Apoptotic Sphingolipid Signaling by Ceramides in Lung Endothelial Cells

Terry R. Medler^{*1}, Daniela N. Petrusca^{*2}, Patty J. Lee³, Walter C. Hubbard⁴, Evgeny V. Berdyshev⁵, Jarrett Skirball¹, Krzysztof Kamocki², Edward Schuchman⁶, Rubin M. Tudor^{1,7}, and Irina Petrache^{1,2,7}

¹Division of Pulmonary and Critical Care Medicine, ⁴Division of Clinical Pharmacology, and ⁷Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland; ²Division of Pulmonary, Allergy, Critical Care and Occupational Medicine, Indiana University, Indianapolis, Indiana; ³Section of Pulmonary and Critical Care Medicine, Yale University School of Medicine, New Haven, Connecticut; ⁵Division of Pulmonary and Critical Care Medicine, University of Chicago, Chicago, Illinois; and ⁶Division of Molecular Genetics, Mount Sinai School of Medicine, New York, New York

The *de novo* pathway of ceramide synthesis has been implicated in the pathogenesis of excessive lung apoptosis and murine emphysema. Intracellular and paracellular-generated ceramides may trigger apoptosis and propagate the death signals to neighboring cells, respectively. In this study we compared the sphingolipid signaling pathways triggered by the paracellular- versus intracellular-generated ceramides as they induce lung endothelial cell apoptosis, a process important in emphysema development. Intermediate-chain length (C_{8:0}) extracellular ceramides, used as a surrogate of paracellular ceramides, triggered caspase-3 activation in primary mouse lung endothelial cells, similar to TNF- α -generated endogenous ceramides. Inhibitory siRNA against serine palmitoyl transferase subunit 1 but not acid sphingomyelinase inhibited both C_{8:0} ceramide- and TNF- α (plus cycloheximide)-induced apoptosis, consistent with the requirement for activation of the *de novo* pathway of sphingolipid synthesis. Tandem mass spectrometry analysis detected increases in both relative and absolute levels of C_{16:0} ceramide in response to C_{8:0} and TNF- α treatments. These results implicate the *de novo* pathway of ceramide synthesis in the apoptotic effects of both paracellular ceramides and TNF- α -stimulated intracellular ceramides in primary lung endothelial cells. The serine palmitoyl synthase-regulated ceramides synthesis may contribute to the amplification of pulmonary vascular injury induced by excessive ceramides.

Keywords: apoptosis; lung; cytokines; signaling; sphingolipids

Excessive lung endothelial cell apoptosis may account for the pathogenesis of nonmalignant lung disease, including emphysema (a chronic obstructive pulmonary disease) and several types of acute lung injury (1–4). The molecular mechanisms leading to pulmonary endothelial cell apoptosis include the up-regulation of ceramides, a class of signaling sphingolipids comprising several species differing by fatty acid chain length and saturation (1, 2, 5). Although several downstream mediators of ceramide-induced apoptosis have been identified, little is known regarding the upstream events leading to ceramide production or the specific involvement of individual ceramide species in mediating apopto-

CLINICAL RELEVANCE

Prevention of lung microvascular endothelial apoptosis modulated by serine palmitoyl transferase might evolve as a therapeutic strategy in emphysema.

sis. Ceramides may be generated via two pathways: the *de novo* pathway, involving upstream activation of the serine palmitoyl transferase (SPT), and the sphingomyelinase pathway, through acid (ASMase) or neutral sphingomyelinase activation. Alternatively, intracellular ceramide levels may also increase by blocking its metabolic clearance. It has been suggested that individual ceramide species could play distinct biological roles (6). Moreover, the specific ceramide species induced by intermediate-chain ceramides or by TNF- α remain undefined.

Using pharmacological inhibitors in a model of murine emphysema, we have shown that ceramide up-regulation via the *de novo* pathway was critical for lung cell apoptosis *in vivo* and that ceramide activation occurred upstream of caspase-3 activation (1). Interestingly, the ASMase (specifically its soluble isoform), but not the neutral sphingomyelinase, was also activated in this model of emphysema and may have accounted for the increased production of endogenous paracellular ceramides in response to exogenous ceramide (1).

These results have raised several questions. First, does the uptake of bioactive (extracellular) ceramide trigger new synthesis of endogenous ceramides to activate caspases in primary lung endothelial cells, and if so, by which pathway? Second, is there a common pathway of ceramide synthesis required for pro-apoptotic signaling in these cells? To address these questions, we analyzed endogenous ceramide species generated in response to extracellular ceramide or TNF- α , a relevant trigger of endothelial apoptosis. Previously, investigators used exogenous ceramide to mimic the action of the intracellular signaling ceramide. Furthermore, due to limited solubility, mostly very-short chain (C₂–C₆) ceramides have been studied in cell culture systems. The premise of our work was that bioactive paracellular pools of ceramides may initiate distinct intracellular signaling events when compared with the intracellular-generated ceramides. We approached these experimental questions using small inhibitory RNA strategies coupled with mass spectrometric measurements of ceramide species, and employing longer-chain C_{8:0} ceramides, which may be more relevant to cellular responses to naturally occurring ceramides. Our results, some of which were previously presented in abstract form (7), indicate that the *de novo* serine palmitoyl transferase (SPT)-activated pathway of sphingolipid synthesis is necessary for pro-apoptotic intracellular ceramide generation in primary mouse lung endothelial cells in response to both extracellular ceramide and TNF- α . We then compared the pattern and kinetics of intracellular ceramide species generated by the two stimuli using mass spectrometry.

(Received in original form July 19, 2007 and in final form January 8, 2008)

*These authors contributed equally to this manuscript.

This work was funded by ATS/Alpha One Foundation Research Grant and NIH RO1 HL077328 (to I.P.); NIH RO1HL66554 (to R.M.T.); and NIH 1 S10 RR16798 for purchase of LC-MS/MS instrumentation (to W.C.H.).

Correspondence and requests for reprints should be addressed to Irina Petrache, M.D., Indiana University, Division of Pulmonary, Allergy, Critical Care and Occupational Medicine, Van Nuys Medical Science Building, 635 Barnhill Drive, MS224, Indianapolis, IN 46202-5120. E-mail: ipetrach@iupui.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 38, pp 639–646, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-02740C on January 10, 2008

Internet address: www.atsjournals.org

MATERIALS AND METHODS

Chemicals and Reagents

N-Octanoyl-D-erythro-Sphingosine (C_8 , as well as standard ceramides including the internal standard C_{17} ceramide utilized for mass spectrometry), 4-nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled C_{12} ceramide, and dihydroceramides standards were from Avanti Polar Lipids (Alabaster, AL). Fumonisin B1 (FB1; Cayman, Ann Arbor, MI) and myricocin (Biomol Research Laboratories, Plymouth Meeting, PA) were used to inhibit *de novo* ceramide synthesis. The following primary antibodies were used: active caspase-3/7 (Cell Signaling Technology, Beverly, MA, and Abcam, Cambridge, MA), caspase-8 (IC12, Cell Signaling), ASMAse (Santa Cruz Biotechnologies, Santa Cruz, CA and from E.S.), SPT (Abgent, San Diego, CA), actin (Calbiochem, La Jolla, CA), vinculin (Calbiochem), and GAPDH (Abcam). Human recombinant TNF- α and all other reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Cell Culture Experiments

Primary mouse lung microvascular endothelial cells were obtained as described (8, 9) and experiments were performed up to passage 18. Cells were maintained in complete culture medium consisting of Dulbecco's modified Eagle's medium (Gibco, Invitrogen Corporation, Carlsbad, CA), 20% FBS, and penicillin/streptomycin (100 U/ml, 0.1 mg/ml; Sigma) at 37°C in 5% CO_2 and 95% air. Experiments were performed at 80 to 100% confluence. Ceramides were first dissolved in ethanol and then diluted in 1% FBS-containing cell culture medium to a final ethanol concentration less than 0.1%. All controls were included this vehicle. Cells were treated with either $C_{8:0}$ ceramide at a final concentration of 10 μ M, or TNF- α (20 ng/ml) in the presence of cycloheximide (1 μ g/ml; Sigma). As with human primary lung endothelial cells (10), the proapoptotic effects of TNF- α in mouse lung endothelial were evident only with the addition of cycloheximide, which we added in all experiments involving TNF- α . Culture media was changed to serum-free media for 1 hour before treatment. After the addition of $C_{8:0}$ ceramide or TNF- α , or vehicle, the serum content was increased to 1% for 24 hours and then cells were harvested and lysed in cell lysis buffer (containing HEPES [10 mM], EDTA [2 mM], CHAPS/NP40 [0.1%], DTT [5 mM], PMSF [1 mM], pepstatin A [10 μ g/ml], leupeptin [20 μ g/ml], and aprotinin [10 μ g/ml]). Similarly, NBD-labeled $C_{12:0}$ ceramide was added for the indicated period of time to cultured endothelial monolayers grown on coverslips. Cells were then washed with PBS, labeled with DAPI, and then fixed in 3% formalin before visualization by fluorescence microscopy.

siRNA Constructs

The siRNA sequences targeting mouse SPT subunit 1 and acid sphingomyelinase were designed using mRNA sequences from Gen-Bank (gi:29244576 and gi:6755581, respectively). Sequences were then BLASTed against the mouse genome to eliminate those with homology to other genes. Scramble sequences were designed and BLASTed to eliminate sequences that were homologous to other mouse genes. For each target, two sequences were chosen to determine the effectiveness of silencing. The chosen sequences are as follows. SPTLC1: 5'-AAT ACTTTGGATAATCAGACT-3'; ASMAse: 5'-AAGTTGATCAAG AGCCAAAAG -3'; scrambled siRNA: 5'-AATTTTACGGTTAAAC GAACCTCTGTCTC-3'. Sense and antisense oligonucleotides were obtained from the Johns Hopkins University Genetic Resources CORE Facility. For construction of the siRNA, the *Silencer* siRNA Construction Kit by Ambion (Austin, TX) was used following the manufacturer's instructions. Endothelial cells (at 30–50% confluence) were transfected using siPortamine (Ambion) as a vehicle for the siRNA duplexes. For determination of transfection efficiency, cells were visualized using fluorescence microscopy after SiGlo (Dharmacon, Lafayette, CO) transfection under conditions similar to those of the targeted siRNAs. Cells were serum-deprived for 4 hours before transfection and remained in serum-free media for an additional 4 hours, followed by addition of full serum media of an equal volume (10% FBS final concentration). Cells were then incubated until optimum silencing was obtained (6 d for SPTLC1 and 3 d for ASMA) before apoptosis experiments were conducted.

Lipid Extraction and Ceramide Species Measurement by Tandem Mass Spectrometry

To determine which ceramide species were present in cultured endothelial cells, we used a modification of the combined liquid chromatography-tandem mass spectrometric (LC-MS/MS) technique by Sullards and Merrill (11). Cellular or lung tissue lipids were extracted and lipid content was assessed by measurements of total lipid phosphorus (P_i) measurements (1). After lipid extraction, ceramides were eluted as previously described (1). The following individual molecular species of ceramides were monitored: 14:0, 16:0, 18:0, 18:1, 20:0, 24:0, and 24:1-ceramides, using $C_{17:0}$ ceramide as internal standard.

Enzymatic Activity Determination

The ASMAse activity was measured at pH 5.0 in the absence of added cations as described (1) using the Amplex E Red Sphingomyelinase Assay Kit (Molecular Probes, Eugene, OR) following the manufacturer's instructions. The rate of labeled product formation over 60 minutes was expressed as the sample's OD rate from which the blank's OD rate was subtracted. Hydrogen peroxide and sphingomyelinase were used as positive controls. The SPT activity was measured by cell pulse-labeling with D_3 -serine and quantitation of dihydrosphingosine and D_2 -analogs of dihydrosphingosine by liquid chromatography tandem mass spectrometry as described previously (12). The approach is based on the incorporation of D_3 -serine into sphingoid bases during sphingolipid *de novo* biosynthesis and detection of M+2 mass shift in the portion of newly formed dihydrosphingosine (we have determined that one deuterium is exchanged with protium during molecule ionization). Briefly, after lipid extraction from cells spiked with C_{17} -sphingosine sphingoid bases were separated using Discovery C18 column (2.1 $\times$ 50 mm, 5 μ m particle size; Supelco, Bellefonte, PA) employing a gradient from methanol/water/formic acid (61/38/1) containing 5 mM ammonium formate to methanol/acetonitrile/formic acid (39/60/1) containing 5 mM ammonium formate at flow rate of 0.5 ml/minute. The sphingolipids were ionized via positive electrospray ionization with detection via multiple reaction monitoring. Standard curves for each sphingoid base were constructed by adding increasing concentrations of the individual analytes to approximately 50 pmol C_{17} -sphingosine employed as the internal standard.

Apoptosis Assays

Executioner caspase (Caspase-3 and/or -7) activity was measured with ApoONE Homogeneous Caspase-3/7 assay kit (Promega, Madison, WI) as described (1). Human recombinant caspase-3 (Calbiochem) was used as a positive control. Apoptotic nucleosomal release was measured with a nucleosomal ELISA kit (Oncogene; Calbiochem) (1). Apopercutent kit (Biocolor Ltd, Accurate Chemical and Scientific Corporation, Westbury, NY) was used following the manufacturer's instructions. After labeling, the cells were visualized by phase-contrast microscopy. Cell count was performed in triplicate and normalized by DAPI-counterstained nuclei.

Western Blotting

Total endothelial cell lysates were loaded in equal protein amounts (10 μ g, unless otherwise noted) determined by BCA (Pierce, Rockford, IL). Proteins were resolved by SDS-PAGE (Novex; Invitrogen), followed by immunoblotting as previously described (1). The chemiluminescent signals were quantified by densitometry (ImageQuant; Amersham, Piscataway, NJ) and normalized by housekeeping proteins (actin, GAPDH, or vinculin).

Statistical Analysis

Mean values between two groups were compared using Student's *t* test. For three or more groups, comparisons were made by ANOVA, followed by *post hoc t* test. Statistical significance was set at $P < 0.05$.

RESULTS

Kinetics of Intracellular Ceramide Production and Caspase-3 Activation in Lung Endothelial Cells Treated with Exogenous Ceramides and TNF- α

Because the longer-chain ceramides' poor solubility limits the exogenous delivery of ceramides, most investigators use short-chain

ceramides. However, the effects of very short-chain ceramides, such as 2-carbon length fatty acid chain ($C_{2:0}$), may be non-physiological and/or nonspecific. We therefore chose ceramides of 8 to 12 carbon-length fatty acid chains, which more closely resemble endogenous ceramides while still being deliverable and permeating endothelial cells, as shown here and in our previous studies (1). We and others have shown that administration of short-chain ceramides triggers apoptosis not only by intracellular exchange of a short-chain fatty acid with long-chain fatty acids, but also by synthesis of endogenous ceramides via cell-specific pathways (1, 13). To investigate which pathway of ceramide synthesis mediates the pro-apoptotic effects induced by extracellular intermediate-chain ceramides in lung endothelium, we first examined the time-course of cellular uptake and apoptosis triggered by $C_{8:0}$ ceramide. These responses were compared with that induced by the $TNF-\alpha$, a cytokine well known to induce endogenous pro-apoptotic ceramide signaling. Treatment of primary mouse lung endothelial cells with exogenous $C_{8:0}$ ceramide induced a rapid elevation in total endogenous ceramides ($C_{14:0}$ and higher), as early as 2 hours and remaining sustained for the first 24 hours of treatment (Figure 1a). Treatment with $TNF-\alpha$ (20 ng/ml) plus cycloheximide (known to cause apoptosis in these cells) elevated endogenous ceramide species between 6 h and 24 hours of treatment (Figure 1a). This up-regulation of endogenous ceramides preceded or was concurrent with the activation of caspase-3 in $C_{8:0}$ ceramide- (Figure 1b) and $TNF-\alpha$ -treated cells (Figure 1c), respectively. Microscopically, both $C_{8:0}$ ceramide and $TNF-\alpha$ treatments induced endothelial cells apoptosis at 16 hours (see Figure E1a in the online supplement). Similar results were obtained with fluorescently labeled $C_{12:0}$ ceramide, which was incorporated by endothelial cells as early as 2 hours. $C_{12:0}$ ceramide triggered typical apoptotic chromatin condensation at 18 hours (DAPI staining, Figure E1b). Next, to identify which enzymatic pathway of sphingolipid synthesis was responsible for the pro-apoptotic effects of exogenous ceramide, we employed siRNA specifically designed to SPTLC1 and ASMase.

Inhibitory Effect of Silencing RNA for SPT and ASMase

SPT, the rate-limiting enzyme in the *de novo* pathway of ceramide synthesis, contains two subunits, of which the SPT subunit 1 (SPTLC1) is essential for enzymatic activity (14). Primary mouse lung endothelial cells were treated with vehicle (SiPortamine), nonspecific fluorescently labeled siRNA (siGlo) (Figure 2a), scrambled siRNA, or SPTLC1 siRNA. As expected, cells treated with the SPTLC1 siRNA exhibited reduced basal SPT protein levels by immunoblot (Figure 2b) and markedly reduced the SPT enzymatic activity (Figure 2b) 6 days after siRNA transfections. In contrast, similar treatment with nonspecific, scrambled siRNA failed to inhibit SPTLC1 protein expression (Figure 2c). The delayed reductions in SPT activity and SPTLC1 protein levels induced by SPTLC1 siRNA are likely explained by a long half-life of SPT protein in these cells. ASMase siRNA, but not control transfection reagent, significantly reduced basal ASMase activity and protein levels (Figure 2d) at 72 hours after transfection. Similar treatment with scrambled siRNA failed to inhibit ASMase protein expression (Figure 2e).

Exogenous Ceramide-Induced Caspase-3 Activation Requires De Novo Sphingolipid Synthesis in Lung Endothelial Cells

In response to $C_{8:0}$ ceramide administration, primary lung endothelial cells significantly activated the executioner caspases-3/7 (Figure 3a) and the precursor caspase-8 (Figure E1e). Pretreatment of endothelial cells with inhibitors of *de novo* sphingolipid synthesis such as SPTLC1 siRNA (Figure 3a) or the pharmaco-

Figure 1. Kinetics of endogenous ceramide and caspase-3 up-regulation induced by exogenous ceramide ($C_{8:0}$ Cer) and $TNF-\alpha$ in primary lung endothelial cells. (a) Endogenous ceramides were measured by mass spectrometry, then normalized by lipid phosphorus (Pi). The data are presented as fold change compared with controls. In shaded circles are shown vehicle controls compared with untreated controls. When compared with controls, both exogenous $C_{8:0}$ ceramide (10 μ M) and $TNF-\alpha$ (20 ng/ml with cycloheximide) increased endogenous ceramide levels (mean $\pm$ SEM; * P < 0.05 versus controls, Student's *t* test). (b) Caspase-3 activity (normalized by protein concentration and expressed as fold change compared with control) in endothelial cells treated with exogenous $C_{8:0}$ ceramide (10 μ M) (mean $\pm$ SEM; * P < 0.05 versus controls, Student's *t* test). (c) Caspase-3 activity (normalized by protein concentration and expressed as fold change compared with control) in endothelial cells treated with $TNF-\alpha$ (20 ng/ml with cycloheximide) (mean $\pm$ SEM; * P < 0.05 versus controls, Student's *t* test).

Figure 2. Effect of targeted siRNA treatment on SPT (serine palmitoyl transferase) and ASMase (acid sphingomyelinase) in lung endothelial cells. (a) Immunofluorescence micrograph of primary mouse lung endothelial cells treated with vehicle alone, siPortamine (left) and vehicle with fluorescently-labeled siRNA, SiGlo (right), showing a high efficiency (> 90%) of siRNA transfection (figures representative of $n = 3$). (b) SPT activity was measured as D₃-serine incorporation (% of total labeled serine administered to cells) by mass spectrometric analysis of labeled dihydrospinganine and sphingosine ($n = 3$). Cells were assessed 6 days after treatment with SPTLC1 siRNA (50 nM). *Inset:* Upper immunoblot of SPT subunit 1 (57 kD) performed 6 days after treatment with SPTLC1 (SPT long-chain base subunit 1) siRNA at the indicated concentrations. *Lanes:* 1, untreated cell lysate; 2, siPortamine; 3, SPTLC1 siRNA, 50 nM; and 4, SPTLC1 siRNA, 100 nM. The lower immunoblot is for actin, shown as loading control. (c) Densitometry measurements of SPTLC1 immunoblots normalized by vinculin density as loading control of untreated cell lysates (CTL) and scramble siRNA (Scr SiRNA). Mean $\pm$ SEM ($n = 3$). (d) ASMase activity was measured fluorometrically at pH 5.0, as described in MATERIALS AND METHODS, then normalized by protein concentration

($n = 3$). The experiments were performed in endothelial cells treated with the indicated concentrations of ASMase siRNA for 72 hours; (* $P < 0.05$, t test). *Inset:* representative ($n = 3$) immunoblot of ASMase (57 kD) 72 hours after treatment with ASMase siRNA at the indicated concentrations. *Lanes:* 1 and 2, untreated cell lysates; 3, Vehicle; 4, ASMase siRNA, 25 nM; and 5, ASMase siRNA, 50 nM. The same membrane was immunoblotted for GAPDH, as loading control. (e) Densitometry measurements of ASMase immunoblots normalized by vinculin density as loading control of untreated cell lysates (CTL) and scramble siRNA (Scr SiRNA). Mean $\pm$ SEM ($n = 3$).

logical inhibitor fumonisins B1 (data not shown) blocked the activation of effector caspases in response to ceramide. In contrast, treatment with ASMase siRNA did not inhibit ceramide-induced caspase-3/7 activity (Figure 3b) or nucleosomal ELISA (data not shown), suggesting that the sustained (16–24 h), proapoptotic responses to exogenous ceramides in lung endothelial cells are primarily mediated by *de novo* sphingolipid synthesis, rather than sphingomyelin hydrolysis. Consistent with the notion

that exogenous C_{8:0} ceramide stimulated *de novo* ceramide synthesis, we measured a 75% increase in dihydroceramides, the ceramide precursors in the *de novo*, SPT-regulated pathway compared with control cells (8.5 nmol/mmol lipid Pi in C₈-treated cells versus 4.8 nmol/mmol lipid Pi in vehicle-treated cells, $P = 0.01$, data not shown). Moreover, the increase in intracellular ceramides (C_{14:0} and above) was blocked by treatment with SPTLC1 siRNA (Figure 3c).

Figure 3. Effect of ceramide synthesis inhibition on effector caspase activation induced by extracellular ceramide in pulmonary endothelium. Primary mouse lung endothelial cells were pretreated with (a) SPTLC1 siRNA (50 nM, 6 d) or (b) ASMase siRNA (50 nM, 72 h) before C₈-ceramide treatment (10 μM, 18 h; Caspase-3/7 activity was normalized by protein concentration; Veh (vehicle siPortamine; mean + SD). Note the robust inhibition of C₈-ceramide-induced caspase activation by the specific inhibition of SPTLC1 compared with the vehicle control. (c) Effect of SPTLC1 siRNA on endogenous endothelial cell ceramide content (C_{14:0} and higher fatty acid chain length) measured by mass spectrometry at 16 hours (mean ± SD, **P* < 0.05 versus control cells; #*P* < 0.05 versus control C₈-ceramide-treated cells).

Comparison with TNF-α-Induced Caspase-3 Activation in Lung Endothelial Cells

The pro-inflammatory cytokine TNF-α triggers endothelial cell apoptosis either alone (e.g., in bovine pulmonary artery endothelial cells) (15–17), or when supplied concomitantly with the protein synthesis inhibitor cycloheximide (15, 18). TNF-α induces endothelial cell apoptosis by stimulating endogenous ceramide synthesis (5, 19). Our primary objective was to determine whether exogenous ceramides induce endogenous ceramide synthesis by enzymatic pathways similar to those of TNF-α. We used SPT and ASMase siRNAs at the concentrations and time points shown above to effectively inhibit ceramide synthesis, before challenging endothelial cells with TNF-α (20 ng/ml plus cycloheximide; see MATERIALS AND METHODS). The targeted genetic (Figure 4a) and pharmacological (data not shown) inhibition of SPT using SPTLC siRNA or inhibitors (fumonis B1 or myriocin), respectively, significantly attenuated TNF-α-induced caspase-3, a marker of apoptosis. In contrast, siRNA-mediated knockdown of ASMase (Figure 4b) did not ameliorate TNF-α-induced apoptosis. The addition of SPTLC siRNA significantly inhibited TNF-α-induced ceramides at 16 hours (Figure 4c, *P* < 0.001). ASMase inhibition decreased baseline ceramide levels, but only minimally attenuated the TNF-α-induced late ceramide elevation (by 17%, *P* = 0.11, Figure 4d). Scramble siRNA or vehicle treatment had a slight, but insignificant inhibitory effect on TNF-α-induced ceramides (Figures 4c and 4d, respectively). These results indicate a negligible contribution of the ASMase pathway to caspase-3 activation and ceramide production induced by TNF-α at 16 hours. They also suggest that both exogenous C_{8:0} ceramide and TNF-α augment *de novo* sphingolipid synthesis to induce caspase-3 activation in lung endothelial cells. We next addressed whether the ceramide species profiles in response to

exogenous ceramide and TNF-α have a pattern similar to that predicted by the activation of a common enzymatic pathway.

Patterns of Intracellular Ceramide Species Synthesized in Response to Extracellular Ceramide and TNF-α

In the context of lung endothelial cell apoptosis, we identified a robust increase in both long-chain (C₁₄–C₁₈) and very long-chain (C₂₀ and higher) ceramides induced by TNF-α and extracellular ceramide (Figure 5a). The TNF-α- or C_{8:0} ceramide-induced synthesis of ceramide species containing very long-chain fatty acids appeared primarily under the control of the *de novo* pathway (Figure 5b). In contrast, the inhibition of ASMase had a very modest inhibitory effect on ceramide synthesis induced by the two treatments, manifested primarily on the long-chain ceramides. Of note, the vehicle employed for intracellular delivery of siRNA, siPortamine, did not significantly change overall ceramide content compared with other control conditions (such as untreated cells or cells treated with ethanol [0.1%] as a vehicle for ceramide), with the exception of C_{24:1}, which was decreased by siPortamine by 29% (not shown). Therefore, the marked decrease in ceramide species induced by SPTLC1 siRNA (Figure 4c) cannot be attributed solely to its vehicle, siPortamine.

Of the ceramide species studied, C_{16:0} and C_{20:0} ceramides demonstrated absolute concentrations similarly regulated by TNF-α and C_{8:0} ceramide. The absolute concentrations of these two ceramide species were elevated by the treatments in an SPT-dependent manner (not shown). However, when measuring the relative expression of ceramide species, C_{16:0} had the single largest increase in abundance relative to the other ceramide species induced by TNF-α and C_{8:0} ceramide (Figure 5c). The relative increase in C_{16:0} induced by TNF-α and extracellular C_{8:0} ceramide was markedly attenuated by SPTLC1 siRNA (Figure 5c), suggesting

Figure 4. Effect of ceramide synthesis inhibition on TNF- α -induced effector caspase activation in pulmonary endothelial cells. Primary mouse lung endothelial cells were pretreated with (a) SPTLC1 siRNA (50 nM, 6 d) or (b) ASMase siRNA (50 nM, 72 h) before TNF- α treatment (20 ng/ml, with cycloheximide). Caspase-3/7 activity was normalized by protein concentration; Veh (vehicle siPortamine). (c, d) Effect of siRNA inhibitory treatments on intracellular ceramides (normalized by lipid phosphorus). Effect of Scramble siRNA (Scr siRNA, 50 nM, 6 d) or SPTLC1 siRNA (50 nM, 6 d) on TNF- α -induced ceramides (c) compared with the effect of ASMase siRNA (50 nM, 3 d) (d). SPTLC1, but not ASMase inhibition significantly reduced ceramide up-regulation in response to TNF- α (20 ng/ml, with cycloheximide, 16 h); (mean $\pm$ SD, * P < 0.05 versus control untreated cells; # P < 0.05 versus control TNF- α -treated cells).

this species of ceramide might be the target for SPT regulation in response to both TNF- α and extracellular ceramide.

DISCUSSION

These results implicate *de novo* ceramide synthesis as a critical component of the pro-apoptotic action of extracellular ceramides on endothelial cells. A similar requirement for *de novo* sphingolipid synthesis was observed for late (16 h) apoptosis induced by TNF- α in lung endothelial cells. Furthermore, the differential profiles of endogenous ceramides released by these two stimuli associate the relative and absolute increases in C_{16:0} ceramides with caspase activation in primary lung endothelial cells. Recently, ASMase-dependent increases in ceramide C_{16:0} have been implicated in TNF- α -induced apoptosis in hepatocytes (20), underscoring the cell-type specificity of these responses. Ceramide up-regulation is relevant to a wide range of diseases, including radiation-induced tissue injury, Alzheimer's disease, and pulmonary emphysema. In the latter, injury of lung capillary endothelial cells by cigarette smoke itself, oxidants, or circulating pro-inflammatory cytokines may initiate the apoptotic destruction of alveolar septae, a distinctive feature of pulmonary emphysema. TNF- α and ceramide have both been shown to cause endothelial cell apoptosis and have both been implicated in the pathogenesis of emphysema (21). TNF- α increases intracellular ROS and ceramide levels, but as previously pointed out by several investigators, the pro-apoptotic signaling events that lie downstream of

ceramide may be distinct from TNF- α (5, 19). This divergence is especially evident in the context of endothelial cell activation by TNF- α , where pro-apoptotic pathways are inhibited and pro-inflammatory pathways are activated (22). In the context of a pro-apoptotic, ceramide-generating TNF- α response, however, TNF- α and ceramides were used almost interchangeably in the study of ceramide-dependent apoptosis. TNF- α -stimulated ceramides may in fact facilitate pro-apoptotic TNF- α effects, through fusion of lipid microdomains and clustering of TNF receptors (23). Therefore, work from others and our laboratories suggested that both TNF- α and extra- (or para)-cellular ceramides may amplify the injury of microvascular endothelial cells. We propose that the injury-amplifying effects of these molecules are mediated by the generation of specific ceramide species.

To better characterize the stimulus-specificity of ceramide synthesis, we employed genetic manipulation using small inhibitory RNAs. Pharmacological inhibitors, while relatively specific, commonly interfere with other cellular pathways and at high concentrations may exhibit cellular toxicity. In contrast, siRNAs are highly specific (24), and compared with other molecular manipulations of enzymatic activities in the sphingolipid pathways (e.g., ASMase knockout) they have the advantage of a timed onset of action, thus limiting the potential effect of long-term accumulated lipids in the cells. Furthermore, siRNA treatments are well tolerated *in vivo* in the lung (4), thus having the prospective for clinical applications. Using this effective tool, we have demonstrated that TNF- α required *de novo* sphingolipid

Figure 5. Patterns of intracellular ceramide species induced by TNF- α or exogenous ceramides in lung endothelial cells. (a) Effect of TNF- α (20 ng/ml with cycloheximide, 16 h) or extracellular C_{8:0} ceramide (10 μ M, 16 h) treatment on endogenous long-chain (C_{14:0} - C_{18:0}) and very long-chain ceramides (C₂₀₋₂₄). (b) Inhibitory effect of SPTLC1 and ASMase siRNAs on long-chain and very long-chain ceramides induced by TNF- α or C_{8:0} ceramide. Note the robust inhibition of very-long-chain ceramides induction by SPTLC1 siRNA ($n = 2$). (c) Pie charts of relative expression profiles of individual ceramide species comprising total ceramides measured by mass spectrometry. Ceramide species expression profiles at baseline and in response to C_{8:0} and TNF- α treatment. Depicted in the lower pie charts are the effects of SPTLC1 siRNA treatment on ceramide species induced by the two stimuli. (d) Changes in the relative abundance of C_{16:0} (% of total ceramides) induced by C_{8:0} and TNF- α treatment and the effect of SPTLC1 siRNA. The control values include untreated control, vehicle-treated control and scramble siRNA (mean $\pm$ SEM; * $P < 0.05$ versus controls, # $P < 0.05$ versus C_{8:0} and TNF- α treatment, respectively, Student's t test).

synthesis, rather than ASMase activity for caspase-3/7 activation. This result is in contrast with findings in HUVECs and other cell types (5, 25–27), and may reflect organ, species, or inhibitor specificity. For example, similar to lung endothelial cells, *de novo* ceramide synthesis is required for tumor-induced apoptosis in dendritic cells (28). Furthermore, our results suggest a prolonged half-life of endothelial SPT protein and perhaps the need for prolonged inhibitory strategies before functional SPT assays are performed. It is conceivable that the *de novo* ceramide synthesis shown here to be essential for triggering apoptosis may be associated with either a very rapid (minutes) and/or late (beyond 18 h) wave of sphingomyelin breakdown, further contributing to lung injury. This hypothesis is supported by work implicating sphingomyelinases in the paracrine amplification of lung apoptosis (1) and in lung inflammation (29). Our results do not rule out the involvement of neutral sphingomyelinase. We focused our work on SPT and ASMase because of their involvement in an apoptosis-dependent murine emphysema model (1). Furthermore, SPT inhibition completely blocked TNF- α and ceramide-induced caspase-3 activation, suggesting its activation was necessary and

sufficient for this response. The relevance of this finding to the *in vivo* effects of the cytokine awaits further investigation.

As novel investigations into individual ceramide species generation and function emerge, more precise mechanistic conclusions about the role of specific ceramide species in disease will be possible. Our data suggest that very long-chain ceramides and C_{16:0} are predominantly synthesized by lung endothelial cells via SPT activation and that they might be specifically involved in the activation of executioner caspases. The role of the shorter-chain ceramides prominently induced by C_{8:0} remains unclear. Distinct ceramide species may differ in subcellular compartmentalization and assume specific signaling roles. Furthermore, individual ceramide species may be differentially regulated through the activation of specific ceramide synthase enzymes. The longevity assurance homolog LASS and various ceramide synthase gene homologs encode distinct ceramide synthase isoforms that become activated downstream of SPT, or are part of the recycling pathway (30, 31). Interestingly, distinct ceramide synthase isoforms are responsible for the generation of specific-length ceramides. For example, the overexpression of ceramide synthase

1 increased C_{18:0}-ceramide levels preferentially, ceramide synthase 2 and 4 increased levels of longer ceramides such as C_{22:0}- and C_{24:0}-ceramides, whereas ceramide synthase 5 and 6 produced predominantly shorter ceramide species (C_{14:0}- and C_{16:0}-ceramides) (32). Future investigations will determine whether specific ceramide synthases are up-regulated differentially by stimuli such as TNF- α or exogenous ceramides.

In conclusion, our data highlight the importance of SPT in the initiation of lung endothelial cell injury and death. To our knowledge, this is the first study of the pro-apoptotic sphingolipid signaling mechanism and ceramide species signature of paracellular and intracellular generated ceramides in lung endothelial cells. This study has relevance to the development of emphysema, since both ceramides and TNF- α are pathogenic in experimental models of emphysema, in which they may cause excessive apoptosis of capillary endothelial cells with loss of alveolar structures. Prevention of excessive lung microvascular endothelial cell death modulated by sustained SPT stimulation might therefore represent an attractive therapeutic target.

Conflict of interest statement: R.M.T. received an unrestricted postdoctoral support grant from Quark Biotech for studies involving RTP801 in cigarette smoke-induced emphysema, \$2,500 for speaker fees in an international conference sponsored by AstraZeneca, and \$1,500 from the Rush Medical Center's CME speakers training workshop titled, Simply Speaking. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors gratefully acknowledge the expert assistance of Patrick Singleton, Ph.D., in the design of siRNA and Kelly Schweitzer, Ph.D., for critical reading of the manuscript.

References

- Petrache I, Natarajan V, Zhen L, Medler TR, Richter AT, Cho C, Hubbard WC, Berdyshev EV, Tudor RM. Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat Med* 2005;11:491-498.
- Kolesnick R, Fuks Z. Radiation and ceramide-induced apoptosis. *Oncogene* 2003;22:5897-5906.
- Haimovitz-Friedman A, Cordon-Cardo C, Bayoumy S, Garzotto M, McLoughlin M, Gallily R, Edwards CK III, Schuchman EH, Fuks Z, Kolesnick R. Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. *J Exp Med* 1997;186:1831-1841.
- Zhang X, Shan P, Jiang D, Noble PW, Abraham NG, Kappas A, Lee PJ. Small interfering rna targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. *J Biol Chem* 2004;279:10677-10684.
- Slowik MR, De Luca LG, Min W, Pober JS. Ceramide is not a signal for tumor necrosis factor-induced gene expression but does cause programmed cell death in human vascular endothelial cells. *Circ Res* 1996;79:736-747.
- Kroesen BJ, Jacobs S, Pettus BJ, Sietsma H, Kok JW, Hannun YA, de Leij LF. Bcr-induced apoptosis involves differential regulation of c16 and c24-ceramide formation and sphingolipid-dependent activation of the proteasome. *J Biol Chem* 2003;278:14723-14731.
- Medler TR, Berdyshev EV, Singleton P, Lee PJ, Tudor RM, Petrache I. Ceramide induces pulmonary endothelial cell apoptosis by stimulation of its own *de novo* synthesis [abstract]. *Am J Respir Crit Care Med* 2005;171:A714.
- Kuhlencordt PJ, Rosel E, Gerszten RE, Morales-Ruiz M, Dombkowski D, Atkinson WJ, Han F, Preffer F, Rosenzweig A, Sessa WC, et al. Role of endothelial nitric oxide synthase in endothelial activation: Insights from enos knockout endothelial cells. *Am J Physiol Cell Physiol* 2004;286:C1195-C1202.
- Zhang X, Shan P, Qureshi S, Homer R, Medzhitov R, Noble PW, Lee PJ. Cutting edge: Tlr4 deficiency confers susceptibility to lethal oxidant lung injury. *J Immunol* 2005;175:4834-4838.
- Pober JS. Activation and injury of endothelial cells by cytokines. *Pathol Biol (Paris)* 1998;46:159-163.
- Sullards MC, Merrill AH Jr. Analysis of sphingosine 1-phosphate, ceramides, and other bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Sci STKE* 2001;2001:PL1.
- Berdyshev EV, Gorshkova IA, Garcia JG, Natarajan V, Hubbard WC. Quantitative analysis of sphingoid base-1-phosphates as bisacetylated derivatives by liquid chromatography-tandem mass spectrometry. *Anal Biochem* 2005;339:129-136.
- Ogretmen B, Pettus BJ, Rossi MJ, Wood R, Usta J, Szulc Z, Bielawska A, Obeid LM, Hannun YA. Biochemical mechanisms of the generation of endogenous long chain ceramide in response to exogenous short chain ceramide in the a549 human lung adenocarcinoma cell line: role for endogenous ceramide in mediating the action of exogenous ceramide. *J Biol Chem* 2002;277:12960-12969.
- Gable K, Han G, Monaghan E, Bacikova D, Natarajan M, Williams R, Dunn TM. Mutations in the yeast lcb1 and lcb2 genes, including those corresponding to the hereditary sensory neuropathy type i mutations, dominantly inactivate serine palmitoyltransferase. *J Biol Chem* 2002;277:10194-10200.
- Polunovsky VA, Wendt CH, Ingbar DH, Peterson MS, Bitterman PB. Induction of endothelial cell apoptosis by tnfr1: modulation by inhibitors of protein synthesis. *Exp Cell Res* 1994;214:584-594.
- Petrache I, Verin AD, Crow MT, Birukova A, Liu F, Garcia JG. Differential effect of mlc kinase in tnfr1-induced endothelial cell apoptosis and barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1168-L1178.
- Petrache I, Birukov K, Zaiman AL, Crow MT, Deng H, Wadgaonkar R, Romer LH, Garcia JG. Caspase-dependent cleavage of myosin light chain kinase (mlck) is involved in tnfr1-mediated bovine pulmonary endothelial cell apoptosis. *FASEB J* 2003;17:407-416.
- Karsan A, Yee E, Harlan JM. Endothelial cell death induced by tumor necrosis factor- α is inhibited by the bcl-2 family member, bcl-2. *J Biol Chem* 1996;271:27201-27204.
- Mallampalli RK, Peterson EJ, Carter AB, Salome RG, Mathur SN, Koretzky GA. Tnf- α increases ceramide without inducing apoptosis in alveolar type ii epithelial cells. *Am J Physiol* 1999;276:L481-L490.
- Osawa Y, Uchinami H, Bielawski J, Schwabe RF, Hannun YA, Brenner DA. Roles for c16-ceramide and sphingosine 1-phosphate in regulating hepatocyte apoptosis in response to tumor necrosis factor- α . *J Biol Chem* 2005;280:27879-27887.
- Churg A, Wang RD, Tai H, Wang X, Xie C, Wright JL. Tumor necrosis factor- α drives 70% of cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med* 2004;170:492-498.
- Janes KA, Gaudet S, Albeck JG, Nielsen UB, Lauffenburger DA, Sorger PK. The response of human epithelial cells to tnfr1 involves an inducible autocrine cascade. *Cell* 2006;124:1225-1239.
- Gulbins E, Grassme H. Ceramide and cell death receptor clustering. *Biochim Biophys Acta* 2002;1585:139-145.
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide rnas mediate rna interference in cultured mammalian cells. *Nature* 2001;411:494-498.
- Sawada M, Kiyono T, Nakashima S, Shinoda J, Naganawa T, Hara S, Iwama T, Sakai N. Molecular mechanisms of tnfr1-induced ceramide formation in human glioma cells: P53-mediated oxidant stress-dependent and -independent pathways. *Cell Death Differ* 2004;11:997-1008.
- Andrieu-Abadie N, Levade T. Sphingomyelin hydrolysis during apoptosis. *Biochim Biophys Acta* 2002;1585:126-134.
- Heinrich M, Neumeyer J, Jakob M, Hallas C, Tchikov V, Winoto-Morbach S, Wickel M, Schneider-Brachert W, Trauzold A, Hethke A, et al. Cathepsin d links tnfr1-induced acid sphingomyelinase to bid-mediated caspase-9 and -3 activation. *Cell Death Differ* 2004;11:550-563.
- Kanto T, Kalinski P, Hunter OC, Lotze MT, Amoscato AA. Ceramide mediates tumor-induced dendritic cell apoptosis. *J Immunol* 2001;167:3773-3784.
- Goggel R, Winoto-Morbach S, Vielhaber G, Imai Y, Lindner K, Brade L, Brade H, Ehlers S, Slutsky AS, Schutze S, et al. Paf-mediated pulmonary edema: a new role for acid sphingomyelinase and ceramide. *Nat Med* 2004;10:155-160.
- Riebeling C, Allegood JC, Wang E, Merrill AH Jr, Futerman AH. Two mammalian longevity assurance gene (lag1) family members, trh1 and trh4, regulate dihydroceramide synthesis using different fatty acyl-coa donors. *J Biol Chem* 2003;278:43452-43459.
- Venkataraman K, Riebeling C, Bodenec J, Riezman H, Allegood JC, Sullards MC, Merrill AH Jr, Futerman AH. Upstream of growth and differentiation factor 1 (uog1), a mammalian homolog of the yeast longevity assurance gene 1 (lag1), regulates n-stearoyl-sphinganine (c18(dihydro)ceramide) synthesis in a fumonisins b1-independent manner in mammalian cells. *J Biol Chem* 2002;277:35642-35649.
- Mizutani Y, Kihara A, Igarashi Y. Mammalian lass6 and its related family members regulate synthesis of specific ceramides. *Biochem J* 2005;390:263-271.