Inhibition of Acid Sphingomyelinase Disrupts LYNUS Signaling and Triggers Autophagy

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Abbreviations

Acid sphingomyelinase (ASM), bicinchoninic assay (BCA), immortalized bronchial epithelial cells (BEAS2b), ceramide (cer), chronic obstructive pulmonary disease (COPD), N-heptadecanoyl-sphingosine (C17:0-Cer), 17-carbon analog of sphingosine (C17-Sph), 17-carbon analog of S1P (C17-S1P), dihydrosphingosine (DHSph), dihydrosphingosine-1-phosphate (DHS1P), fluorescence units (FU), primary human lung microvascular endothelial cells (HLMVEC), primary human pulmonary artery endothelial cells (HPAEC), horseradish peroxidase (HRP), primary human umbilical vein endothelial cells (HUVEC), lysosome-associated membrane protein 1 (LAMP1), microtubule-associated protein one light chain three beta (LC3B), lysosomal nutrient sensing complex (LYNUS), mammalian target of rapamycin (mTOR), multiple reaction monitoring (MRM), phosphate buffered saline (PBS), quantitative Polymerase Chain Reaction (qPCR), Ras homolog enriched in brain (RHEB), sphingomyelin phosphodiesterase 1 (SMPD1), Sphingosine (Sph), sphingosine-1-phosphate (S1P), transcription factor EB (TFEB), voltage-dependent anion channel (VDAC).
Abstract (154 words)

Activation of the lysosomal ceramide-producing enzyme acid sphingomyelinase (ASM) by various stresses is centrally involved in cell death and has been implicated in autophagy. We set out to investigate the role of the baseline ASM activity in maintaining physiological functions of lysosomes, focusing on lysosomal nutrient-sensing complex (LYNUS) – a lysosomal membrane-anchored multiprotein complex that includes the mammalian target of rapamycin (mTOR) and the transcription factor EB (TFEB). ASM inhibition with imipramine or \(\text{SMPD1} \) siRNA in human lung cells, or by transgenic \(\text{Smpd1}^{+/c}\) haploinsufficiency of mouse lungs, markedly reduced mTOR- and P70-S6 kinase Thr 389-phosphorylation and modified TFEB in a pattern consistent with its activation. Inhibition of baseline ASM activity significantly increased autophagy with preserved degradative potential. Pulse labeling of sphingolipid metabolites revealed that ASM inhibition markedly decreased sphingosine and sphingosine-1 phosphate (S1P) levels at the level of ceramide hydrolysis. These findings suggest that ASM functions to maintain physiological mTOR signaling and inhibit autophagy and implicate sphingosine and/or S1P in the control of lysosomal function.

Keywords: Lysosome, Sphingolipids, Membrane, Endothelial cells, Lung, Sphingosine, mTOR
Introduction

The activation above homeostatic levels of acid sphingomyelinase (ASM), a lysosomal phosphodiesterase typically involved in apoptosis via production of ceramide, has been recently implicated in stress-induced macro-autophagy (referred to as autophagy henceforth)(1, 2). Despite increased understanding of the involvement of ASM’s brisk activation in the stress response, little is known about the role of baseline ASM activity in maintaining lysosomal function during physiological conditions. The causal link between loss of ASM function and the lipid storage disorder Niemann-Pick’s disease revealed a requirement of ASM for proper cellular function and highlighted the essential role of ASM in sphingomyelin breakdown and lysosomal membrane homeostasis (3). Docked at the lysosomal membrane, the lysosomal nutrient sensing complex (LYNUS) controls protein synthesis and autophagy, an essential mechanism of cellular survival during stress and starvation (4). In turn, aberrant autophagy has been implicated in the pathogenesis of several lung diseases, including chronic obstructive pulmonary diseases (5). Therefore, understanding the molecular determinants of autophagy is of clinical importance.

The lysosomal and secreted forms of ASM – both being encoded by the sphingomyelin phosphodiesterase 1 (SMPD1) gene – catalyze, optimally at acidic pH, the hydrolysis of sphingomyelin into ceramide and phosphorylcholine. ASM activity is rapidly increased during stress, generating excess ceramide that, in turn, interacts with specific signaling molecules to trigger second messenger effects. In addition, ceramide incorporates and alters the biophysical properties of membrane bilayers such as fluidity and stiffness by dislodging cholesterol from membrane lipid rafts, impacting acyl chain order parameters of neighboring lipids, and modifying membrane bilayer composition (6). Since ceramides produced in the lysosome by ASM are not shuttled to other subcellular compartments, they may directly influence the coalescence of microdomains required for lysosomal signaling (7). However, ASM forms a molecular complex with acid ceramidase, which metabolizes ceramide to sphingosine, a molecule itself implicated in autophagy via alterations of lysosomal calcium efflux (8). We investigated if ASM is required for LYNUS function of
inhibition of autophagy during homeostatic conditions, hypothesizing that inhibition of ASM is sufficient to trigger autophagy associated with disrupted LYNUS signaling.

During plentitude of ATP, amino acids, and growth signals, the mammalian target of rapamycin (mTOR), a serine/threonine kinase component of LYNUS, stimulates protein synthesis (4). This fundamental function of LYNUS requires docking to the lysosomal membrane, ensured by its farnesylated component, the Ras homolog enriched in brain (RHEB) (4). Upon loss of growth signals, mTOR becomes inactive and one of its phosphorylation targets in LYNUS, the transcription factor EB (TFEB) translocates to the nucleus, where it upregulates transcription of genes involved in lysosomal biogenesis and autophagy, including its own (9). When activated above homeostatic levels, such as during stress, ASM triggers autophagy with impaired lysosomal degradation (decreased autophagic flux), coupled with decreased mRNA transcripts for TFEB targets (2). Whether this effect is due to production of excess ceramide or to other sphingolipid perturbations remains unclear. Such alterations in sphingolipids may either have second messenger signaling effects—e.g., excess ceramide engages signaling similar to that induced by amino acid depletion (10)—or could alter the lysosomal membrane lipid composition and fluidity, potentially affecting LYNUS anchoring.

Utilizing human primary lung human lung endothelial cells, human lung epithelial cells, and murine lungs, we show that inhibition of homeostatic ASM activity is sufficient to inactivate signaling of LYNUS components and to initiate autophagy with degradative potential associated with marked reductions in sphingosine—rather than ceramide—levels.
Materials and Methods

Reagents

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich.

Cell Culture

All experiments were performed in full (containing all growth supplements) media unless otherwise stated. Human pulmonary artery endothelial cells (HPAEC) (Invitrogen, C0085C) were cultured in Medium 200 (Invitrogen, M200500), with low serum growth supplement (Invitrogen, S00310). Primary human lung microvascular endothelial cells (HLMVEC) (Invitrogen, CC2527) were cultured in an EGM-2MV BulletKit (Invitrogen, CC3202). Primary human umbilical vein endothelial cells (HUVEC), a kind gift from Dr. Mathias Clauss (Indiana University), were cultured in VasculLife VEGF Medium Complete Kit (LifeLine, LL0003). THP-1 monocytes (ATCC, TIB202) were cultured in RPMI (Invitrogen, 11875-119), supplemented with 10% fetal bovine serum (FBS) (ThermoFisher Scientific, sh30910-03HI). Immortalized bronchial epithelial cells (BEAS2b) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose and pyruvate (Invitrogen, 11995-073), supplemented with 10% FBS and 1% penicillin/streptomycin (Sigma, P4333-100ML). All cells were incubated at 37°C with 5% CO2 and 100% humidity.

ASM inhibition with imipramine

When cells were 80-90% confluent, media was replaced and imipramine or vehicle was added for the indicated time, when cells were washed with 5 mL PBS at 4 ºC and scraped in 1.5 mL PBS at 4 ºC. Cells were then spun at 16,000 g for 10 minutes and the cell pellets were snap-frozen in liquid nitrogen until assayed.

ASM activity

Lysosomal ASM activity was measured with an Amplex Red Sphingomyelinase Activity kit (Invitrogen, A12220) or radioactivity. Briefly, the kit utilizes an indirect two-step reaction that produces a fluorescent readout of ASM activity. Cells were washed in ice-cold phosphate buffered saline (PBS) at end of treatment, scraped in ice-cold PBS and centrifuged at 16,000 g for 10 min. Supernatant was removed, and
the remaining cell pellet was snap frozen in liquid nitrogen. At time of assay, pellets were hydrated with a reaction buffer for lysosomal ASM activity, incubated with 5 mM sphingomyelin, 2 U/mL horseradish peroxidase, and 8 U/mL alkaline phosphatase. Kinetic fluorescence measurements were read using a wavelength of 590 nm over a period of two hours at 37°C in a SpectraMax m2e microplate reader using SoftMax Pro software.

For the radioactive assay, cells pellets were lysed in 75 µL of a buffer containing 25 mM TRIS (pH = 7.6), 5 mM EDTA, 0.2% Triton-x, phosphatase inhibitors, and protease inhibitors. To 4 mL of the same buffer used to lyse cells, 3.08 µL of 14C-choline methyl sphingomyelin @ 55 mCi/mmol and 0.1 mCi/mL was added to create a substrate buffer (1.4 pmol/µL). To 103 µL of the reaction buffer, 35 µL of lysate, 12 µL of 0.2 M acetic acid, and 50 µL of substrate buffer was added, mixed by vortex and allowed to react at 37°C for 2.3 hours. To stop the reaction, 250 µL of a 2:1 (chloroform:methanol) solution was added and vortexed. To this, 800 µL of a 2:1 (chloroform:methanol) plus 250 µL of water was added and vortexed. From the aqueous phase, 200 µL was extracted and added to 800 µL of MicroScint PS (Perkin-Elmer, 6013631) and radioactivity quantified on a Topcount scintillation counter.

**Western Blotting**

Cells were washed with ice-cold PBS, gently scraped in ice-cold PBS, centrifuged at 16,000 g for 10 minutes, the supernatant was removed and the pellets were snap frozen in liquid nitrogen. Cell pellets were thawed at 4°C in cell lysis buffer containing 1% triton-x (EMD, 9002931), 150 mM NaCl (ThermoFisher, BP3581), and 50 mM Tris (pH 7.6; Invitrogen, 15504020) and then were vigorously vortexed five times during a 1 hour period, followed by centrifugation at 4°C for 10 minutes and the use of the supernatant for assays as whole cell lysate.

Protein concentration was determined using a bicinchninic assay (BCA) (Pierce, 23227). Equal amounts of protein (2-20 µg) were diluted in Laemmli 4X buffer (reducing) (Boston Bioproducts, NC9099736) and resolved in Criterion 12+2 well 4-20% TGX gels (Bio-Rad, 5671093). A semi-dry transfer apparatus (Bio-Rad, 1703848) was employed to transfer proteins to a polyvinylidene fluoride membrane (EMD, IPVH00010). Membranes were probed with the following antibodies: anti-β-actin
(A5441), anti-microtubule-associated protein one light chain three beta (LC3B) (Sigma, L7543), anti-vinculin (Abcam, ab10858), anti-GAPDH (Abcam, ab9485), anti-phospho P70-S6 kinase (Thr 389) (Cell Signaling Technologies, 9205) and anti-phospho mTOR (Ser 2448) (p-mTOR) (Cell Signaling Technologies, 5536). Appropriate secondary antibodies (goat anti-rabbit/mouse, horseradish peroxidase (HRP) conjugate) (GE Healthcare, 45001175 /45001187) were used in conjunction with ECL prime or Luminata Forte (ThermoFisher, RPN2232/EMDMillipore, WBLUF0500) for chemiluminescent reaction. Images were taken with a ChemiDoc (Bio-Rad) XRS system with ImageLab software.

**Densitometry**

Density quantification of protein bands in Western blots was performed with ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, http://imagej.nih.gov/ij/, 1997-2012). Quantification of proteins of interest was performed relative to the intensity of the respective loading controls and this ratio was set equal to one for the experimental control (e.g. vehicle-treated or untreated) group.

**Transfections**

Nucleofector Kits for Primary Endothelial Cells (Lonza, VVPI-1001) in conjunction with Amaxa Nucleofector to transflect HPAEC with siGenome siRNA (Dharmacon, siGlo/non-targeting pool 1/non-targeting pool 2/SMPD1, D0016300205/D-001206-13/D-001206-14/M-006676-01-0020) using the U-017 program, per manufacturer’s protocol. Concentration of siRNA listed was that achieved in 150 µL of the inside cuvette, prior to electroporation. After electroporation, cells were added to tissue culture dishes in 10 mL of full (containing all growth supplements) media. Media was changed 18 to 24 hours after electroporation. Cells were harvested at 48 or 72 hours after transfection, as indicated.

**Quantitative Polymerase Chain Reaction (qPCR)**

Cells were washed with ice-cold PBS, gently scraped in ice-cold PBS, centrifuged at high speed for 10 min., supernatant removed and pellet snap frozen in liquid nitrogen. RNA was extracted with a GenElute mammalian total RNA minikit (Sigma, RTN70-1KT) per manufacturer’s protocol, and cDNA was reverse transcribed with a high capacity cDNA reverse transcription kit with RNase inhibitor (Applied Biosystems, 4374966). Using SYBR select master mix (Applied Biosystems, 4472908) and
**SMPD1/PGK1** primers (Qiagen, PPH02494A/PPH02049A), 25 ng of cDNA was amplified on an ABI 7500 real-time PCR System (Applied Biosystems).

**Immunofluorescence**

HPAEC cells were cultured (125,000 cells per well) on 18 mm glass cover slips (VWR, 48380046) residing in 12-well dishes (Costar, 3513) coated with a gelatin-based coating solution (Cell Biologics, 6950) and were, after treatment and fixation with formaldehyde, exposed to rabbit anti-TFEB polyclonal antibody, Alexa Fluor 555 conjugated (Bioss, bs-5137R-A555), and mounted with SlowFade Gold Antifade reagent with DAPI (Invitrogen, S36939) to microscope slides (ThermoFisher, 12-544-3). Images were taken on a Nikon Eclipse 80i microscope.

BEAS2b that stably express eGFP-LC3B were cultured in 4 well Lab-Tek (ThermoFisher Scientific, 154526) slides (50,000 cells per well). After treatment and fixation with formaldehyde, they were probed for lysosome-associated membrane protein1 (LAMP1) by being exposed to rabbit anti-LAMP1 (Cell Signaling Technology, 9091), Alexa Fluor 594 (ThermoFisher, A20185), and mounted with SlowFade Gold Antifade reagent with DAPI (Invitrogen, S36939) to cover slips (ThermoFisher, 12-544-14) and imaged on a Nikon Eclipse 80i microscope.

**Electron Microscopy**

After washing with ice-cold PBS (Fisher), cells were fixed with glutaraldehyde (Sigma, G7776) and formaldehyde (ThermoFisher, 28908), both at 2% in 0.1 M phosphate buffer for 30 minutes, at room temperature. After two rounds of washing with PBS, cells were gently scraped in PBS, centrifuged for 10 minutes and further processed by the Electron Microscopy Core Facility, Indiana University School of Medicine which included post fixing, embedding, cutting, and mounting on slides. Images were taken with Tecnai G2 12 Bio Twin (FEI, Hillsboro) equipped with an AMR CCD (Advanced Microscopy Techniques).

**Stable Clone Generation**

BEAS2b cells were co-transfected (Amaxa program U-017) with mammalian expression plasmid (pEF6/V5-His) expressing C-terminally tagged ASM(11), a kind gift from Dr. Yusuf Hannun (Stony
Brook University) and mammalian expression plasmid expressing N-terminally tagged eGFP-LC3B under the control of a cytomegalovirus promoter (12) (a kind gift from William Jackson, Stanford University). To verify punctate formation with autophagic stimuli, cells were transiently transfected with eGFP-LC3B plasmid and treated with chloroquine (Supplemental Figure S1). Selection pressure was applied with Geneticin (Santa Cruz, sc29065) for several passages. The co-transfected cells were passaged several times and the over-expression of eGFP-LC3B confirmed by immunoblot (Supplemental Figure S1).

**Differential Centrifugation**

Cells were cultured until 80% to 90% confluent, then treated as indicated, followed by media removal and ice-cold Accutase (10 mL) addition to dishes. All reagents and centrifuges were kept at 4°C during all procedures. Cells were collected by pipetting and were spun at 500 g for 5 minutes. Pellets were suspended in 1 mL of PBS and spun again at 500 g for 5 min. After removing supernatants, the pellets were suspended in solution “B” from Axis-Shield Application S53 plus phosphatase and protease inhibitors. An aliquot was immediately taken and spun at 500 g for 5 minutes to prepare whole cell samples. After 25 minutes, each of the 10 samples generated was homogenized separately (30 strokes) in a 1 mL Dounce homogenizer with pestle “B.” Samples were then spun at 16,000 g for 5 min. The supernatant was labeled cytosolic fraction and the remaining pellet was labeled organelle fraction. The organelle fraction was then suspended in 10% v/v with solution “C” as per S53. A 14 mL gradient with 2 mL discontinuous steps was made with v/v dilutions of OptiPrep as follows: 10% (sample), 12%, 14%, 16%, 18%, 20%, 25% and spun at 150,000 g for 20 h. Fractions were collected from the bottom using a 3 mL syringe with a 4-inch, 16 gauge blunt needle. Six fractions were collected, each 150 µL, starting with fraction six and either added to 4X Laemmli (Boston Bioproducts, NC9099736) for Western blotting, or diluted with 10 mL of PBS and spun at 150,000 g for 10 min. The supernatant was decanted and 1 mL of methanol was used to prepare the pellet for lipid extraction.

**Lipid extraction**
Methanol, water and acetonitrile (HPLC grade) were purchased from Burdick and Jackson (Muskegon, MI). Sphingosine (Sph), dihydrosphingosine (DHSph), a 17-carbon analog of sphingosine (C17-Sph), sphingosine-1-phosphate (S1P), dihydrosphingosine-1-phosphate (DHS1P), a 17-carbon analog of S1P (C17-S1P), N-acylated (C14:0-, C16:0-, C18:1-, C18:0-, C20:0-, C24:1-, C24:0-) sphingosines (Ceramides, Cer), N-heptadecanoyl-sphingosine (C17:0-Cer), N-acylated sphingosyolphosphorylcholines (sphingomyelins, N-12:0-, N-16:0-, N-18:0-, N-24:1-, and N-24:0-), and glucosylceramides (N-16:0-, N-24:1-, N-24:0-) were obtained from Avanti Polar Lipids (Alabaster, AL). N-D3-16:0-glucosylceramide and N-D3-16:0-lactosylceramide were from Matreya LLC (State College, PA). The standards were dissolved in methanol (sphingoid base phosphates were dissolved with the addition of a trace amount of concentrated hydrochloric acid) and stored at -20°C. Pyridine (acetylation grade) and acetic anhydride were products of Alltech Associates (Deerfield, IL).

Cellular lipids were extracted with a modified Bligh and Dyer procedure with the use of 0.1N HCl for phase separation (13). C17-S1P (30 pmols), C17-Sph (30 pmols), N-C17:0-Cer (30 pmols), N-12:0-sphingosylphosphorylcholine (12:0-SM, 100 pmols), N-D3-16:0-glucosylceramide (25 pmol) and N-D3-16:0-lactosylceramide (25 pmol) employed as internal standards, were added during the initial step of lipid extraction. The extracted lipids were dissolved in ethanol and aliquots were taken out to determine total phospholipid content as described by (13). Samples were concentrated under a stream of nitrogen, transferred to autosampler vials, and subjected to LC-MS/MS.

**Pulse labeling of sphingolipid metabolites**

Cells (HPAEC) were incubated (for 5- or 8 hours, as indicated) in complete EBM-2 medium, supplemented with L-[U-13C,15N]-serine (50 mg/L; Cambridge Isotope Laboratories, Andover, MA). Imipramine was added to cells at 3- or 5 hours after the beginning of cell labeling, respectively. At the end of incubation, medium was removed and lipids were extracted as described above. Quantitation of label-incorporated sphingolipids was achieved by LC-MS/MS by monitoring [M+3] isotopes in precursor and product ions as described below.

**LC-MS/MS**
Analyses of sphingoid base-1-phosphates, ceramides, sphingoid bases, sphingomyelins, glycosylceramides and lactosylceramides were performed by electrospray ionization tandem mass spectrometry (ESI-LC/MS/MS). The instrumentation employed was Sciex 6500 QTRAP hybrid triple quadrupole linear ion-trap mass spectrometer (AB Sciex, Redwood City, CA) equipped with an Ion Drive Turbo V ionspray ionization source interfaced with a Shimadzu Nexera X2 UHPLC system. All lipid molecules and their derivatives were separated using Ascentis Express RP-Amide 2.7 µm 2.1 x 50 mm column and gradient elution from methanol:water:formic acid (65:35:0.5, 5 mM ammonium formate) to methanol:chloroform:water:formic acid (90:10:0.5:0.5, 5 mM ammonium formate). S1P and DHS1P were analyzed as bis-acetylated derivatives with C17-S1P as the internal standard employing negative ion ESI and multiple reaction monitoring (MRM) analysis as described (14). Ceramides and sphingoid bases were analyzed with C17-ceramide and C17-Sph as internal standards using positive ion ESI and MRM analysis (13). To facilitate sphingomyelin analysis and to avoid stable isotope overlap between phosphatidylcholines and sphingomyelins, lipids were hydrolyzed using a methylamine reagent for 2 hours at 55ºC (15). Reagents were evaporated with a nitrogen stream. The residual non-saponified lipids were then dissolved in 0.2 ml of methanol and subjected to LC-MS/MS analysis of sphingomyelins. Sphingomyelins were detected as positive ions in MRM mode by a transition from the corresponding molecular ion to the m/z of 184 (phosphocholine). Sphingomyelin quantification was achieved by creating standard curves of variable amounts of sphingomyelin standards (N-16:0-, 18:0-, 24:1, and 24:0-sphingomyelins) versus a fixed amount of N-12:0-sphingomyelin (internal standard). The linearity and the correlation coefficients of the standard curves were obtained via linear regression analysis. Quantification of sphingolipid molecular species for which there were no standards available was performed using best approximation from the available analogs with most similar structure.

Mouse Experiments

All experiments were approved by the IACUC (Indiana University School of Medicine, Indianapolis, IN) and conformed to the Public Health Service policy on humane care and use of laboratory animals as outlined by the Institute for Laboratory Animal Research guide of care and use of laboratory animals.
Three month-old female $Smpd1^{+/+}$, $Smpd1^{+/c}$, or $Smpd1^{c/c}$ mice (C57Bl/6 background strain) – obtained from Dr. Edward Schuchman and Dr. Erich Gulbins – were used to harvest perfused lungs, which were snap-frozen in liquid nitrogen. Lung tissue was homogenized in the cell lysis buffer used for Western blotting. Homogenized tissue was sonicated for two seconds at 30% power, centrifuged at high speed, and supernatant was removed as whole lung lysate.

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).
Results

Effect of ASM inhibition on LYNUS signaling

First, using a fluorometric ASM activity assay, we measured the baseline activity of ASM in primary human pulmonary artery endothelial cells (HPAEC) and confirmed the marked inhibitory effect of imipramine (50 µM; 4 h) (Supplemental Figure S2), a tertiary amine tricyclic antidepressant known to increase the proteolytic degradation of lysosomal ASM (16). Pharmacological inhibition of ASM with imipramine significantly decreased the phosphorylation status of P70-S6 kinase rapidly (within one hour) and sustainably (for up to 24 hours), as visualized by Western blot and quantified by densitometry (Fig. 1A-C). ASM inhibition also reduced mTOR phosphorylation (Supplemental Figure S3), but did not affect the phosphorylation of Akt (Supplemental Figure S3). Since ASM inhibition with imipramine may have non-specific effects, we corroborated these results, by targeting the transcript of the sphingomyelin phosphodiesterase gene (SMPD1) with siRNA (1-3.33 µM, 48-72 h). Compared to three non-targeting pools of siRNA that included fluorescently labeled siRNA (siGLO), SMPD1 siRNA reduced SMPD1 transcript levels by ~50%, as measured by qPCR (Fig. 1D) and markedly inhibited ASM activity measured fluorimetrically (by ~85%, Fig. 1E) or by radioactive assay (by ~65%, data not shown). ASM knockdown with SMPD1 siRNA reduced the phosphorylation of both mTOR and P70-S6 kinase as detected by Western blot and quantified (P70S6k) by densitometry (Fig. 1F and G). We next interrogated if ASM inhibition activated TFEB—a transcription factor docked at LYNUS, which requires dephosphorylation for translocation to the nucleus. ASM inhibition was associated with loss of the “smear” appearance of immunoblotted TFEB seen in control conditions, with the appearance of a lower molecular weight, more resolved band, indicating loss of phosphorylation (Fig. 1H). ASM inhibition was associated with an apparent increase in both cytosolic and nuclear TFEB immunostaining, as detected by epifluorescence (Fig. 1I). Together, the inhibition of mTOR and its downstream target P70-S6 kinase, TFEB dephosphorylation, and increased TFEB in the nucleus indicate that ASM inhibition decreases LYNUS function.
ASM inhibition triggers autophagy

Since mTOR inhibits autophagy, we next determined if baseline ASM activity in normal growth conditions is required not only to maintain LYNUS activity, but also to inhibit autophagy.

Autophagosome abundance was first measured by the amount of intracellular LC3B-II. Pharmacological inhibition of ASM with imipramine (50-100 µM for 2-24 hours) increased LC3B-II levels, as detected by Western blot and quantified by densitometry (Fig. 2A-D). Similarly, ASM knockdown with siRNA led to significant increases in LC3B-II as detected by Western blot and quantified by densitometry (Fig. 2E and F). To determine if this effect extends to other types of cells than HPAEC, we used similar conditions of ASM inhibition (imipramine, 50 µM, 4 hours) to treat other human endothelial cells such as HLMVEC, HUVEC, human THP-1 monocyte cells, and the lung epithelial cell line BEAS2b, and noted that all exhibited similar increases in LC3B-II (Fig. 2G and H). In human immortalized bronchial epithelial BEAS2b cells, we utilized electron microscopy to visualize morphological changes triggered by ASM inhibition. Cells exhibited relatively few intra-cytoplasmic autophagic bodies during baseline conditions (Fig. 2I). In contrast, cells treated with the ASM inhibitor accumulated multiple large autolysosomal bodies with more heterogeneous content (Fig. 2I).

ASM inhibition induces autophagy with lysosomal degradative potential

To clarify whether the increased LC3B-II abundance following ASM inhibition is the result of increased production of autophagosomes versus that of reduced clearance of autophagosomes produced at a basal rate, experiments were performed in HPAEC in the presence or absence of chloroquine – which increases lysosomal pH, diminishes the activity of acidic proteases, reduces autophagosome-lysosome fusion, and decelerates the degradation of LC3B-II associated with autophagosomes (autophagic flux). Compared to cells treated with imipramine alone, those treated with both imipramine and chloroquine exhibited further increases in LC3B-II levels as well as p62 levels (Fig. 3A). This result suggests that autophagosome turnover is present in ASM-inhibited cells and that LC3B-II accumulation is more likely due to increased autophagosome formation rather than due to a significant blockage in autophagic flux (17).
As complementary approach to determine lysosomal degradation of autophagy following ASM inhibition, we used BEAS2b cells stably expressing eGFP-LC3B (Fig. 1B). The colocalization of eGFP-LC3B (green) and LAMP1 (red) –a marker of endosomes and lysosomes – were assessed by immunofluorescence microscopy following ASM inhibition in the presence of chloroquine. Vehicle-treated cells exhibited cytosolic diffuse green immunofluorescence with minimal co-localization with LAMP1-positive (red) endolysosomal vesicles (Fig. 3B). Imipramine treatment increased the number of punctate, GFP-containing autphagic vesicles that co-localized with LAMP1, suggesting autophago-lysosomal fusion (Fig. 3B). Cells co-treated with imipramine and had more intracellular GFP punctate vesicles suggesting non-fused autophagosomes (Fig. 3B) when compared to imipramine treatment alone (Fig. 3B). Together, these results suggest that ASM inhibition induces autophagy with degradative potential.

**ASM inhibition leads to decreased sphingosine levels**

Since the sphingolipid metabolism is highly dynamic and organelle-specific, we next determined levels of sphingolipid metabolites following ASM inhibition in whole cells and in lysosome-enriched fractions obtained by isopycnic separation. To analyze proximal changes in sphingolipids following ASM inhibition, we selected to measure these at the earliest time point (30 min) of detectable LYNUS alterations such as decreased TFEB phosphorylation. Following subcellular fractionation, we noted that fraction six (ordered by increasing density) contained both the mature form of cathepsin D and LAMP1, indicating lysosome enrichment (Fig. 4A). Fraction six also contained LC3B-II (Fig. 4A), as well as the nucleus marker histone h3 and the mitochondrial marker voltage-dependent anion channel (not shown). Following imipramine treatment, neither whole cell lysates, nor the lysosome-enriched sub-cellular fraction exhibited decreased ceramides (Fig. 4B-C); instead, ceramides were modesty increased on account of less abundant species (Supplemental Figure S4). Similarly, inhibition of ASM with imipramine did not significantly change sphingomyelins within this timeframe (Supplemental Figure S4) or for up to 24h (data not shown). The most remarkable change in sphingolipid species following
pharmacological ASM inhibition was a significant decrease in relative sphingosine levels (Fig. 4B). As complementary approach, we transfected HPAEC with non-targeting pool 1, non-targeting pool 2 or SMPDI siRNA (1-3.33 μM; 48-72 hours), and noted that similar to imipramine ASM knockdown significantly reduced sphingosine concentrations compared to control conditions (Fig. 4C).

To further study how ASM inhibition caused sphingosine depletion, we performed pulse labeling of de novo synthesized sphingolipids with U-[\(^{13}\)C,\(^{15}\)N]-serine. Cells were allowed to incorporate the label for 5- or 8 hours, of which, the last 2- or 3 hours, respectively were in the presence of vehicle or imipramine. The quantitation of labeled and non-labeled sphingolipid levels by tandem mass spectrometry established that ASM inhibition had modest effects on sphingomyelin levels and indeed did not reduce but rather increased ceramide levels, at the expense of markedly decreasing sphingosine and S1P production (Fig 4D). Since sphingolipid metabolites such as dihydroceramide, dihydrosphingosine, and glycosylceramides did not significantly change following imipramine incubation (Fig 4D), these data indicate that inhibition of ASM was intimately linked to inhibition of acid ceramidase. It should be also noted that it was mostly the levels of short chain ceramides (14:0; 16:0, and 18:0) that were increased following imipramine (Supplemental Figure S4), which was confirmed in pulse labeled experiments, where 16:0 ceramide species but not 24:1 (Supplemental Figure S4) or 24:0 (data not shown) were increased as a result of imipramine treatment, suggesting that the effect of the metabolic block at ASM/acid ceramidase level is linked to the preferential accumulation of newly formed short-chain ceramide molecules.

**ASM inhibition increases LC3B-II levels and decreases sphingosine levels in vivo**

To investigate if the marked effects of ASM inhibition on autophagy are recapitulated in vivo, we measured for LC3B-II by Western blot in whole lung lysates of Smpd1\(^{-/-}\) mice, known to exhibit 50% reductions in ASM activity compared to wild-type mice (18). Smpd1\(^{-/-}\) lungs had increased abundance of LC3B-II, compared to Smpd1\(^{+/+}\) littermates (Fig. 5A). Sphingosine levels were significantly lower in the
lungs of Smpd1^{+/−} mice compared to Smpd1^{+/+} mice indicating that sphingosine abundance depends on ASM (Fig. 5B) and that the elevation in LC3BII following ASM inhibition is associated with decreased sphingosine both \textit{in vitro} and \textit{in vivo}.
Discussion

Our study demonstrates for the first time an important homeostatic functional relationship between ASM and LYNUS activity, indicating that a baseline ASM activity is required for mTOR signaling and autophagy inhibition. Our data indicate that upon ASM inhibition, mTOR fails to phosphorylate P70-S6k, TFEB becomes dephosphorylated and translocates to the nucleus, events associated with overall increased TFEB abundance and with signs of increased autophagy with degradative potential. Although the precise mechanism underlying the functional connection between ASM-LYNUS remains to be elucidated, our data suggest that it may be linked to the critical role of ASM to sustaining sphingosine levels in the lysosome. These new findings that implicate basal ASM activity in fundamental lysosome functions complement a large body of knowledge relating ASM hyperactivation to lysosome (dys)function during multiple conditions.

ASM inhibition reduced the phosphorylation of mTOR at Ser-2448, which typically occurs through Akt pathway signaling and is required for mTOR catalytic activity. Since the phosphorylation of Akt was unaffected following ASM inhibition, the effect is likely to occur downstream of Akt. The phosphorylation of mTOR at Ser-2448 is also dependent, through a feedback loop, on the phosphorylation (activation) of its downstream target P70-S6k (19), which was markedly decreased by ASM inhibition. It is therefore likely that ASM activity participates is the feedback signaling between P70-S6k and mTOR Ser-2448 phosphorylation during homeostasis. How this occurs, remains elusive. We hypothesized that these effects may be ascribed to signaling or structural effects of sphingolipids metabolized by ASM. Because we noted increased, rather than decreased ceramide upon ASM inhibition, this may inhibit mTOR activity via the SET-PP2A signaling axis (20), but the measured increases were rather modest. However, since lysosomal ASM-generated ceramides are spatially constrained within lysosomes, they may contribute to lysosomal membrane biophysical properties, such as stiffness. These membrane perturbations may affect docking and signaling by interfering with the insertion of farnesylated...
complex anchors or by rearranging microdomains. Nevertheless, since sphingosine was the most marked and consistent affected sphingolipid following ASM inhibition, it is likely that the decreased sphingosine content in the lysosome may have significantly altered the lysosomal calcium efflux, ultimately affecting TFEB phosphorylation through calcineurin (21). Alternatively, subsequent reductions in S1P levels may be implicated, although increased rather than decreased intracellular S1P have been associated with triggering autophagy (22). The pro-autophagic effect of S1P production and the importance of the subcellular localization of S1P accumulation have been recently described in neuronal cells (23-25). This body of work suggests that the autophagy triggered by ASM inhibition, which was associated with decreased S1P levels occurs via a different mechanism than by S1P overproduction.

The consistent decrease in sphingosine upon ASM inhibition has been previously reported and suggests either reduced substrate (ceramide) for acid ceramidase to produce sphingosine, or indicate that inactivation of ASM, which is known to form a physical complex with acid ceramidase, directly inhibits the ceramidase activity (26). We could not document decreased substrate (ceramide) availability for acid ceramidase and our pulse-label experiments indicate that ASM inhibition rapidly causes marked inhibition of acid ceramidase activity, as well. Since sphingosine readily crosses lysosomal membranes, reduced sphingosine, which could generate ceramide via recycling in the endoplasmic reticulum may compensatory stimulate de novo ceramide synthesis or reduce ceramide utilization for complex sphingolipids synthesis (27) (see schematic in Supplemental Figure S5). Pulse-labeling analyses did suggest modest stimulation of de novo synthesis of long chain fatty acid ceramide species (C14:0- C18:0) of which the most abundant is the palmitoyl (C16:0) ceramide. These effects may explain why ceramide levels were not found decreased for up to 72 hours following ASM inhibition and implicate decreased sphingosine as a driver of autophagy, rather than increases in previously described autophagic drivers S1P or ceramide. Furthermore, the subcellular localization of lower levels of sphingosine may be important, since an inhibitor of de novo sphingolipid production in the endoplasmic reticulum did not increase LC3BII levels (28), whereas we show here that inhibition of sphingosine production in the lysosome...
effectively triggered lysosomal autophagy. However, the sphingolipid metabolism is notoriously reactive to perturbations, and we recognize that we have not analyzed the kinetics of all sphingolipid species changes following ASM inhibition. Another limitation of our study is the lack of direct correlation of the magnitude of SMPD1 mRNA decrease following knockdown with the marked enzymatic activity inhibition that followed this approach, especially in the absence of documented ASM protein levels, which are known to have a relatively short half-life, of only five hours (16).

Our results, using chloroquine, immunofluorescence of LC3-BII and lysosome markers, and electron microscopy, indicate that ASM-inhibition triggered lysosomal autophagy with preserved flux, which is defined as degradation of autophagosomes within lysosomes. Furthermore, the finding that LC3B-II was absent in the less dense subcellular fractions of control cells but present in all fractions of ASM-inhibited cells indicate the presence of elongating phagophores (29), which suggests rapid induction of autophagy following ASM inhibition. Electron microscopy images demonstrated not only increased number of structures suggestive of autophagic vesicles following ASM inhibition, but showed that the content of these vesicles was more heterogeneous, a feature previously described to reflect various stages of cargo degradation in autophagolysosomes (30). This finding, in the context of the rest of our other data, may indicate that autophagic induction and pleiotropic fusion may occur simultaneously following ASM inhibition. The autophagic activity following ASM inhibition was associated with lysosomal integrity, as appreciated by immunofluorescence with LAMP1 antibodies, ocridine orange staining (data not shown), and electron microscopy and with preserved cell viability, as assessed by Annexin V and propidium iodide staining and thymidine incorporation (data not shown). Our results, obtained in primary cells, immortalized macrophages, or lung epithelial cell lines differ from those reported in a cancer cell line that overexpressed LC3-GFP, which showed that desmethylclomipramine, a metabolite of clomipramine that structurally resembles imipramine, blocked autophagic flux (31). Such discrepancy may be related to differences in baseline ASM or mTOR activities among different cell types, rather than the use of a different pharmacological inhibitor. Our results do not contradict the report that autophagosomes may
move slower in the absence of ASM due to decreased calcium efflux from the lysosome (32), since our data indicate that ASM inhibition induces autophagy and that the autophagic flux is present, and we cannot dispute that such flux may occur at slower rates that in other conditions of autophagy.

In conclusion, our study demonstrates that lysosomal ASM is an important determinant of homeostatic mTOR signaling and LYNUS function, which suggests that this sphingolipid metabolic enzyme is an important regulator of autophagy. The clinical implications of these results relate to the widespread use of antidepressants in various clinical settings, and their potential to affect conditions where initiation of autophagy with degradative potential is desirable.
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Figure 1. Effects of ASM inhibition on LYNUS signaling in human pulmonary artery endothelial cells (HPAEC). (A-C). Representative Western blots (A-B) and quantification by densitometry (C) of phosphorylated P70-S6 kinase (p-P70-S6k) and vinculin (loading control) following treatment with ASM inhibitor imipramine or vehicle (water) for the indicated time (hours) and concentrations (μM). (D-E). SMPLD1 mRNA expression measured by qPCR and ASM activity detected by fluorometry using a fluorescently-labeled substrate (Amplex Red) following treatment with transfection reagent (mock), control siRNA: siGlo (n=1), or non-targeting (NT) pools 1 or 2, siRNAs or SMPLD1 siRNA (each 3.33 μM; 72 h); UT, untreated cells. (F-G). Phosphorylated mTOR (p-mTOR; at Ser 2448) and p-P70-S6k abundance detected by western blotting (E); quantified by densitometry relative to actin loading control (F) following indicated siRNA. Panels C and F show individual data points of independent experiments, means and SEM (Standard error of the mean). *** p ≤ 0.001 (ANOVA; Tukey Post hoc); ** p ≤ 0.05 (Student’s t-test), respectively. (I). Western blot of TFEB following imipramine treatment (50 μM; for the indicated time). Note absence of smear-like TFEB protein detection in ASM-inhibited cells. (I). Representative fluorescence micrographs following immunostaining for TFEB (red) and nuclear staining (DAPI, blue; n=3). Note increased TFEB immunostaining in both the nucleus (yellow arrowhead) and cytoplasm (white arrow) in ASM-inhibited cells at 4 h following addition of imipramine.
Figure 2. Effect of ASM inhibition on autophagy. (A-E). Representative Western blots (A, C, E) and quantification by densitometry (B, D, F) of LC3B-I and -II protein abundance relative to vinculin (loading control) in HPAEC following ASM inhibition with imipramine at the indicated concentrations (A-B, 4 h time (C-D, 50 μM); or with SMPD1 siRNA (E-F, 3.33 μM; 72 h). (G). LC3B-I and -II and vinculin (loading control) detected by Western blot in primary human lung microvascular endothelial cells (HLMVEC), primary human umbilical vein endothelial cells (HUVEC), human monocytes (THP-1), or immortalized bronchial epithelial cells (BEAS-2B) following imipramine treatment (50 μM; 4 h). (H). LC3B-I and -II protein abundance (normalized to vinculin) in BEAS-2B cells treated with imipramine (+) measured by densitometry. (I). Representative electron microscopy micrographs of BEAS-2B cells following ASM inhibition with imipramine (50 μM; 6 h). Note multiple few small intracytoplasmic bodies in control cells (κ arrow) compared to multiple large intracytoplasmic bodies with heterogeneous density in ASM inhibited cells (black arrows). B, D, F, and H graphs show individual data points from independent experiments, means ± SEM, ***p ≤ 0.001 (ANOVA; Tukey post hoc).
Figure 3. Effect of ASM inhibition on lysosomal degradative potential. (A). Representative Western blot of p62, LC3B-II, and vinculin (loading control), following treatment with the ASM inhibitor imipramine (50 μM; 4 hours) and low-dose chloroquine (5 μM; 4 hours) or rapamycin (Rapa; 1 μM; 4 hours). (B). Representative immunofluorescence images of bronchial epithelial cells (BEAS-2B) expressing eGFP-LC3B (green) immunostained with LAMP1 antibody (red; white arrows) following treatment with imipramine (50 μM; 4 h) and/or chloroquine (50 μM; 4 h). Note increased green punctae in imipramine-treated cells (indicating increased autophagosomes) and relatively low red signal (indicating non-fused lysosomes); intense green punctate in imipramine-chloroquine co-treated cells (yellow arrowhead, indicating increased undigested autophagosomes); increased red immunofluorescence (white arrow) along with green punctae (yellow arrowhead), indicating autophagosomes that do not fuse with lysosomes in chloroquine treated cells.
4. Effect of ASM inhibition on sphingolipids. (A). Representative immunoblots of markers of the endosomal marker LC3B-II in the indicated subcellular fractions (1-6, ordered by increasing density; obtained by isopycnic separation) of cells treated with imipramine (50 μM; 30 min) or control vehicle. Note increased abundance of the endosomal marker LC3B-II in all imipramine-treated fractions. (B). Relative levels of ceramide and sphingosine in HPAEC exposed to ASM inhibitor imipramine compared to cells exposed to vehicle, assessed by tandem mass spectrometry. Each data point represents an independent experiment; mean±SEM; * p ≤ 0.05 imipramine vs. control. (C). Absolute sphingosine levels in HPAEC transfected with non-targeting pool 1, pool 2 or SMPD1 siRNA (1-3.33 μM; 48-72 hours). Each data point represents an independent experiment; mean±SEM; * p ≤ 0.05. Abbreviations: SM: sphingomyelin; DHC: dihydroceramide; Cer: ceramide; LC: long chain species; VLC: very long chain species. (D). Levels of U-[N]-serine-labeled and unlabeled sphingolipids measured by tandem mass spectrometry following treatment with vehicle (Veh) or imipramine (ASM inh) for the indicated time. Horizontal bar represents
Figure 5. Effect of ASM inhibition on lung autophagy and sphingosine levels *in vivo* (A-B).

Representative immunoblot of LC3B-I and -II and vinculin (loading control) in whole lung lysates of mice expressing baseline levels (Smpdl+/+) or deficient (Smpdl+/− and Smpdl−/−) in ASMase (each lane represents a separate mouse lung lysate of each genotype). (B). Sphingosine levels in whole lungs of Smpdl+/+, Smpdl+/−, and Smpdl−/− mice. Graph shows individual data from distinct animals, means and SEM, * = p ≤ 0.05, *** = p ≤ 0.001 (ANOVA; Dunnett’s post hoc).