LASS5 is the predominant ceramide synthase isoform involved in de novo sphingolipid synthesis in lung epithelia

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Abstract **Ceramide is a key bioactive mediator that inhibits surfactant phosphatidylcholine (PtdCho) synthesis in lung epithelia. Ceramide availability is governed by sphingomyelin (SM) hydrolysis, but less is known regarding its de novo synthesis. In this study, we observed that ceramide synthesis within murine lung epithelia was associated with high-level ceramide synthase (dihydroceramide synthase) activity. Longevity assurance homolog 5 (LASS5) was the predominant ceramide synthase isoform detected in lung epithelia, whereas relatively lower level expression was detected for the other five mammalian homologs. Pulmonary LASS5 was developmentally regulated, but its expression was spatially and gender nonspecific. Exogenously expressed LASS5 in lung epithelia was membrane-associated, triggering increased ceramide synthesis, whereas knockdown studies using fumonisin B_1 or LASS5 small, interfering RNA reduced ceramide synthase activity by 78% or 45%, respectively. Overexpression of LASS5 also reduced PtdCho synthesis, but maximal inhibition was achieved when LASS5 was coexpressed with a plasmid encoding a neutral sphingomyelinase involved in SM hydrolysis. These results demonstrate that LASS5 is the major ceramide synthase gene product involved in sphingolipid production that may also regulate PtdCho metabolism in pulmonary epithelia.**—Xu, Z., J. Zhou, D. M. McCoy, and R. K. Mallampalli. **LASS5 is the predominant ceramide synthase isoform involved in de novo sphingolipid synthesis in lung epithelia.** ***J. Lipid Res.*** **2005.** 46: **1229–1238.**

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Ceramide has emerged as a key signaling molecule involved in the inflammatory response, cell growth and differentiation, apoptosis, and cell senescence (1–4). In the lung, ceramide produces pulmonary injury in response to injurious cytokines and inflammatory mediators (1). Ceramide potently induces programmed cell death in lung cells and inhibits the biosynthesis of phosphatidylcholine (PtdCho), the predominant phospholipid of alveolar surfactant (1, 5–8). Thus, there has been growing interest in investigations of the metabolic pathways for the generation of ceramide that might lead to newer strategies in manipulating the content of these bioactive sphingolipid products in mammalian cells.

Prior studies have demonstrated that ceramides are metabolically derived, in part, from sphingomyelin (SM) hydrolysis (9). In this pathway, one of several membrane-associated sphingomyelinases (SMases) catalyze the rapid hydrolysis of SM to ceramide in response to a physiologic stress signal. These signals include tumor necrosis factor-α (TNF-α) and platelet-activating factor, which activate SMases, resulting in ceramide formation (9, 10). Furthermore, interruption of SMase activation using genetic or pharmacologic strategies attenuates lung injury, underscoring the observation that the SM hydrolysis pathway is a critical bioeffector mechanism for ceramide formation (10, 11).

Unlike SM hydrolysis, there is more limited information regarding ceramide generation via the de novo biosynthetic pathway. The first step in this pathway involves the condensation of serine with palmitoyl-CoA catalyzed by serine palmitoyltransferase (SPT; EC 2.3.1.50), generating 3-ketosphinganine. This product is immediately reduced to sphinganine by the enzyme 3-ketosphinganine reductase (EC 1.1.1.102). Acylation of sphinganine to dihydroceramide (or to ceramide if sphingosine is available) is catalyzed by ceramide synthase (dihydroceramide synthase, sphinganine *N*-acyltransferase, sphingosine *N*-acyltransferase; EC 2.3.1.24). Dihydroceramide, a physiologically inactive intermediate, is converted to bioactive ceramide by dihydroceramide desaturase. In this pathway, the molecular regulation of ceramide synthase may be of particular in-

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Abbreviations: CCT, CTP:phosphocholine cytidylyltransferase; FB1, fumonisin B_1; LASS1, longevity assurance homolog 1; PtdCho, phosphatidylcholine; siRNA, small interfering RNA; SM, sphingomyelin; SMase, sphingomyelinase; SPT, serine palmitoyltransferase; TNF-α, tumor necrosis factor-α.

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terest, as recent studies indicate that this enzyme is a metabolic target for TNF-α (12–14).

Ceramide synthase has been partially purified, but very little is known about its enzymology and regulation (15). The enzyme localizes to the endoplasmic reticulum, and its activity is inhibited by fumonisins, fungal inhibitors that produce pulmonary edema (16, 17). Ceramide synthase effectively catalyzes *N*-transacylation of either sphinganine to form dihydroceramide or sphingosine to form ceramide and uses a multitude of fatty acyl-CoA species. These properties are indicative of the enzyme's relative substrate nonselectivity or reflect the existence of multiple isoenzymes (15). Indeed, in mammalian cells, three known functional isoforms exist: longevity assurance homolog 1 (LASS1), LASS4, and LASS5. These isoforms are mammalian homologs of the yeast longevity assurance gene 1, proteins that regulate life span (18). Overexpression of these isoforms in human embryonic kidney (293T) cells confers ceramide synthetic activity, although each isoform exhibits varying substrate selectivity (18, 19). Both LASS4 and LASS5 are expressed ubiquitously in murine tissues, in contrast to LASS1, the expression of which is more restricted; mRNAs for all three isoforms are also detected in the lung (18). Additional variants that display a high degree of homology to these mammalian isoforms have been identified [LASS2, LASS3, and LASS6 (18)]. However the physiologic role of these latter isoforms is not yet known. In view of the pulmonary expression of LASS isoforms coupled with the inhibitory effects of sphingolipid metabolites on PtdCho synthesis in alveolar cells, we hypothesized that *i*) ceramide synthase isoforms are differentially expressed in the lung and are developmentally regulated, and *ii*) overexpression of ceramide synthase will coordinately modulate de novo ceramide synthesis and PtdCho synthesis.

METHODS

Materials

The monoclonal anti-V5 peroxidase-conjugated antibody, fumonisin B_1 (FB1), Ni-CAM HC resin columns, protease inhibitor cocktail, and TRI reagent were obtained from Sigma Chemical (St. Louis, MO). The MLE-12 cell line was obtained from the American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium/F12 medium was from the University of Iowa Tissue Culture and Hybridoma Facility (Iowa City, IA). Radiochemicals, including L-[palmitoyl-1-^{14}C]CoA, L-[3-^{3}H]serine, D-*erythro*-[3-^{3}H]sphingosine, [^{14}C]ceramide (*N*-oleoyl-D-sphingosine, oleoyl-1-^{14}C), and [methyl-^{3}H]choline chloride, were from American Radiolabeled Chemicals (St. Louis, MO). The AR-2000 imaging scanner was purchased from BioScan (Washington, DC). Precast 10% SDS-PAGE gels, prestained SDS-PAGE low-range standards, immunoblotting membranes, and protein concentration assay kits were from Bio-Rad (Hercules, CA). The ECL Western blotting detection system was from Amersham Biosciences (Piscataway, NJ). The TaqMan reverse transcription reagents, SYBR Green PCR master mix, and rodent GAPDH control reagents were obtained from Applied Biosystems (Foster City, CA). The dicer small, interfering RNA (siRNA) Generation Kit and the GeneSilencer siRNA Transfection Reagent were from Gene Therapy Systems (San Diego, CA). The Qiaquick Gel Extraction Kit was from Qiagen (Valencia, CA). The murine expression sequence tag for LASS5 [Integrated Molecular Analysis of Genomes and their Expression (IMAGE) number 4238182] was obtained from Open Biosystems (Huntsville, AL). Pfu DNA polymerase was from Stratagene (La Jolla, CA). The pcDNA3.1/V5-HisB and pCR-Blunt II-TOPO plasmids and *Escherichia coli* TOP10 competent cells were obtained from Invitrogen (Carlsbad, CA). Bacterial neutral SMase-13 was a kind gift from Dr. Lina Obeid (20). FuGENE6 transfection reagent was purchased from Roche Diagnostics (Indianapolis, IN). Restriction enzymes were from New England Biolabs (Beverly, MA). Centricon Plus-30 was from Millipore (Bedford, MA). All DNA sequencing was performed by the University of Iowa DNA Core Facility (Iowa City, IA).

Animals, tissue preparation, and cell culture

Mice (C57BL/6) were housed in the Animal Care Center at the University of Iowa. Primary alveolar type II epithelial cells and fibroblasts were isolated from 8 week old mice as described (21, 22). Gestationally timed pregnant mice, newborn mice (2–3 days old), or adult mice were killed with pentobarbital (150 mg/kg ip). Lungs were harvested and minced into ~1 mm sections and homogenized in buffer A (20 mM HEPES, pH 7.4, 1 mM PMSF, and 250 mM sucrose) containing protease inhibitor cocktail or tissues harvested in TRI reagent (see below). The homogenate was centrifuged at 500 *g* at 4°C for 5 min to remove unbroken tissue, whole cells, and large debris. The supernatant was used as crude enzyme preparation. Primary alveolar type II epithelial cells or MLE cells were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 2% FBS and penicillin (100 U/ml)/streptomycin (100 μg/ml) in 60 mm plastic dishes. All studies conducted on primary epithelial cells were performed within 24 h of isolation. In some studies, cells were incubated with FB1 at a final concentration of 20 μM that was added into the culture medium for 24 h before harvesting.

Cell fractionation

Cells were harvested as described above, sonicated, and centrifuged at 500 *g* for 10 min. The supernatant was further fractionated by ultracentrifugation at 80,000 *g* at 4°C for an additional 1 h. The yielded membranous pellet was resuspended in buffer A supplemented with protease inhibitor cocktail and stored at −20°C for subsequent analysis.

Ceramide synthase activity

Ceramide synthase (dihydroceramide synthase) activity was assayed in vitro by detection of [palmitoyl-1-^{14}C]CoA incorporation into ceramide using the sphingoid bases, sphinganine or sphingosine (19). All assays were done in triplicate. The reaction mixture contained 80 μM sphingosine (or 80 μM sphinganine), 78.6 μM palmitoyl-CoA containing 20 nCi [palmitoyl-1-^{14}C]CoA, and 100 μg of crude cellular lysate in 20 mM HEPES buffer, pH 7.4, containing 250 mM sucrose, 1 mM PMSF, and 20 μM BSA. The total reaction mixture (100 μl) was incubated at 37°C for 30 min. The reaction was terminated by the addition of 2 ml of methanol. Total cellular lipids were extracted using Bligh-Dyer extraction (23). The chloroform layer was dried, resuspended in 50 μl of chloroform-methanol (2:1, v/v), and applied to Whatman LK6 TLC plates (18). Sphingolipids were resolved using the solvent mixture chloroform-methanol-2 N ammonium hydroxide (40:10:1, v/v/v) using [^{14}C]ceramide (*N*-oleoyl-D-sphingosine, oleoyl-1-^{14}C) as a standard (18).

The TLC plates were quantified with an AR-2000 imaging scanner. One unit of enzyme activity was defined as the amount of 1 nmol ceramide formed per minute under the incubation conditions used. Ceramide synthase activity was linear with regard to protein concentrations ranging from 20 to 100 μg in the reac-

tion mixture, which was the effective concentration used in these studies. Neutral SMase activity was assayed as described (21).

Sphingolipid and PtdCho biosynthesis

Nearly confluent cultures of primary type II epithelia and MLE cells were radiolabeled with [3-^{3}H]serine or D-*erythro*-[3-^{3}H]sphingosine (1 μCi/60 mm dish) or with [methyl-^{3}H]choline chloride (1 μCi/60 mm dish) for up to 6 h. Cellular lysates were harvested in 1:1 methanol-water. Total cellular lipids extracted from equal amounts of cellular protein using the method of Bligh and Dyer (23) were applied to Whatman LK6 TLC plates, and the major sphingolipid intermediates were resolved using the solvent mixture chloroform-methanol-2 N ammonium hydroxide (40:10:1, v/v/v) (18). PtdCho was resolved using the solvent system chloroform-methanol-ammonium hydroxide (65:35:5, v/v/v). Radioactivity into individual lipids was determined by comigration with known standards [e.g., using *N*-oleoyl-D-sphingosine (sphingosine-3-^{3}H) standard] and lipids quantitated by scintillation counting using the AR-2000 imaging scanner as described (24). PtdCho mass was assayed by measuring lipid phosphorus content on the PtdCho spots resolved by TLC (25).

RNA isolation and real-time PCR analysis

Murine lung tissues were minced into ~1 mm pieces and homogenized with a blender; homogenates and MLE cells were prepared in 1 ml of TRI reagent for total RNA isolation according to the manufacturer's protocol. First-strand cDNA synthesis was performed using TaqMan Reverse Transcription Reagents (26). Real-time PCR analysis was performed as before using the following primers for expression: for LASS1, 5′-CACACACATCTTTCGGCCC-3′ (sense) and 5′-ACCTGGCAGCATCTCTAGGC-3′ (antisense); for LASS2, 5′-TCTCCTGGTTTGCCAATTACG-3′ (sense) and 5′-CCGGGCAGGGACCCTCATCA-3′ (antisense); for LASS3, 5′-GAGCGCCAGGTTGAAAGATG-3′ (sense) and 5′-GGAATTTCTGCAGCCTGCA-3′ (antisense); for LASS4, 5′-AATGGGATGGCTCGAGGG-3′ (sense) and 5′-TGCTGCTGCCCGGCT-3′ (antisense); for LASS5, 5′-GCAATGGTGCCAACTGCAT-3′ (sense) and 5′-TCCCCTGCTCTTCAGCCA-3′ (antisense); and for LASS6, 5′-TTTTGGCTTCCGCACAATG-3′ (sense) and 5′-GCGGAAAGGTGGCTTCCT-3′ (antisense) (27). Real-time RT-PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using SYBR Green PCR Master Mix according to the manufacturer's protocol. A reference housekeeping gene, GAPDH, was included in the real-time PCR as a control. Standard curves were generated for target genes and compared with GAPDH using serial dilutions of mRNA and were found to be linear from 0.08 to 50 ng of RNA in the reaction mixture. This range included effective concentrations used in the experiments. The PCR consisted of 10 μl of SYBR Green PCR Master Mix, 400 nM forward and reverse primers, and 1 μl of template cDNA (from 20 ng of RNA) in a total volume of 20 μl. Cycling was performed using the default conditions of the ABI PRISM 7000 SDS software: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The relative quantity of the target gene was normalized to that of the reference gene and expressed as normalized relative units.

Construction of a LASS5 expression vector

The mouse expression sequence tag (IMAGE number 4238182) was sequence verified by the University of Iowa DNA Facility and shown to contain the full-length cDNA sequence of the murine LASS5 gene (National Center for Biotechnology Information accession number AK010241).

A cDNA fragment encoding the LASS5 open reading frame was amplified using Pfu DNA polymerase using the template described above with the following primers: 5′-AAGCTTATGGCGACTGCAGCAGCGGAAA-3′ (sense) and 5′-GAATTCTCACAGGAGTGTAGATGTGGGGAG-3′ (antisense). PCR was carried out using the following conditions: one cycle at 98°C for 2 min and 30 cycles of 45 s at 98°C, 45 s at 60°C, and 2 min at 72°C. The PCR product was isolated from 1% agarose gels using the Qiaquick Gel Extraction Kit. The purified PCR product (~1,250 bp) was directionally cloned into pCR-Blunt II-TOPO for subsequent transformation into competent *E. coli* TOP10 cells. The integrity of the plasmid insert was verified by DNA sequencing. The amplified fragment was digested with *Hind*III and *Eco*RI, separated and isolated from agarose gels, and ligated to pcDNA3.1/V5-HisB that was previously digested with the same enzymes.

Transient transfections

Transient transfection was performed with liposome-mediated DNA transfer using FuGENE6. MLE cells cultured in serum-free Dulbecco's modified Eagle's medium/F12 medium in 60 mm dishes were transfected with 4 μg of an empty vector (pcDNA3.1/V5-HisB), a plasmid encoding murine LASS5, or a bacterial neutral SMase. Cells were harvested 24 h after transfection.

Purification of LASS5

MLE cells transiently transfected with His-tagged LASS5 plasmid were harvested in buffer A (containing 0.1% Triton X-100). Cell lysates were centrifuged at 27,000 *g* at 4°C for 30 min. The resulting supernatant was applied to Ni-CAM HC resin columns, and the enzyme was purified according to the manufacturer's instructions. The activity-enriched fraction was pooled, concentrated, and desalted with the addition of buffer A (15 ml) followed by concentration (three times) using Centricon Plus-30 filters.

Western blotting

Equal amounts of proteins were electrophoresed using precast 10% SDS-PAGE gels and run using prestained low-range molecular weight standards. Proteins were electrotransferred to nitrocellulose membranes as described previously (25). Proteins were immunodetected using monoclonal anti-V5 peroxidase-conjugated antibodies or anti-β-actin antibodies (1:4,000 dilution). Immunoreactive material was visualized by ECL.

LASS5 gene silencing

Template DNA (620 bp at a region spanning nucleotides 241–860 in the open reading frame of LASS5 cDNA) for transcription was generated using LASS5-pCR-Blunt II-TOPO as a DNA template and with the T7 promoter-coded primers 5′-GCGTAATACGACTCACTATAGGGAGACGTGAGGATGCTGTTTGAGC-3′ (sense) and 5′-GCGTAATACGACTCACTATAGGGAGACACGTCTGGCATAATTGGCC-3′ (antisense) by PCR: one cycle at 94°C for 3 min, then 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. LASS5 dicer siRNA was generated using the recombinant Dicer Enzyme in the Dicer siRNA Generation Kit according to the manufacturer's instructions. Transfection of siRNA was carried out using the GeneSilencer siRNA Transfection Reagent using 200 ng of scrambled or LASS5 siRNA in 60 mm dishes. Twenty-four hours after transfection, MLE cells were harvested for further analysis.

Protein assay

Protein determination was performed using the Bio-Rad protein assay kit with BSA serving as a standard.

Statistical analysis

Statistical analysis was performed using one-way ANOVA with a Bonferroni adjustment for multiple comparisons or a Student's *t*-test (28). Data are presented as means ± SEM with $P < 0.05$ indicating significance.

RESULTS

Pulmonary de novo sphingolipid synthesis

We first examined the biosynthesis of biologically active sphingolipids within murine lung epithelia. MLE and primary type II alveolar epithelia were radiolabeled with [3-^{3}H]serine, a substrate for sphingolipid biosynthesis, and incorporation of this label into ceramide, sphingosine, and SM was assayed. In both MLE cells (**Fig. 1A**) and type II cells (Fig. 1B), radioactivity within ceramide versus sphingosine and SM was observed to be greatest. To specifically investigate whether lung cells metabolically convert sphingosine to individual sphingolipids, cells were also pulsed with [3-^{3}H]sphingosine (Fig. 1C, D). Compared with labeling within sphingosine, SM, or sphingosine-1-phosphate, both MLE cells and type II cells synthesized large amounts of ceramide from sphingosine independent of the duration of pulse labeling. These results demonstrate that murine lung epithelia predominantly synthesize ceramide from sphingolipid precursors using a de novo pathway. They also support the existence of a functional *N*-acyltransferase for ceramide formation from sphingosine.

Ceramide synthase expression in murine lung

Because lung epithelia effectively converted sphingoside to ceramide and exhibited high-level de novo ceramide synthesis, comparative analysis was performed to examine various ceramide synthase isoforms. Real-time PCR was used to detect mRNAs of six known mammalian isoforms of ceramide synthase: LASS1, LASS2, LASS3, LASS4, LASS5, and LASS6 (**Fig. 2**). In primary type II epithelial cells, murine lung fibroblasts, and MLE cells, LASS5 was the predominant species identified (Fig. 2A, B). Lung cells also exhibited ceramide synthase activity in the range of ~0.3 nmol/min/mg protein (or ~18,000 pmol/h/mg protein), specific activities that were ~25-fold higher than values detected previously for SPT in these cells (Fig. 2B) (21). However, unlike SPT activity, ceramide synthase activity differed little between type II cells and pulmonary fibroblasts (Fig. 2B) (21). Similar to LASS5 mRNA expression in primary isolates and MLE cells, LASS5 was the predominant isoform in male and female whole lung (Fig. 2C), and there were no gender differences in the expression of this species (Fig. 2D). Because lung sphingolipids are developmentally regulated (21) and LASS5 was comparatively expressed at high levels in murine lung cells, we assayed LASS5 mRNA levels and activity during the fetal, newborn, and adult stages (Fig. 2E, F). LASS5 mRNA displayed a modest peak at day 17 in fetal lung, thereafter decreasing to lower levels in adult lung tissue (Fig. 2E). The ontogeny for changes in transcripts generally paralleled enzyme activity with lung maturation, in further support of the notion that LASS5 is the major species conferring ceramide synthase activity.

Cloning and expression of LASS5

The studies described above indicate that in pulmonary epithelia, LASS5 was detected as the major species of cer-

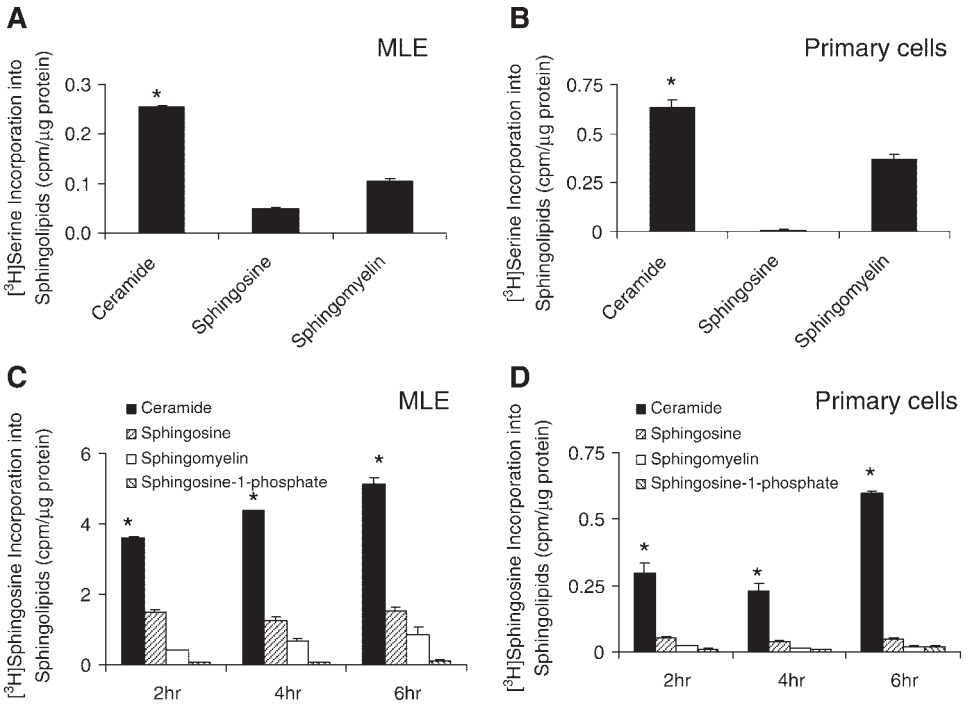

Fig. 1. Sphingolipid biosynthesis in murine lung epithelia. MLE cells (A, C) or primary murine type II alveolar epithelial cells (B, D) were pulsed with L-[3-^{3}H]serine (A, B; 6 h) or D-*erythro*-[3-^{3}H]sphingosine (C, D; 1 μCi/dish) for 6 h (A, B) or various times (C, D), and cell lysates were harvested, lipids extracted, and sphingolipids resolved using TLC. Incorporation of radioactivity into individual sphingolipids was determined using TLC. Data represent means ± SEM from three separate experiments and are expressed as cpm/μg protein. * $P < 0.05$ for ceramide versus other sphingolipids.

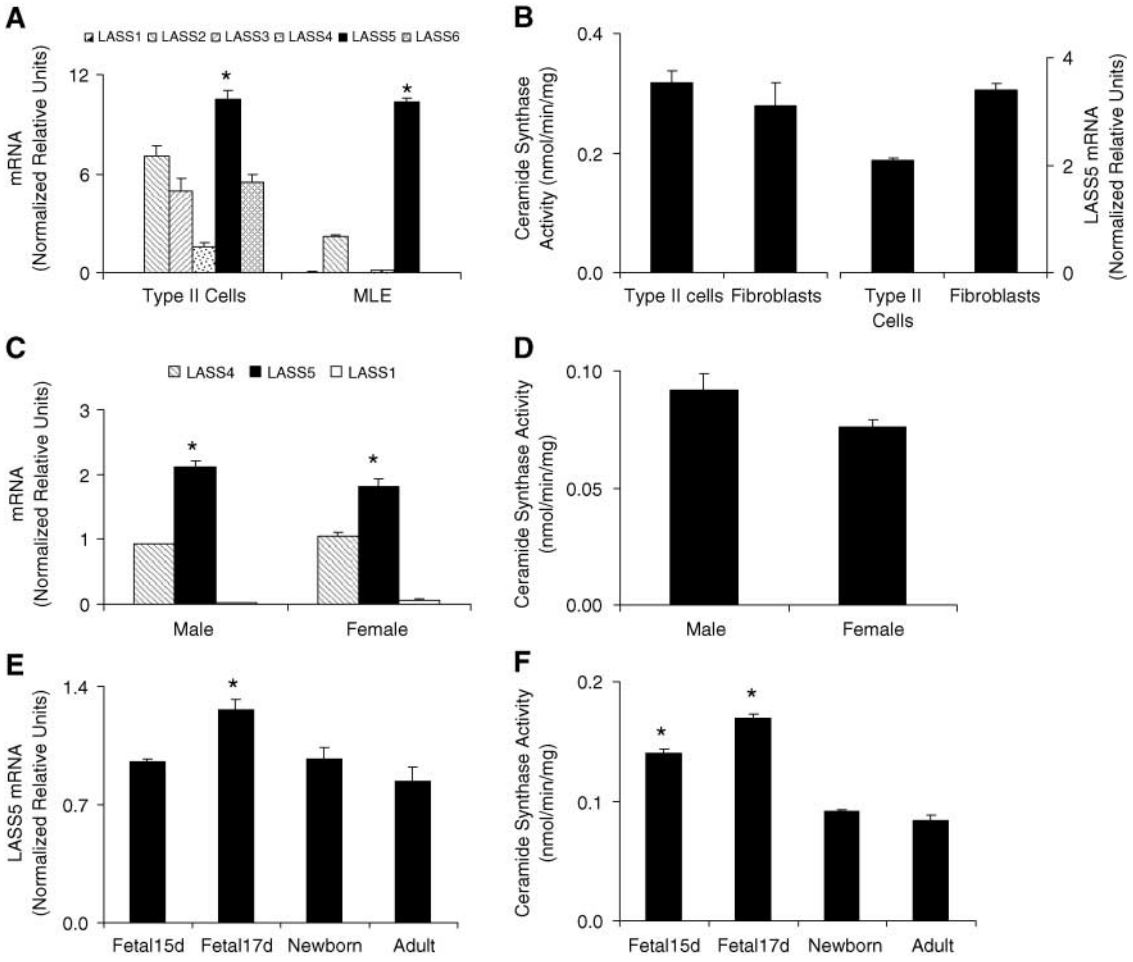

Fig. 2. Ceramide synthase expression in the lung. Real-time PCR was used to detect mRNAs of ceramide synthase longevity assurance homologs (LASS) in primary murine type II alveolar epithelial cells and MLE cells (A), within fibroblasts (B, right), in mouse whole lung from males and females (C), and during lung development (E). Data represent means ± SEM from three separate experiments and are normalized to the housekeeping gene GAPDH. * $P < 0.05$ for LASS5 versus other homologs (A, C) or during other periods of development (E). Ceramide synthase activity was assayed in type II cells and fibroblasts (B, left), in mouse male and female whole lungs (D), and during lung development (F). Data represent means ± SEM from three separate experiments, and enzyme activities are expressed as nmol/min/mg protein. In F, * $P < 0.05$ for fetal activities versus adult whole lung activity.

amide synthase. Thus, to further investigate this isoform, we cloned LASS5 into a vector encoding a V5-epitope tag to readily detect its expression in murine lung epithelia (**Fig. 3A**). Transient transfections of MLE cells with LASS5 plasmid led to a dose-dependent increase in expression of the immunoreactive product with a coordinate increase in enzyme activity (Fig. 3B). LASS5 was partially purified after transfection in cells, as indicated by the increased immunoreactive enzyme by immunoblotting (Fig. 3C) and a ~16-fold increase in enzyme activity (Fig. 3D). After in vivo expression, 82% of ceramide synthase activity and the bulk of immunoreactive enzyme were detected within the membrane pellet (Fig. 3E, F).

Manipulation of ceramide synthase activity

To further examine the role of LASS5 in regulating endogenous ceramide synthase activity, we used pharmacologic and genetic strategies to knock down sphingolipid biosynthesis. Cells transiently transfected with a control plasmid or LASS5 plasmid were incubated with or without FB1, a ceramide synthase inhibitor (**Fig. 4A**). Indeed, murine lung epithelia harbored a FB1-sensitive ceramide synthase that was inhibited whether or not cells were transfected with LASS5 (Fig. 4A). FB1 decreased ceramide synthase activity in LASS5-transfected cells without decreasing levels of overexpressed enzyme mass (Fig. 4B). Thus, FB1 reduced both endogenous activity and, after the introduction of LASS5 plasmid exogenously. Because of the potential nonspecificity of these pharmacologic approaches, we attempted to knock down LASS5 expression using siRNA (Fig. 4C, D). Cells exposed to LASS5 siRNA, but not scrambled siRNA, exhibited a 45% reduction in enzyme activity (Fig. 4C). These changes were associated with a similar reduction in LASS5 mRNA levels without alterations in LASS4 or LASS1 transcripts (Fig. 4D). FB1 and LASS5 siRNA also effectively reduced [3-^{3}H]serine incorporation into ceramide, although the magnitude of these effects differed between these agents (Fig. 4A, C, right panels). Taken together, these data suggest that overexpression of LASS5 stimulates ceramide synthesis, whereas downregulation of its expression

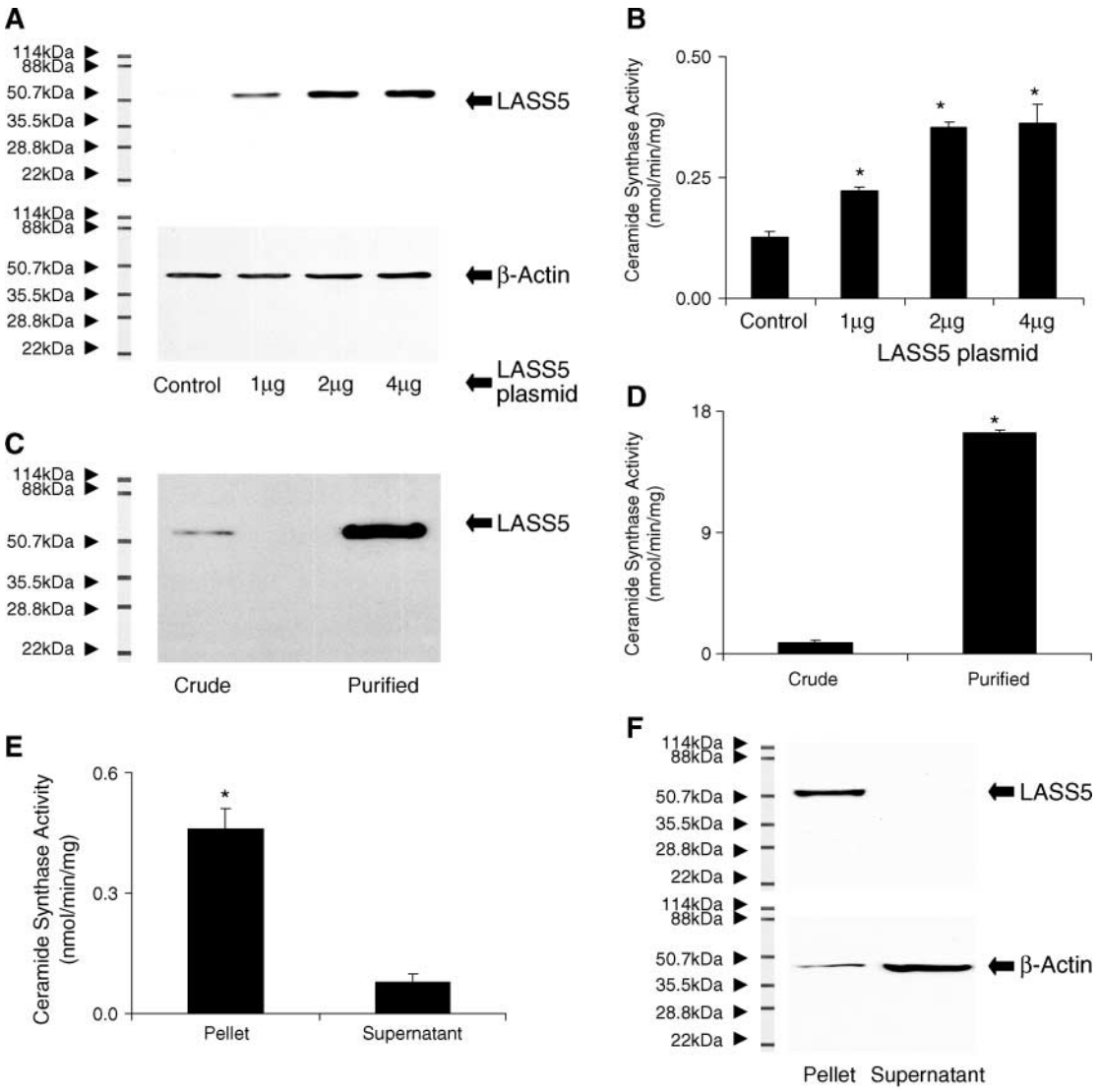

Fig. 3. Expression and partial purification of LASS5 in lung epithelia. A: Top, MLE cells were transiently transfected with various amounts of a plasmid construct encoding LASS5 fused to a V5 epitope tag, and 24 h later cells were harvested and processed for immunoblotting for V5. Bottom, Cells were also probed for immunoreactive β-actin content. The data are representative of one of three independent experiments. B: Ceramide synthase activity was assayed in transfected cells, and data represent means ± SEM of enzyme activities that are expressed as nmol/min/mg protein. C, D: MLE cells were transiently transfected with a plasmid construct encoding His-tagged LASS5, the cells were harvested and centrifuged, and the resulting supernatants were applied to Ni-CAM HC resin columns to partially purify LASS5. The activity-enriched fraction was pooled, concentrated, and desalted using Centricon Plus-20 filters before immunoblotting for LASS5 (C) or determination of ceramide synthase activity (D) in crude cell lysates or the purified fraction. E, F: MLE cells were transiently transfected with LASS5 plasmid as described above, cells were harvested, and the membranous pellet and soluble (supernatant) fractions were isolated by sequential centrifugation as described in Methods. These fractions were then used to determine ceramide synthase activity (E) or immunoreactive V5 epitope (LASS5) or β-actin content by immunoblotting (F). * $P < 0.05$ versus control (B, D) or supernatant fraction (E).

at least partially inhibits the production of the bioactive sphingolipid.

LASS5 regulates PtdCho synthesis

One important action of ceramide is its inhibitory effect on the biosynthesis of PtdCho. Ceramide can be generated in cells by the rapid breakdown of SM, catalyzed by SMases. We investigated whether overexpression of SMase or LASS5 was sufficient to inhibit PtdCho synthesis. Cells were transiently transfected with a plasmid encoding neutral bacterial SMase or LASS5, or cotransfected with both plasmids, and radiolabeled choline incorporation into PtdCho was then determined (Fig. 4E). LASS5 expression alone reduced [^{3}H]choline incorporation into PtdCho by 26%, whereas coexpression of LASS5 with SMase produced a nearly 40% reduction in PtdCho synthesis. In these studies, SMase overexpression produced a 36% increase in neutral SMase activity (data not shown). These results indicate that LASS5 is sufficient to inhibit phospholipid synthesis, but maximal suppression is achieved in combination with the activation of SM hydrolysis. These effects of LASS5 were associated with a 27% decrease in the mass of PtdCho in epithelial cells (data not shown).

As an alternative approach to demonstrate that PtdCho

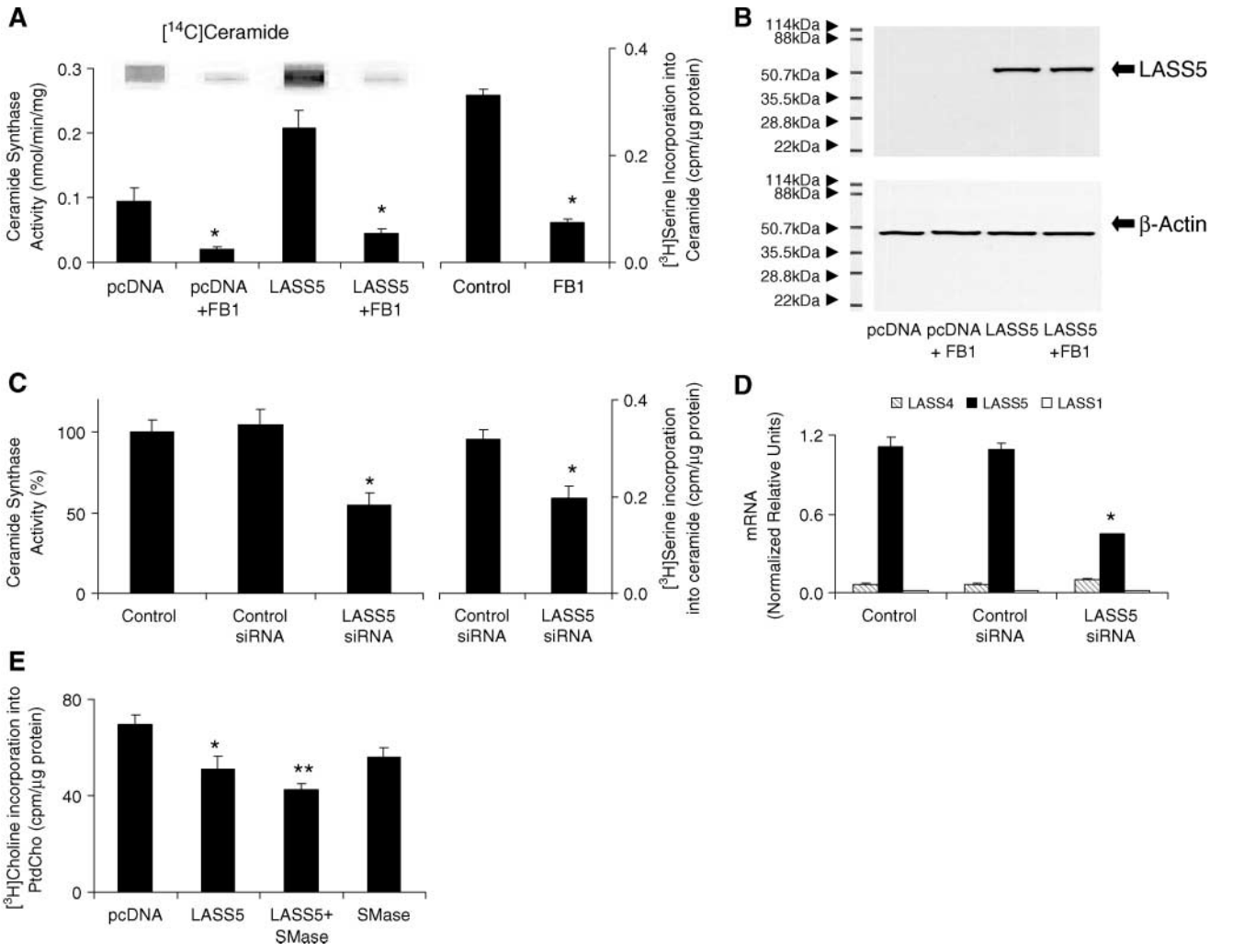

Fig. 4. Manipulation of ceramide synthase activity in lung epithelia. A: MLE cells were transfected with a mock plasmid (4 μg) or a plasmid encoding LASS5 (4 μg). Ten minutes later, cells were exposed to medium alone or in combination with fumonisin B_1 (FB1; 20 μM) for an additional 24 h. Lysates were harvested for ceramide synthase activity (left), and a representative scan of ceramide spots on TLC plates is shown in the inset. In separate studies, cells were pulsed with L-[3-^{3}H]serine (1 μCi/dish for the last 3 h) in medium alone or in combination with FB1 as described above, and incorporation of radioactivity into ceramide was then determined (right) as in Fig. 1. Data represent means ± SEM from three separate experiments where * $P < 0.05$ in inhibitor-treated groups versus respective control groups. B: A representative immunoblot is shown for LASS5 expression in the presence of FB1 with or without plasmid transfection from samples obtained in A (left). C: Cells were transfected with LASS5 small interfering RNA (siRNA; 200 ng/dish) or scrambled siRNA (200 ng/dish). Twenty-four hours after transfection, cells were harvested for ceramide synthase activity (left) and incorporation of radioactivity into ceramide (right). Data represent means ± SEM from three separate experiments where * $P < 0.05$ in LASS5 siRNA-treated groups versus respective control groups. D: Untransfected control cells, or cells transfected with scrambled siRNA or LASS5 siRNA, were harvested and processed for real-time PCR analysis for ceramide synthase homologs as in Fig. 2. * $P < 0.05$ in LASS5 siRNA versus LASS4 or LASS1. E: Effect of LASS5 on phosphatidylcholine (PtdCho) synthesis. Cells were transiently transfected with a mock vector (pcDNA3.1; 4 μg), LASS5 (4 μg), a bacterial neutral sphingomyelinase (SMase; 4 μg), or cotransfected using LASS5 and SMase (4 μg each). Cells were pulsed with [methyl-^{3}H]choline chloride (1 μCi/dish) for the final 2 h of culture. Cells were harvested and processed for incorporation of radioactivity into PtdCho using TLC. Data represent means ± SEM from three separate experiments and are expressed as cpm/μg protein. * $P < 0.05$ for LASS5 versus control by ANOVA; ** $P < 0.01$ for LASS5 plus SMase versus control by ANOVA.

synthesis is linked to SM synthesis, untransfected cells or cells transfected with scrambled siRNA or LASS5 siRNA were assayed for rates of PtdCho production. Compared with control cells, cells transfected with LASS5 siRNA exhibited a 43% increase in rates of PtdCho synthesis [51 ± 10 cpm/μg protein (control), 63 ± 9 cpm/μg protein (scrambled siRNA), and 73 ± 10 cpm/μg protein (LASS5 siRNA)]. Although these effects did not reach statistical significance (n = 4, $P = 0.09$ for LASS5 siRNA versus control), the results, coupled with the LASS5 knockdown studies, suggest that PtdCho synthesis and SM synthesis are physiologically linked.

DISCUSSION

Ceramide is a key lipid mediator in the lung. We and others have provided evidence that it is the putative bio-

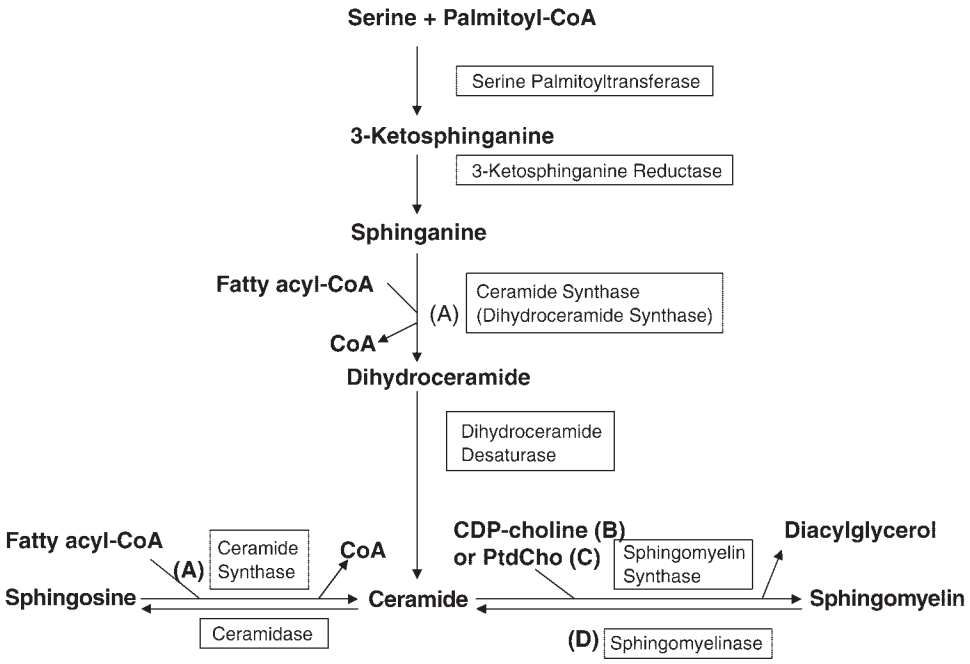

Fig. 5. Cartoon illustrating the major pathways for sphingolipid metabolism. The pathways discussed in this study include the de novo pathway involving the conversion of sphinganine to dihydroceramide or sphingosine to ceramide catalyzed by ceramide synthase (A). Ceramide generated in this manner can then undergo enzymatic condensation with phosphocholine derived from either CDP-choline (B) or PtdCho as a donor (C) to generate sphingomyelin (SM). These condensation reactions are catalyzed by SM synthase or PC:ceramide phosphocholine transferase, respectively. Finally, SM can undergo hydrolysis by acidic or neutral SMases, generating ceramide (D).

molecule inhibitory for surfactant phospholipid synthesis (6, 8, 29–32). Thus, the primary aim of this study was to ascertain whether ceramide, generated via a de novo pathway, coordinately inhibits PtdCho production in lung epithelia. The new observations from these studies include the following: *i*) that lung epithelia engage in high-level de novo ceramide synthesis using sphingoid bases; *ii*) that D-*erythro*-[3-^{3}H]sphingosine in lung cells is rapidly incorporated into ceramide, indicative of a robust *N*-acyltransferase mechanism catalyzed by a ceramide synthase; *iii*) that LASS5 is the predominant isoform conferring ceramide synthase activity that was developmentally regulated in the lung; and *iv*) that PtdCho synthesis is dependently regulated with de novo ceramide synthesis.

Although ceramide generated by SM degradation has been extensively studied in several systems, investigations of ceramide synthesis are more limited as many of the enzymes used in this pathway have yet to be isolated and characterized. Both MLE cells and primary type II cells exhibited significant incorporation of [3-^{3}H]serine and D-*erythro*-[3-^{3}H]sphingosine into ceramide, suggestive of the ability to rapidly acylate sphingoid bases. Our TLC solvent system does not reliably distinguish between dihydroceramide and ceramide, but ceramide was detected as a major bioactive species in pulmonary epithelia using high-performance liquid chromatography (32). Thus, it is likely that ceramide is produced either from sphingosine or from sphinganine involving a ceramide synthase. In this respect, specific activities for ceramide synthase within type II alveolar cells appear much higher than activities for SPT, suggesting that SPT may be rate regulatory in the de novo sphingolipid pathway (21). Confirmation of this will require measurements of pool sizes of sphingolipid intermediates. Nevertheless, high-level ceramide synthase activity in lung epithelia might be important, as this enzyme could serve as a potential therapeutic target for inhibition, thereby affecting the mass of bioactive sphingolipids.

LASS5 was the major ceramide synthase species not only in type II cells but also within lung fibroblasts and alveolar macrophages (data not shown). Specifically, LASS5 expression was greater than the expression of two mammalian isoforms that were demonstrated to exhibit ceramide synthase activity (LASS1 and LASS4) and also related genes showing a high degree of homology to ceramide synthase (LASS2, LASS3, and LASS6). The expression of LASS5 varied somewhat throughout lung development, with a modest yet significant peak of mRNA levels during the canalicular (day 17) phase. This phase is characterized by airway branching morphogenesis and initial widening of early alveolar units at the expense of interstitial tissue (33). Therefore, in view of ceramide's role in apoptosis, it is possible that LASS5 expression might participate in programmed cell death associated with interstitial condensation and alveolar expansion during lung maturation (34).

In the only comparative study analyzing LASS4, LASS5, and LASS1 to date, LASS5 was observed to exhibit bifunctionality, with some preference for sphinganine over sphingosine as a sphingoid base substrate, whereas LASS4 used

either sphingosine or sphinganine (18). A unique observation by Riebeling et al. (18) was that LASS4 or LASS5 overexpression in 293T cells in combination with exposure to FB1, a competitive ceramide synthase inhibitor, increased ceramide and dihydroceramide levels above the values observed after LASS4 or LASS5 expression alone. These data suggest FB1 resistance and indicate that LASS overexpression somehow stimulates endogenous ceramide synthases or that in 293T cells FB1 regulates a product that activates yet unidentified LASS isoforms. In contrast with this study, FB1 effectively reduced both enzyme activity and ceramide synthesis in MLE cells after LASS5 overexpression, and effects were recapitulated to some degree using LASS5 siRNA (Fig. 4). LASS5 siRNA inhibitory effects on ceramide synthesis were substantial but not totally effective, reflecting transfectional inefficiency or compensatory effects from other pulmonary ceramide synthase isoforms. The same group also observed that LASS5 overexpression increased palmitate-containing sphingolipid products (18). Although palmitate-containing sphingolipids are relatively minor components, these latter results are intriguing because type II cells are highly enriched with phospholipids (specifically PtdCho) that are acylated with this fatty acyl moiety at the *sn*-1 and *sn*-2 positions within the glycerol backbone. Thus, it is possible that ceramide synthase activation in the lung might limit the availability of palmitoyl-CoAs used for incorporation in surfactant phospholipids.

One biologic effect of ceramide is its ability to impair PtdCho synthesis. This is indicated by studies showing that short-chain cell-permeable ceramide analogs inhibit [methyl-^{3}H]choline incorporation into PtdCho and the activity of CTP:phosphocholine cytidylyltransferase (CCT), the rate-regulatory enzyme involved in PtdCho production (6, 31, 35). Mice deficient in SMase also have increased surfactant PtdCho levels (36). However, naturally occurring long-chain ceramides have limited inhibitory effects on CCT function in vitro, raising the issue of whether endogenous sphingolipids inhibit PtdCho synthesis (8). To determine whether ceramides generated endogenously from either SM hydrolysis or via de novo synthesis are inhibitory for PtdCho production, we used genetic strategies involving transient transfection of plasmids encoding SMase or LASS5. Indeed, LASS5 expression in vivo alone was sufficient to reduce PtdCho synthesis, but optimal inhibition was achieved with activation of both the SM hydrolysis and ceramide synthetic pathways. Conversely, treatment of cells with LASS siRNA tended to stimulate PtdCho synthesis. It is possible that the observed decrease in PtdCho synthesis after LASS5 overexpression might be attributed to an exchange mechanism whereby increased ceramide generation in cells stimulates PC:ceramide phosphocholine transferase activity. This enzyme transfers phosphocholine from PtdCho to the acceptor, ceramide, generating SM (**Fig. 5**) (21, 37). However, our preliminary data reveal that PC:ceramide phosphocholine transferase activity in MLE cells was not significantly altered after LASS5 transfection [20.4 ± 0.9 pmol/h/g protein (control plasmid) and 23 ± 0.8 pmol/h/g protein (LASS5 plasmid); n = 3, P = NS]. These data as a whole show interdependency of PtdCho synthesis with SM synthesis at the ceramide synthase step.

In conclusion, our studies reveal that de novo ceramide synthesis in lung epithelia is catalyzed primarily by a constitutively active LASS5 isoform; expression of this enzyme also downregulates surfactant PtdCho biosynthesis. Additional studies will be needed to investigate whether these enzymes act directly or indirectly by increasing other sphingolipid metabolites that regulate targets with the PtdCho pathway.

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