to decrease acid ceramidase activity in the membrane tissue fraction by 20% ($P = 0.07$). These results suggest that betamethasone may be decreasing sphingosine mass by inhibiting the hydrolysis of sphingomyelin.

Effects of betamethasone on *de novo* sphingosine synthesis

The levels of endogenous sphingosine in tissues may also be regulated by *de novo* synthesis. This can occur by initially generating sphinganine or an N-acylsphinganine which can then be converted into sphingosine [49]. We assayed the activity of serine palmitoyltransferase since this is the first committed

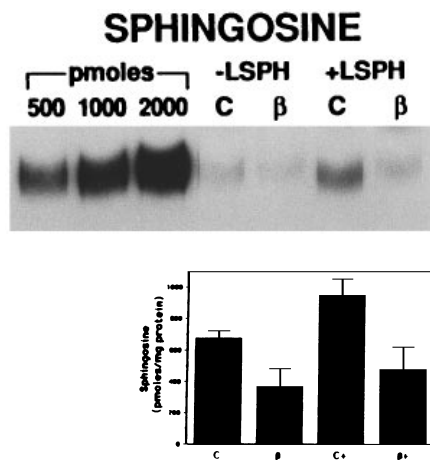


Figure 4 Effect of betamethasone on the generation of sphingosine from lysosphingomyelin in lung membranes

Sphingolipids from 0.5 mg of lung membrane were extracted and the amount of [^3H] C_2 -ceramide product was assayed directly (without incubation of extracts with lysosphingomyelin [-LSPH]), or after incubation of membranes for 1 h at 37 °C with 1000 pmol of lysosphingomyelin (+LSPH). The N-acylated sphingosine product was assayed in control (C) and betamethasone (β)-treated membrane preparations. Above left: various amounts of sphingosine standards (500–2000 pmol) were acylated and chromatographed as described previously. Below: densitometric analysis of the autoradiograms was performed in order to quantify the levels of sphingosine in control and hormone-treated lung extracts, in the presence or absence of LSPH.

enzyme required for the *de novo* synthesis of long-chain sphingoid bases. Betamethasone did not significantly change the specific activity of the microsomal enzyme [459 ± 50 pmol/h per mg (control) versus 472 ± 78 pmol/h per mg (betamethasone)] when activity was assayed optimally in the presence of palmitoyl-CoA.

Generation of sphingosine from lysosphingomyelin (lyso-SP)

It has been demonstrated that sphingosine in hepatic membranes may be generated from the direct cleavage of the phosphocholine head group from lyso-SP [50]. In order to evaluate this potential mechanism for the effects of glucocorticoids, we incubated control and betamethasone-treated lung membrane extracts in the presence and absence of 1000 pmol of lyso-SP. As shown in Figure 4, compared with control extracts, betamethasone produced a 45% decrease in the amount of sphingosine. In addition, when control lung membranes were incubated for 1 h in the presence of lyso-SP, the amount of sphingosine detected increased substantially over similar control extracts which were analysed without the inclusion of sphingolipid substrate. These data suggest that lung membranes are capable of actively generating sphingosine from lyso-SP. By contrast, betamethasone extracts incubated in the presence of exogenous lyso-SP did not display similar increases in the levels of sphingosine compared with betamethasone extracts that were not incubated with lyso-SP. Thus, the glucocorticoid appears to inhibit phosphocholine hydrolysis of lyso-SP precursor.

Kinetic effects of sphingosine on CT activity

The direct effects of sphingosine or sphinganine on lung CT activity were evaluated by adding commercially available preparations of these lipids to partially purified CT *in vitro*. We compared the effects of these sphingolipids on CT activity alone, or when combined with phosphatidylglycerol, a known activator

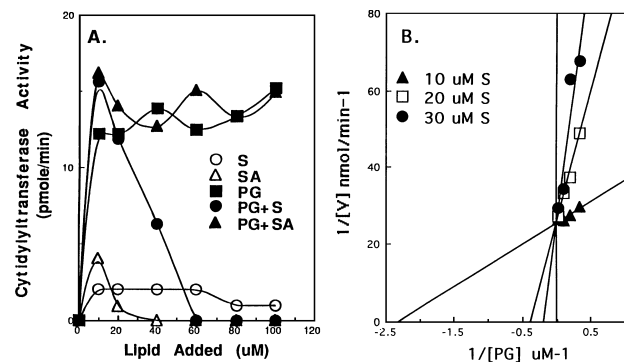


Figure 5 Effects of sphingolipid bases on lung CT activity *in vitro*

(A) Commercially available suspensions of sphingosine (S) and sphinganine (SA) were prepared at various concentrations and added to partially purified CT (20 μg) either alone, or as a vesicular preparation in combination with a fixed amount (20 μM) of the enzyme activator, phosphatidylglycerol (PG), in the assay mixture. (B) Double-reciprocal plots demonstrating competitive inhibition of CT activity in the presence of various concentrations of sphingosine. CT activity was assayed in the presence of 3–60 μM PG in the reaction mixture. The data are expressed as $1/V$ (nmol/min) of CT activity versus the reciprocal of PG concentration. The results are representative of three separate experiments.

of CT (Figure 5A). The addition of either sphingosine or sphinganine to CT resulted in minimal changes in CT activity over a broad range of concentrations. Phosphatidylglycerol produced a marked increase in CT activity at relatively low concentrations in the assay mixture. The addition of increasing amounts of sphinganine with a fixed amount of phosphatidylglycerol (20 μM) did not alter activation of CT by the anionic phospholipid. In contrast, when increasing molar ratios of sphingosine to a fixed amount of phosphatidylglycerol (20 μM) were added together as a vesicular preparation in the reaction mixture, sphingosine above 10 μM produced a dose-dependent decrease in CT activity, suggesting inhibition (Figure 5A). Similar results were obtained with phytosphingosine (results not shown).

Kinetic studies revealed that sphingosine was a competitive, reversible, inhibitor of CT with respect to the lipid activator, phosphatidylglycerol (Figure 5B). Double-reciprocal plots demonstrated that in the presence of various concentrations of sphingosine and increasing amounts of phosphatidylglycerol in the assay mixture, sphingosine altered the K_m ; however, it did not affect the V_{max} .

Effects of L-cycloserine on surfactant metabolism

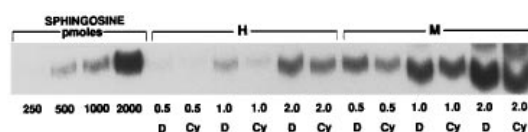
In an attempt to further investigate whether sphingolipid metabolites may have a direct role in regulating CT activity *in vivo*, we administered L-cycloserine, an agent which has previously been shown to inhibit sphingosine biosynthesis by inhibiting serine palmitoyltransferase activity [51]. After three doses of L-cycloserine, CT activity increased in lung membranes by a modest 17% relative to control levels. However, 5 doses of L-cycloserine (50 mg/kg *i.p.* given over 3 days) stimulated membrane-associated CT activity from 87 ± 5 pmol/min per mg to 151 ± 5.3 pmol/min per mg (a 74% increase) compared with rats treated with diluent (Table 4). In separate studies, the sphingolipid inhibitor was also observed to increase the amount of DSPC in alveolar lavage nearly 52% compared with control animals (Table 4). Throughout the drug treatment period, the animals remained healthy and there were no observed signs of toxicity. In addition, the agent had no significant effect on dry

Table 4 Effect of L-cycloserine on parameters of surfactant lipid synthesis in adult rat lung

L-Cycloserine (50 mg/kg i.p.) or diluent was administered to adult male rats and microsomal-associated CT activity was assayed after either three or five doses of this agent. The levels of DSPC in alveolar lavage were also measured (as described in the Materials and methods section) after five doses of L-cycloserine (50 mg/kg). Each experiment consisted of three control and three L-cycloserine-treated animals. Statistical analysis was performed using Student's *t*-test.

Parameter	Control	L-Cycloserine
CT activity (nmol/min per mg)		
3 Doses	77.6 ± 1.8	91.0 ± 0.9
5 Doses	87.0 ± 5.0	151.0 ± 5.3*
DSPC content (nmol of lipid P/mg of protein)	38.8 ± 1.7	58.8 ± 2.0*

* *P* < 0.001 versus control.

**Figure 6** Effect of L-cycloserine on lung sphingosine content

L-Cycloserine (50 mg/kg i.p., [Cy]) or diluent (D) were administered to adult male rats (50 mg/kg i.p. × 3 doses over 3 days) and the levels of sphingosine were measured in lung homogenate (H) and membrane (M) preparations. Lipids were extracted from various amounts of lung protein (0.5–2 mg of protein) and acylated with [³H]acetic anhydride, resolved using TLC, and autoradiographed as described in the Materials and methods section. Left: various amounts of sphingosine standards (250–2000 pmol) were also acylated and chromatographed with lung lipid extracts.

lung weight (control = 0.53 ± 0.06 g versus L-cycloserine = 0.49 ± 0.06 g). Finally, the administration of L-cycloserine (50 mg/kg i.p., three doses) produced a substantial decrease in the levels of sphingosine both in lung homogenate and membrane preparations (Figure 6). Although L-cycloserine is a general inhibitor of enzymes that utilize pyridoxal 5'-phosphate as a cofactor, these results suggest that depletion of tissue sphingosine by an alternative mechanism (i.e. inhibition of sphingoid base biosynthesis) also mimics the effects of glucocorticoids by stimulating surfactant synthesis.

DISCUSSION

The biochemical mechanism(s) by which glucocorticoids stimulate surfactant phospholipid synthesis have remained elusive. Although these agents have been shown to be useful for the prevention of the neonatal respiratory distress syndrome, a condition associated with a primary surfactant deficiency, glucocorticoids are also in use to treat a number of adult lung disorders, many of which have recently been implicated with a functional deficiency of surfactant [3–7]. Thus, these studies were conducted in the adult lung model. To date, all prior studies which have utilized fetal lung tissue have demonstrated that glucocorticoids increase surfactant phospholipid synthesis by increasing CT activity. In this study, we demonstrate that CT in the adult lung is also glucocorticoid-responsive. More importantly, we present evidence that these agents activate CT, in part, by decreasing the levels of endogenous free sphingosine, an inhibitor of the enzyme.

Glucocorticoid stimulation of CT activity in adult lung tissue differs from prior results observed in fetal lung [14]. First, unlike in fetal lung, where the hormone stimulates the cytosolic enzyme, in these studies the effects of betamethasone were primarily observed on the microsomal enzyme. These differences may be related to the fact that most of the cytosolic enzyme in fetal tissue is physiologically inactive, whereas in the adult lung the cytosol contains the majority of activity, which primarily exists as an active high-molecular-mass species [18,22,23]. Moreover, when enzyme activity measurements in these studies were expressed on a pmol per mg basis, the effects of the hormone on CT activation in adult tissue were relatively modest, compared with a 2–3-fold increase in activity seen in fetal lung [14]. However, it has been demonstrated that significant changes occur both during lung maturation and in the setting of glucocorticoid treatment with regard to the relative amounts of cellular and extracellular components. For example, the number of type II cells/mg of lung protein in adult lung decreases to one-third of that observed in fetal lung [47]; these changes are largely attributed to a marked increase in extracellular matrix, as connective tissue comprises nearly 60% of dry lung weight in the adult lung [46]. Further, it has been demonstrated that glucocorticoids decrease lung DNA content and [³H]thymidine incorporation into DNA, indicative of inhibitory effects on cell proliferation [48]. Thus, when we expressed our data as pmol/min per mg of DNA, betamethasone elevated CT activity to levels typically seen in fetal lung tissue.

Betamethasone stimulation of CT activity was not due to an increase in the amount of enzyme mass. Although the hormone produced an increase in CT mRNA in these studies, these effects appeared to be relatively small and were not associated with an increase in immunoreactive CT. The increase in CT message by betamethasone observed using our methods, however, is consistent with prior results in fetal lung explant culture where glucocorticoids also appear to exert modest pretranslational effects on CT mRNA [52,53]. On the basis of these results, we cannot exclude the possibility that the hormone may have altered either the translational efficiency of CT mRNA or the degradation of enzyme protein in lung tissues. Alternatively, it is possible that betamethasone may have produced a small increase in the amount of enzyme at a time point other than when we performed our immunoblot analysis.

We investigated a post-translational activation mechanism for the effects of glucocorticoids on CT activity, since our data did not indicate that betamethasone altered the levels of immunoreactive enzyme. In several experimental models, glucocorticoids have been shown to increase the amount of sphingomyelin [14,30,31,54]. Betamethasone also increases microsomal-associated sphingomyelin in the lung, suggesting that the hormone might have inhibitory effects on the generation of the downstream sphingomyelin hydrolysis product, sphingosine. This sphingoid base has been reported to be an inhibitor of liver CT [26]. Indeed, in these studies sphingosine proved to be a competitive, reversible inhibitor of lung CT with respect to the enzyme activator phosphatidylglycerol; these effects were specific to sphingosine and were not observed with sphinganine. In addition, preliminary studies using lung cytosolic CT have demonstrated that sphingosine is a competitive inhibitor of the enzyme with respect to the substrate, CTP (R. G. Salome and R. K. Mallampalli, unpublished work). More importantly, in these studies we observed that the long-term *in vivo* administration of betamethasone produced a substantial decrease in lung sphingosine. These results were confirmed using two separate assay systems which were sensitive for detection of endogenous sphingosine in the picomolar range. Interestingly, the amount of

sphingosine in whole lung rat tissue was in the low picomolar range, which is in agreement with the levels reported previously in murine lung using a method of derivatization of sphingoid bases with *o*-phthalaldehyde and HPLC [27]. As anticipated, much higher levels of the sphingoid base was detected in the microsomal fraction. The effects of betamethasone on sphingosine content were also more pronounced in the lung microsomal fraction. The extent to which these changes are physiologically significant remains to be established since high concentrations of sphingosine were required in our *in vitro* studies to achieve inhibition of CT activity.

Betamethasone decreased lung sphingosine, in part, by inhibiting sphingomyelin hydrolysis. Although the activities of sphingomyelinase and ceramidase have been reported in whole lung tissue [29,55], the regulation of these hydrolases in the lung has not been studied. Only low levels of sphingomyelinase activity were observed at a neutral pH in lung tissue, with most of the activity identified when the enzyme was assayed at an acid or lysosomal pH optima. The effects of betamethasone were accordingly observed on acid sphingomyelinase activity, which decreased by 33% after hormonal treatment. In addition to sphingomyelinase, the glucocorticoid also tended to decrease acid ceramidase activity and significantly inhibit alkaline ceramidase activity (Table 3). Since the intracellular levels of long-chain bases may also be regulated by *de novo* synthesis of *D*-erythro-sphinganine, which may then be modified directly to sphingosine [49], we assayed the activity of serine palmitoyltransferase. Betamethasone did not alter the activity of this enzyme. However, betamethasone did inhibit the activity of a phosphocholine hydrolase (Figure 4), suggesting that an alternative route for sphingosine formation may be both physiologically relevant and hormonally regulated. Finally, these results differ from the effects of dexamethasone in 3T3-L1 fibroblasts where the hormone was observed to increase neutral sphingomyelinase activity and elevate sphingosine content [54]. However, these differences can be reconciled, in part, by the fact that the latter study investigated the effects of glucocorticoids on sphingomyelin hydrolysis after short-term exposure using a different model system; perhaps these disparities are also indicative of the complexity involved in the response of the sphingolipid system to the effects of glucocorticoids.

The topographical relationships between these various parameters of phospholipid and sphingolipid metabolism are also important. The endoplasmic reticulum (ER) is enriched with phosphatidylcholine, the site of surfactant lipid synthesis [56]. In addition, the membrane form of CT has been localized to the ER [57], Golgi [58], and the nuclear membrane [59], whereas the cytosolic form may actually represent enzyme that originates from the nuclear membrane [60]. Sphingomyelin, on the other hand, is present at high levels in the plasma membrane, but is also detected in the ER [61]. The enzymes that catalyse the initial steps involved in the biosynthesis of sphingomyelin, including serine palmitoyltransferase, have also recently been localized to the ER [62]. However, the intracellular site(s) of sphingomyelinase, ceramidase and the phosphocholine hydrolase have not been conclusively identified, although our microsomal preparations, which contain both ER and plasma membranes, appear to express activities for these enzymes and are enriched with sphingosine (Figure 3). It is conceivable, therefore, that the effects of betamethasone on phosphatidylcholine synthesis and sphingolipid metabolism are co-ordinated primarily within the ER, because it contains several of these elements involved in lipid metabolism; it is less likely that these processes occur within the plasma membrane because it appears to lack CT [57].

Despite the recent introduction of inhibitors of sphingomyelin

hydrolysis, limited experience is available with regard to the specificity and suitability of these agents for *in vivo* studies [37,63]. *L*-Cycloserine, an inhibitor of sphingolipid biosynthesis, has been proposed as potential therapy for various glyco-sphingolipid storage disorders in view of the fact that this agent, when administered *in vivo*, depletes tissues of sphingosine [51,64]. We selected this agent in order to test our hypothesis that metabolic inhibitors of sphingosine formation, similar to glucocorticoids, will increase CT activity, and thus surfactant synthesis. The long-term administration of *L*-cycloserine in the present study produced a substantial decrease in the mass of sphingosine in lung tissues (Figure 6). Further, after five doses of *L*-cycloserine, microsomal CT activity increased by 74%. The agent also produced a significant increase in alveolar lavage DSPC, similar to the effects of glucocorticoids.

In summary, these studies suggest that glucocorticoids activate CT by decreasing the availability of inhibitory sphingolipid metabolites in the lung. These effects of glucocorticoids on parameters of surfactant lipid synthesis are also reproduced by the administration of a metabolic inhibitor, which decreases the levels of free sphingosine in tissues. In view of the fact that elevated sphingolipid metabolites have been observed in surfactant-deficient disorders such as acute lung injury [65], these studies lend support to the supposition that the sphingolipid system has physiological significance as it relates to surfactant metabolism. These results also raise the possibility that the pharmacological manipulation of sphingolipid metabolism may serve as a new strategy for stimulating surfactant synthesis in the future.

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